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The fundamental biology of basophils in health and disease

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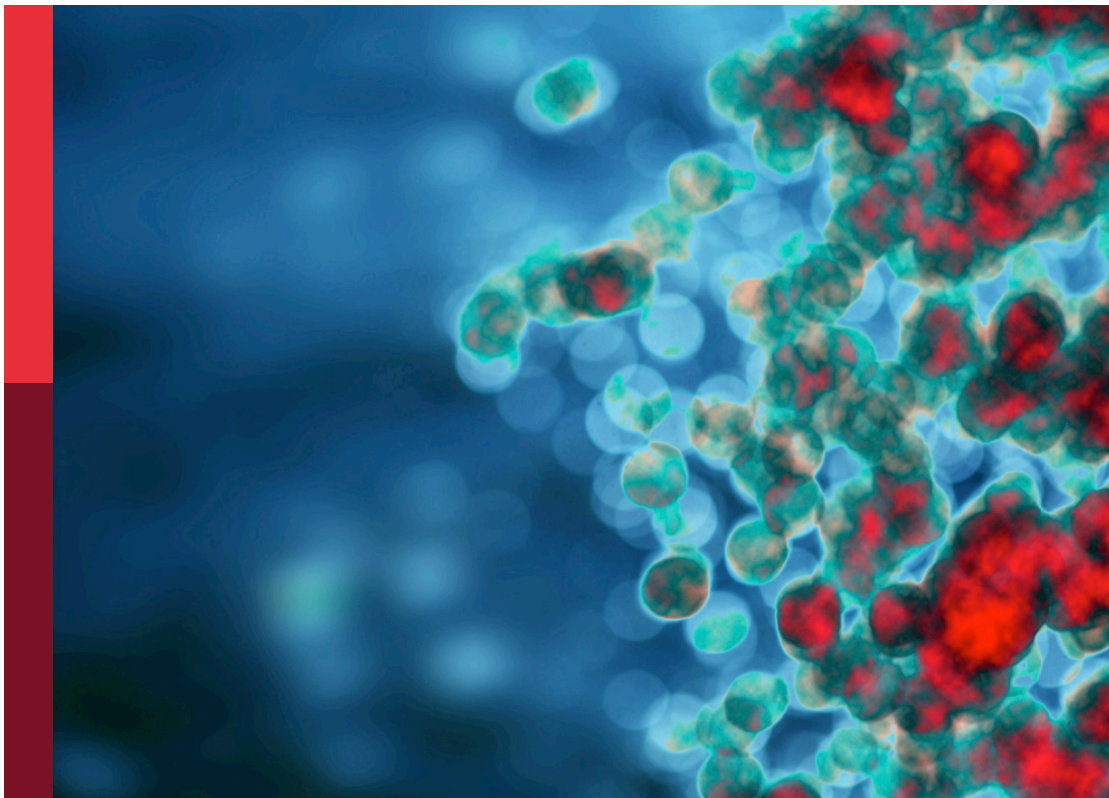

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The fundamental biology of basophils in health and disease

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Editorial: The fundamental biology of basophils in health and disease

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Editorial on the Research Topic
The fundamental biology of basophils in health and disease

1 Quick history of basophils research and emerging hot topics

Basophils are one of the rarest immune cell types, representing less than 1% of circulating leucocytes in humans. They were discovered more than 140 years ago by Paul Ehrlich, but basophils research has suffered from their rarity and from "shifting trends" in immunology. Indeed, from 1985 to 2009, the number of publications on basophils stalled (Figure 1A), while "newer" immune cells grabbed a steady focus (i.e., "Dendritic cells"). The year 2009 saw a renewal of basophils research, which may have arisen from several anterior breakthroughs, beginning with thorough descriptions of the regulation and dynamics of basophil degranulation (1–6), of their expression of IL-4 (7, 8), and of human basophils promoting B cell IgE production without exogenous IL-4 (9) in the 1980s-1990s. The democratization of flow cytometry in the 2000s enabled better protocols of purification and deeper characterization of the human basophil (10, 11), which fostered the development of the Basophil Activation Test (BAT) (12). Mice basophil research showed that basophils are a primary source of IL-4 in helminth infection (13), mediate delayed hypersensitivity reactions after intravenous IgE sensitization and intradermal allergen challenge (14), and promote *in vivo* antibody responses (15), Th2 responses (16), and IgG-driven anaphylaxis (17). This formed the announcement of a "rebirth" of basophils research in 2009, with numerous major publications characterizing how basophils are activated and promote Th2 responses (18–27).

From 2009 to 2019, the number of publications citing basophils rose steadily, with many discoveries deciphering the fundamental biology of basophils and their contribution to health or disease. This was supported by the generation of specific basophil-deficient mice (28, 29) and conditionally basophil-deficient mice (30, 31), which allowed unambiguous demonstrations of the various roles of basophils (32). Nowadays, we have a better understanding of several aspects of basophils biology, including their differentiation (and a pre-mature basophil state) (33–35), their heterogeneity (36–38),

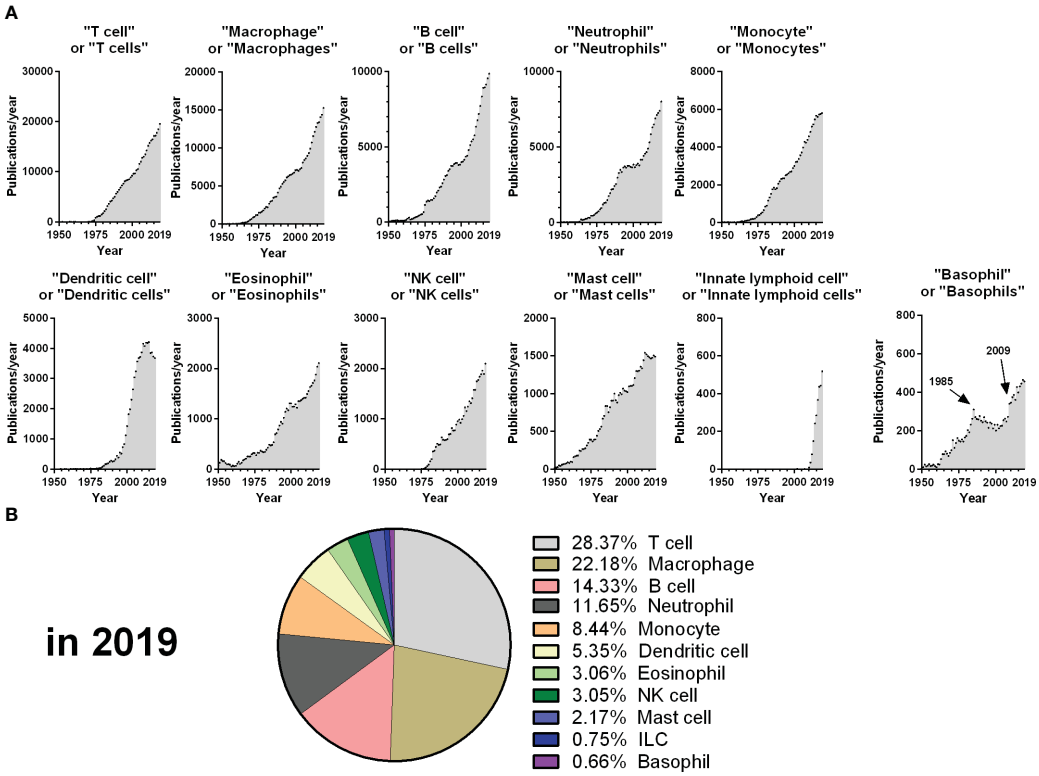

FIGURE 1
Evolution of publications from 1950 to 2019 for each main immune cell type. **(A)** The number of publications per year retrieved from the Pubmed database from 1950 to 2019 is depicted for each query, corresponding to the dot plot titles. **(B)** Percentage of publications retrieved from each query from the sum of all the queries represented in A for the year 2019. The year 2019 was chosen as an endpoint as the volume and dynamics of publications changed drastically from 2020 due to the COVID-19 pandemic.

their responsiveness to various ligands (24, 27, 37–39), their expression of chemokine receptors (40, 41), and the mechanisms by which they can present antigens (42, 43). The controversies regarding how basophils promote the priming of T cells are underlined by Möbs et al.

A deleterious role of basophils seems evident in several allergic diseases of the skin (atopic dermatitis and chronic spontaneous urticaria), the airways (asthma and chronic rhinosinusitis), or the gut and in some anaphylactic reactions. Basophils are also detrimental in various autoimmune diseases (i.e., systemic lupus erythematosus) and chronic inflammatory or fibrotic diseases of the lungs (chronic obstructive pulmonary dysfunction), the gut (inflammatory bowel diseases), the kidneys (ischemia/reperfusion-induced fibrosis), or the heart (allograft-induced fibrosis) (32). Wiebe et al. underlined how basophils contribute to pruritus in allergic and inflammatory or autoimmune skin diseases. An updated description of the contribution of basophils to non-allergic and non-parasitic diseases, with a focus on autoimmune and chronic inflammatory disorders, has been reviewed by Poto et al. Basophils show complex capabilities to promote tumor progression or, inversely, tumor suppression. An updated description of the potential prognostic value of circulating basophils counts and a summary of their functions in various cancer or cancer models has been presented in another manuscript by Poto et al.

These complex roles in cancer highlight that basophils can also promote health and homeostasis in a broad array of conditions: they display unique interactions with hematopoietic and non-hematopoietic cells during lung development (38); they secrete both retinoic acid (44), IL-10 (45), and cleave extracellular ATP (46) to reduce inflammation; and they promote the resolution of infectious and sterile inflammation in the skin, liver, lungs, or heart (32, 47). Basophils have also emerged as being protective in infectious models beyond ectoparasite infections, including in a mouse model of sepsis (48) and of malarial infection (49, 50).

2 Original research, brief reports, and hypotheses

Despite these exciting discoveries, basophils remain the least studied of the main immune cells, representing less than 1% of these publications in 2019 (Figure 1B). In this context, the aim of this Research Topic was to aggregate original manuscripts exploring emerging hot topics in basophils research, which will be presented below.

IgE crosslinking induces several signaling events controlling intracellular calcium mobilization and degranulation. Hansen Selnø et al. explored the expression of the sarcoplasmic reticulum Ca2+ ATPase (SERCA2) in human basophils and its function. SERCA2

expression is strongly inversely correlated with anti-IgE-induced histamine release, and pharmaceutical inhibition or activation of SERCA proteins controls the amplitude of basophil histamine release. Thus, SERCA2 appears as a new negative regulator of basophil degranulation.

Basophil responsiveness to IgE decreases among patients suffering from chronic spontaneous urticaria (CSU), supposedly due to the presence of autoreactive antibodies against IgE or its receptor. However, Matsubara et al. showed that the response of CSU patients' basophils to the anaphylatoxin C5a is unaltered. This suggests targeting the C5a/C5aR axis may be of critical value in patients refractory to anti-FcεRIα treatments.

In chronic inducible urticaria, urticaria is induced by specific stimuli, such as ultraviolet light exposure. Mizuno et al. showed that the circulating basophils of these patients are more activated than those of healthy controls (as CSU patients) but without any IgE hyporesponsiveness. This highlights differences in the pathophysiology of these distinct conditions.

Peripheral basopenia in CSU patients is associated with disease activity, and basophils are found in patients' skin lesions. Kishimoto et al. confirmed previous reports underlining a reversal of basopenia upon treatment of CSU patients with Omalizumab or anti-histamine. Then, using an oxazolone-induced contact dermatitis model, they demonstrated that the migration of circulating basophils to skin lesions provoked a transient basopenia. This supports the concept that clinical observations of basopenia reflect an active basophils extravasation.

El Hachem et al. explored the mechanisms governing basophils extravasation in the skin in FITC-induced dermatitis. They revealed that basophil migration was critically dependent on the secretion of IL-3 by T cells. They also demonstrated that IL-3-stimulated human and mice basophils relied on an autocrine retinoid acid production to drive their expression of specific integrins and mice basophil extravasation. Overall, these results strongly suggest that T cell IL-3 drives basophils autocrine secretion of retinoic acid to enable their extravasation in the inflamed skin.

The unique properties of skin-homing basophils have been described in a hypothesis and theory article by Shibuya and Kim, which suggests these basophils may have a unique identity, acquired during hematopoiesis and/or through late imprinting by the action of TSLP and epithelial-derived alarmins as mice lung basophils do under the control of IL-33 and GM-CSF (38). Skin-infiltrating basophils may externalize MRGPRX2, a receptor involved in pseudoallergic reactions and neuroimmune interactions.

MRGPRX2 expression by basophils has been the subject of some controversy. In this Research Topic, Toscano et al. explored the expression and function of this receptor on basophils from patients allergic to birch pollen or hypersensitive to moxifloxacin. Circulating basophils express only very low levels of functional surface MRGPRX2, but this is very quickly externalized by specific activation (anti-IgE, fMLP) or non-specific activation (i.e., purification). Thus, the reactivity of patients' basophils to MRGPRX2 ligands can be studied but only when using carefully controlled conditions.

Basophils are known to participate in allergic airway inflammation and allergic asthma but have mainly been studied following allergen challenge or asthma exacerbation. Here, Iype et al. analyzed the expression of activation markers on stable asthmatics basophils and reported that they express more surface CD25 but no other activation markers. As human basophils activated by IL-2 secrete type 2 cytokines, and IL-2 is associated with asthma, this pathway seems important in asthma chronicity and pathophysiology.

The development of efficient helminth vaccines is an ongoing challenge in immunology. Thuma et al. cloned a new immunogenic protein secreted by the model helminth *Nippostrongylus brasiliensis* (*Nb*) during infection, Nb-LSA1a. Immunization with Nb-LSA1a induces specific IgG1 and protective immunity against *Nb* infection in wild-type but not basophil-deficient mice. This strongly suggests helminth vaccination strategies should benefit from inducing basophil-dependent immunity.

Overall, the manuscripts submitted to this Research Topic underline current and emerging trends in basophils immunology: the regulation of their degranulation via FcεRIα or MRGPRX2; their roles in chronic urticaria, pruritic diseases, asthma, or cancer; the controversies surrounding their regulation of T cell polarization and their potency in promoting anti-helminth protective immunity; the mechanisms controlling their extravasation and peripheral basopenia; and, finally, the concept of mature basophils harboring distinct specific identities.

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A newly identified secreted larval antigen elicits basophil-dependent protective immunity against *N. brasiliensis* infection

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Hookworms infect more that 400 million people and cause significant socio-economic burden on endemic countries. The lack of efficient vaccines and the emergence of anthelminthic drug resistance are of major concern. Free-living hookworm larvae infect their hosts *via* the skin and live as adult worms in the small intestine where they feed on host tissue and blood. Excretory/secretory (E/S) products, released by helminths as they migrate through their host, are thought to play a key role in facilitating infection and successful establishment of parasitism. However, E/S products can also elicit protective immune responses that might be harnessed for vaccine development. By performing Western blots with serum of *Nippostrongylus brasiliensis* (Nb) infected mice as a model for human hookworm infection, we identified a largely overlapping set of IgG1- and IgE-reactive antigens in E/S from infective L3 stage larvae. Mass spectrometry analysis led to the identification of a new protein family with 6 paralogues in the Nb genome which we termed Nb-LSA1 for "*Nippostrongylus brasiliensis* larval secreted protein 1". The recombinantly expressed 17 kDa family member Nb-LSA1a was recognized by antibodies in the serum of Nb immune mice. Immunization of mice with Nb-LSA1a in alum elicited a strong IgG1 response but no detectable antigen-specific IgE. Most importantly, immunized mice were largely protected against a challenge Nb infection. This effect was dependent on the presence of basophils and occurred before the parasites reached the intestine. Therefore, basophils appear to play a critical role for rapid control of infection with L3 stage larvae in mice immunized with a single secreted larval protein. A better understanding of basophil-mediated protective immunity and identification of potent larval antigens of human hookworms could help to develop promising vaccination strategies.

KEYWORDS

hookworms, basophils, SCP/TAPS protein, CAP domain, immunization

Introduction

About a quarter of the human world population is infected with helminths, especially in low economic countries with poor sanitary conditions. Hookworms alone account for more than 400 million infections and cause major socioeconomic problems in endemic countries (1). Hookworm infections can result in anemia, malnutrition and intellectual disability of children. Anthelmintic drugs such as mebendazole or pyrantel can be used to efficiently reduce the worm burden but reinfections rapidly occur after deworming. In addition, there is evidence for increasing drug resistance to some anthelmintics (2). Despite major research efforts, there are no vaccines available yet for any human helminth infection (3).

The major human hookworm species are *Necator americanus* and *Ancylostoma duodenale* which live as adult worms for up to 10 years in the lumen of the small intestine and feed on blood and host tissue. Hookworms infect their hosts as free-living L3 larval stage by penetration of the skin. Next, they reach the lung *via* the bloodstream, enter the alveolar space, get coughed up and swallowed, to finally reach the lumen of the small intestine. Here, they mature to adult worms, feed on host tissue and blood, and females produce eggs that are excreted to the environment where the L1-L3 larval stages develop. Hookworms secrete a huge variety of different proteins which are poorly characterized but likely facilitate entry and persistence in their hosts. Such secretomes contain mainly three categories of proteins: proteases and protease inhibitors, sperm-coating proteins/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) including Venom Allergen-Like (VAL) or Activation-associated Secreted Proteins (ASPs) (Pfam acc. no. PF00188), and proteins with domains of unknown function (4). SCP/TAPS proteins are often immunogenic and therefore include candidates for vaccine development (5).

Although vaccines are unlikely to eradicate hookworm infections, it has been calculated that in combination with anthelmintics they would reduce the disability adjusted life years (DALYs) about 6-fold in a 10 years time frame as compared to administration of anthelmintics alone (6). The development of an efficient hookworm vaccine remains a major challenge and requires detailed understanding of molecular and cellular events required for an efficient and protective immune response. Ideally, protective immunity should be achieved in the skin to prevent larval migration to the lung and intestine.

Infection of mice with *Nippostrongylus brasiliensis (Nb)* is widely used to investigate potential mechanisms of protective immunity against hookworms. *Nb* is genetically related to *N. americanus* and has a similar life cycle. Although more prevalent in rats, *Nb* has also been isolated from wild mice (*Mus musculus*) (7). *Nb* elicits a strong type 2 immune response during primary infection and promotes worm expulsion from the intestine within 10 days by a "weep-and-sweep" mechanism that requires IL-13-elicited activation of goblet cells and smooth muscle cells. During a secondary infection, most L3 larvae are trapped in the skin or lung and only few parasites reach the intestine (8). We and others could show that protective immunity against secondary infection is provided by antibodies, basophils and alternatively activated macrophages (9–14).

In this study we performed Western blots with immune serum of *Nb*-infected mice followed by mass spectrometry of secreted proteins from infective *Nb* L3 stage larvae to identify new antigens with the potential to elicit protective immunity against *Nb* infection. We identified a 17 kDa protein which belongs to a new subfamily of SCP/TAPS proteins. This Nb-LSA1a protein elicited a strong IgG1 response but no detectable IgE upon immunization of mice. Importantly, Nb-LSA1a immunized mice showed a strong reduction in adult worm and egg counts. This protective effect occurred before larval stages reached the lung and was not observed in basophil-deficient Mcpt8Cre mice. These findings indicate that antibodies against Nb-LSA1a and perhaps other cross-reactive antigens activate basophils and prevent larval transit from skin to the lung.

Materials and methods

Mice

Mcpt8Cre mice on C57BL/6 background were bred and maintained in the Franz-Penzoldt Center in Erlangen and kept under specific pathogen free conditions. In Mcpt8Cre mice basophils are specifically and constitutively deleted as a result of Cre toxicity (15). C57BL/6 mice were obtained from Charles River Laboratories.

Ethics statement

Animal experiments were approved by the Local Government of Lower Franconia and performed in accordance with German animal protection law and European Union guidelines.

Parasite infection and enumeration of eggs and worms

For *N. brasiliensis* (*Nb*) infection mice were subcutaneously (s.c.) injected with 500 L3 stage larvae as previously described (15). To assess parasite fecundity, fecal egg counts were determined on day 7 post infection (p.i.) using a modified MacMaster counting chamber. Worm burden in the lung was

analyzed on day 2 p.i. by enumeration of larvae that migrated out of the harvested lung tissue.

Preparation of *N. brasiliensis* antigens

For preparation of *Nb* excretory/secretory proteins (NES) from L3 stage larvae or adult worms, the larvae were collected from the culture plates (L3) or intestine of infected mice (adults) and washed extensively (PBS/PenStrep). For collection of NES-containing supernatants, 10,000 larvae/mL for L3 or 100 worms/mL for adults were cultured in 1% glucose in PBS for 48-72h in 24-well plates at 37°C and 5% CO_2. NES was passed through a 0.2 µm filter and stored at -80°C until used. *N. brasiliensis* somatic extract (NEX) was prepared by homogenization of L3 larvae on ice with stainless steel beads in PBS (TissueLyser, Qiagen, Hilden, Germany) followed by centrifugation and recovery of supernatant.

His-tagged Nb-LSA1a protein expression and purification

For expression of His-tagged Nb-LSA1a protein, fullength cDNA was cloned in pcDNA3.1 (+) C-HA vector (Supplementary Figure S1). Transient transfection of HEK293T cells was performed at a cell confluency of 70-90% using 20 µg plasmid and standard calcium phosphate transfection technique (250 mM $CaCl_2$ and HEPES-buffered saline). Supernatant containing His-tagged Nb-LSA1a protein was stored at -20°C until Ni-NTA purification (HisPur™ Ni-NTA Spin Columns, Thermo Fisher Scientific, Waltham, MA). For immunization experiments purified Nb-LSA1a protein or collected supernatant was used as indicated.

Mouse immunization

Female C57BL/6 or Mcpt8Cre mice were immunized with Nb-LSA1a (purified or supernatant), NES or control (buffer used for Ni-NTA purification of protein or supernatant from empty vector transfected HEK293T cells) by intraperitoneal injection (i.p.) with 200 µL Imject Alum (Thermo Fisher Scientific). Nb-LSA1a or NES protein was used at 5-10 µg/mouse for prime and 1 µg/mouse for boost immunizations. Immunizations were performed on day 0, then boosted on day 7, before infection with *Nb* on day 14.

Sample processing for LC-MS/MS

NES samples (1-10 µg) were prepared in 5x Laemmli buffer without β-mercaptoethanol (non-reducing condition), heated (95°C, 5 min) and analyzed by SDS-PAGE. Following visualization of proteins using colloidal Coomassie Brilliant Blue, gel lanes were cut into 6 slices covering approx. the mass range between 10 to 95 kDa. Slices were washed and destained by alternatingly incubating them with 10 mM NH_4HCO_3 and 50% (v/v) acetonitrile (ACN)/10 mM NH_4HCO_3 (10 min at room temperature (RT) each). Cysteine residues were reduced (5 mM TCEP/10 mM NH_4HCO_3, 30 min at RT) and alkylated (50 mM 2-chloroacetamid/10 mM NH_4HCO_3; 30 min at RT) followed by proteolytic digestion of proteins using trypsin (60 ng per slice; overnight at 37°C). Peptides were eluted with 0.5% (v/v) trifluoroacetic acid (TFA)/50% (v/v) ACN, dried *in vacuo*, resuspended in 30 µl 0.1% TFA and desalted with in-house prepared STAGE tips prior to LC-MS analysis.

LC-MS/MS analysis

Reversed-phase liquid chromatography-mass spectrometry was performed using the UltiMateTM 3000 RSLCnano system (Dionex LC Packings/Thermo Fisher Scientific, Dreieich, Germany) coupled online to a Q Exactive Plus (Thermo Fisher Scientific, Bremen, Germany) instrument. The UHPLC system was equipped with two C18 µ-precolumns (Ø 0.3 mm × 5 mm; PepMap, Thermo Fisher Scientific) and an Acclaim PepMap™ analytical column (ID: 75 µm x 500 mm, 2 µm, 100 Å, Dionex LC Packings/Thermo Fisher Scientific). Peptides eluting from the LC column were transferred to a fused silica emitter for electrospray ionization using a Nanospray Flex ion source with DirectJunctionTM adaptor (Thermo Fisher Scientific) and applying a spray voltage of 1.5 kV and a capillary temperature of 200°C. The MS instrument was externally calibrated using standard compounds and equipped with a nanoelectrospray ion source and a stainless steel emitter (Thermo Fischer Scientific). MS parameters were as follows: MS scan range, *m/z* 375–1,700; resolution, 70,000 (at *m/z* 200); target value, 3 x 10^6 ions; max injection time, 60 ms; TOP12-higher-energy collisional dissociation of multiply charged peptides; NCE of 28%; target value of 1 x 10^5, maximum injection time of 120 ms; dynamic exclusion time of 45 s.

Bioinformatics

For this study, the MaxQuant 1.6.10.43 was used with the UniProt database for *Nippostrongylus brasiliensis*, Taxonomy ID 27835, (release 2020_05; 22636 protein entries). The precursor mass tolerance was set to 20 ppm for the first search and to 4.5 ppm for the main search. Trypsin was set as proteolytic enzyme (≤2 missed cleavages). Oxidation of methionine and acetylation of the protein N-terminus was allowed as variable modifications and cysteine carbamidomethylation as fixed modification. A false discovery rate (FDR) of 1% was applied on both peptide

(on modified peptides separately) and protein lists. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (16) partner repository with the dataset identifier PXD035568.

AlphaFold

The three-dimensonal structure of Nb-LSA1a was predicted using AlphaFold v2.0 (without homologous structure templates and using a selected portion of the BFD database) (17, 18). The prediction is colored by model confidence band and the accuracy of the AlphaFold model was scored as highly accurate with a predicted local distance difference (pLDDT>90) on a scale from 0 to 100.

1D and 2D gel electrophoresis and Western blot

NES and NEX samples (1-10 µg), purified Nb-LSA1a protein or supernatant of transfected cells was subjected to reducing and non-reducing SDS-PAGE using precast gels (10-12% Mini-PROTEAN TGX, Biorad, Hercules, CA) and blotted onto a PVDF membrane according to manufacturer´s instructions (Trans-Blot Turbo System, Biorad). Therefore, samples were prepared in Laemmli buffer containing either 5% (reducing) or no β-mercaptoethanol (non-reducing). Membranes were blocked in 5% milk powder in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) overnight at 4°C, before being incubated with indicated mouse serum samples (1:10 dilution in 3% bovine serum albumin (BSA)/PBS) overnight at 4°C. After extensive washing in TBST, bound immunoglobulin was detected by incubation with HRP-conjugated anti-mouse IgG (Fcγ fragment specific, Jackson ImmunoResearch, Ely, UK), 1:5000 diluted in 5% milk powder/TBST for 1 h at RT. Alternatively, blots were incubated with rat anti-mouse IgE or rat anti-mouse IgG1 (SouthernBiotech, Birmingham, AL), 1:200 in 5% milk powder/TBST) for 2 h at RT, followed by HRP-conjugated goat anti-rat (Jackson ImmunoResearch), 1:5000 in 5% milk powder/TBST for 1 h at RT. For detection of the His-tag, the blot was incubated with polyclonal rabbit anti-His antibody (Cell Signaling, Danvers, MA), 1:1000 3% BSA/PBS for 2 h at RT. Detection followed by HRP-conjugated donkey anti-rabbit (Jackson ImmunoResearch, 1:5000 in 5% milk powder/TBST) and membrane was developed as above. For 2D SDS-PAGE, proteins are separated by isoelectric focusing (IEF) using precast gels (SERVAGel) prior to standard separation by size (SDS-PAGE). In contrast to standard SDS-PAGE, the used NES samples were desalted (Zeba spin columns, Thermo Fisher Scientific) and directly eluted in IEF sample buffer and loaded onto the gel. Subsequent western blotting was carried out as described above.

ELISA

Detection of IgE and IgG1 levels in the serum of naïve and infected mice was determined as follows: Purified mouse anti-IgE (clone R35–72, BD Biosciences, Franklin Lakes, NJ) or a commercial IgG1 ELISA kit (SouthernBiotech) was used for coating. As secondary reagents anti-mouse IgE-AP or IgG1-AP (SouthernBiotech), followed by development with pNPP substrate (SouthernBiotech) was applied. For detection of parasite-specific IgE or IgG1, a 10-20 µg/mL NES protein suspension (Supplementary Figure S2) was coated on 96-well polystyrene plates overnight (4°C), blocked with 3% BSA/PBS for 2 h and then incubated for 2 h with serum dilutions. Parasite-specific antibodies were determined using the secondary reagents described above. For Nb-LSA1a-specific ELISA, 96-well polystyrene plates were coated with a 10-20 µg/mL Nb-LSA1a suspension. Absorption was measured at 405 nm on a Multiskan FC photometer (Thermo Fisher) and blank wells were used for background subtraction.

Statistical analysis

Statistical analysis was performed with Sigmaplot (Version 12.3, Systat Software) using Mann-Whitney U-test. Data is always indicated as mean + standard error (SEM). Levels of significance: *$p < 0.05$, **$p < 0.01$. n.s. = not significant.

Results

Immune serum from secondary Nb-infected mice stains a discrete set of parasite-secreted antigens

Infective L3 stage larvae of *Nb* secrete a large variety of proteins and other molecules (collectively termed *Nippostrongylus brasiliensis* excretory/secretory products, NES) some of which may play a critical role for entry of L3 larvae into the host organism *via* the skin barrier and for successful establishment of parasitism within their hosts. We therefore reasoned that identification of immunogenic proteins in NES could help to develop a vaccination strategy and dissect the mechanisms of protective immunity against the early stage of infection in the skin.

As a first step we determined total and NES-specific IgE and IgG1 levels in the serum after primary and secondary *Nb* infection of mice on C57BL/6 background. While total IgE and IgG1 levels increased after primary infection, we could not detect NES-specific IgE or IgG1 in the serum by ELISA (Figure 1A). This could be due to bystander activation of unspecific B cells or production of low-affinity antibodies.

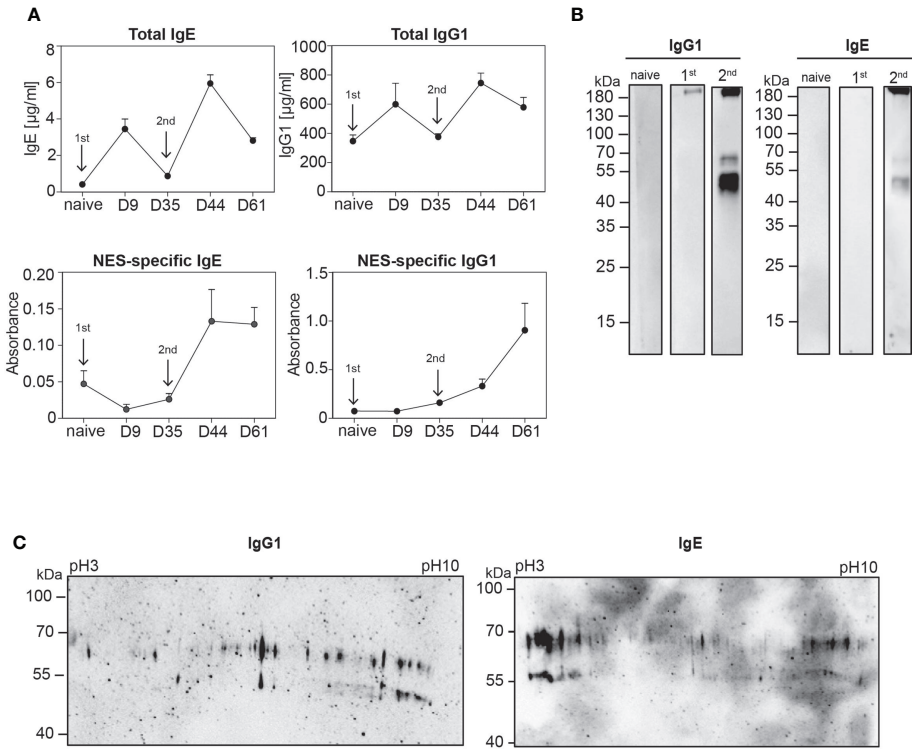

FIGURE 1
Polyclonal IgE and IgG1 antibodies from secondary *Nb*-infected mice recognise a discrete set of *Nb* antigens. **(A)** Graphs display kinetic of total (upper row) and NES-specific (lower row) serum IgE and IgG1 antibodies during the course of *Nb* infection, collected at indicated time points after primary (1st) and secondary (2nd) *Nb* infection (arrows) of wild-type mice or from naïve mice. Mean+SEM with 2-6 mice per group. **(B)** NES from L3 stage larvae were separated by standard SDS-PAGE under non-reducing conditions. Western blots were performed with serum from naïve, 1st or 2nd *Nb*-infected mice, following detection with either anti-mouse IgE or anti-mouse IgG1. **(C)** Representative 2D Western blots of NES hybridized with serum from mice after 2nd *Nb* infection, followed by detection with either anti-mouse IgE or anti-mouse IgG1.

However, after secondary infection NES-specific IgE and IgG1 antibodies were clearly detectable by ELISA (Figure 1A). Next, we performed Western blot analysis. There was no antigen-specific IgG1 or IgE response to NES detectable in the serum of naïve mice while the serum after primary *Nb* infection showed a faint staining for secretions of adult worms (L5) and preparations of whole worm homogenates at ~100 kDa and above 180 kDa (Figure 1B and Supplementary Figure S3). This may indicate that only some antigen multimers are detected by low-affinity antibodies after primary infection. However, a discrete and overlapping set of NES antigens was recognized by both IgG1 and IgE antibodies from secondary *Nb*-infected mice with a broad signal between 45 and 55 kDa and additional signals at 70 kDa and above 180 kDa (Figure 1B). Importantly, this discrete band pattern was only detectable in non-reducing conditions which preserves inter- and intramolecular disulfide bonds of the proteins. We then further separated NES proteins by size and charge using two-dimensional gel electrophoresis (2D SDS-PAGE) followed by Western blotting to reveal the complexity of the detected NES antigens. We identified several spots at about 55 or 70 kDa separated by the pH gradient suggesting that the detected antigens consist of numerous proteins with similar size but different charge (Figure 1C). Interestingly, the 70 kDa spots basically mirrored the charge-based distribution of the 55 kDa spots. This may indicate differences in glycosylation although further analysis would be required to confirm this assumption.

Identification of a new venom/allergen-like protein family in NES of L3 larvae

To further analyze NES components and identify individual antigens, we performed Liquid Chromatography Mass Spectrometry (LC-MS/MS) of eluted gel slices in the area of interest based on the Western blot analysis. We identified a total of 76 proteins, of which the top 25 most abundant proteins are listed in Figure 2A. Only three proteins showed a match with already described proteins exhibiting peptidase activity (legumain, aminopeptidase), as well as a protein disulfide-isomerase, while all others were uncharacterized proteins. Some of the uncharacterized proteins contained domains

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Mass spec ID	MW [kDa]	Gene ID	Blast annotation	Conserved domains	Intensity	NP	Nb-LSA1 family member
ID72	16,696	NBR_0001642601	n/a	DUF4983	8,3E+08	12	**Nb-LSA1a**
ID75	12,231	NBR_0001700401	n/a	none	6,8E+08	5	
ID80	22,631	NBR_0001886101	Keratin-like protein	DUF148	2,5E+08	9	
ID24	49,49	NBR_0000106401	Uncharacterized protein	Double CAP	1,2E+08	22	
ID53	54,89	NBR_0001136301	Protein disulfide-isomerase	Thioredoxin	4,9E+07	11	
ID71	27,626	NBR_0001642501	n/a	none	4,1E+07	10	**Nb-LSA1c**
ID49	60,188	NBR_0000992501	n/a	Peptidase S28	3,0E+07	6	
ID35	87,229	NBR_0000665601	n/a	Tyrosinase copper-binding	3,0E+07	6	
ID40	26,607	NBR_0000880301	n/a	none	2,7E+07	4	
ID39	47,071	NBR_0000861301	Legumain	Peptidase C13	2,1E+07	5	
ID79	14,654	NBR_0001849801	n/a	Phospholipase A2	1,9E+07	5	
ID52	247,74	NBR_0001102901	n/a	Serpin domain	1,6E+07	7	
ID70	79,952	NBR_0001636201	n/a	CUB domain	1,4E+07	4	
ID73	16,68	NBR_0001642701	n/a	none	1,4E+07	12	**Nb-LSA1b**
ID31	118,37	NBR_0000584601	n/a	Aspartic peptidase	1,1E+07	6	
ID65	21,74	NBR_0001394101	n/a	none	1,1E+07	5	
ID62	83,456	NBR_0001353101	Aminopeptidase	Peptidase M1	0,9E+07	6	
ID47	49,332	NBR_0000964701	n/a	Histidine phosphatase	0,9E+07	2	
ID68	80,705	NBR_0001554501	A2M_N_2 domain-containing	Macroglobulin, DUF599	0,8E+07	4	
ID78	44,572	NBR_0001821701	SCP domain-containing	Double CAP	0,7E+07	4	
ID20	24,652	NBR_0000724201	SCP domain-containing	Single CAP	0,7E+07	8	
ID41	22,391	NBR_0000881801	n/a	none	0,6E+07	3	
ID77	22,495	NBR_0001768801	SCP domain-containing	Single CAP	0,6E+07	7	
ID83	22,705	NBR_0002066901	ERAP1_C domain-containing	ERAP1-like (pfam11838)	0,5E+07	2	
ID36	23,595	NBR_0000724101	SCP domain-containing	Single CAP	0,4E+07	5	

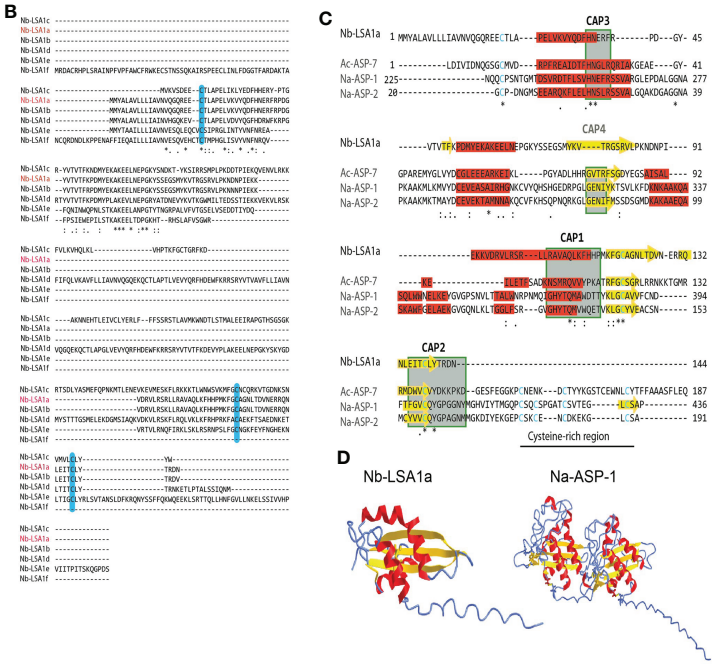

FIGURE 2
Top 25 most abundant proteins in the secretory proteins of *Nb* L3 stage larvae (NES). **(A)** Table summarizes the top 25 most abundant proteins in NES, representative for 2 separate LC-MS/MS runs. Mass spec identification numbers (ID) were assigned to distinguish between different proteins. Gene ID is taken from WormBase ParaSite database after Blast search for *Nb* genome annotations (Taxonomy ID 27835). Conserved domains were identified using PfamScan (EMBL-EBI) and CD-Search (from NCBI). Peptide intensity score is visualized by color code. MW, Molecular Weight; NP, Number of unique peptides; n/a, not available. **(B)** Multiple sequence alignment of five Nb-LSA1a paralogues. The deduced amino acid sequence of the NBR_0001642601 gene, assigned Nb-LSA1a (red letters), was aligned to its five paralogues, using Clustal Omega. The following consensus symbols are used for amino acid alignment: `*´ indicates identical alignment, `:´ indicates that substitutions are conserved, `.´ means weak similarity of substitutions; Cysteine residues are shaded in light blue. **(C)** Comparison of Nb-LSA1a sequence with selected CAP-domain proteins of known structure. Alignment of Nb-LSA1a with Ac-ASP-7 (PDB entry 3s6s), Na-ASP-1 (PDB entry 3nt8) and Na-ASP-2 (PDB entry 1u53). CAP domains 1-4 are shown in green boxes, Cysteine residues are shown in blue. The Cysteine-rich region, not present in Nb-LSA1a, is indicated by a black line. The same consensus symbols for amino acid alignment as in B were used. Sequence alignments were generated manually based on initial Clustal Omega prediction, secondary structures are shaded in red (α-helix) and yellow (β-strand) according to AlphaFold Protein Structure Prediction. **(D)** AlphaFold prediction and experimental structure for Nb-LSA1a and Na-ASP-1 with the same color-coding as in C. * indicates identitical alignment and ** or *** therefore simply means that two or three identical alignments are next to each other.

found in serine proteases (PF05577), histidine phosphatases (PF00328), copper-binding tyrosinase (PF01549), or macroglobulin (PF01835). ID68, a macroglobulin-related protein, may confer endopeptidase inhibitor activity. ID52 contains a Serpin domain (PF00079), characteristic for serine protease inhibitors whose role in nematodes is still poorly defined. ID70 contains a CUB domain (PF00431) often found in peptidases. The most frequently represented group of proteins

in the NES products belonged to the SCP/TAPS superfamily (19). This superfamily also contains members of the Venom Allergen-Like (VAL) or Ancylostoma Secreted Protein (ASP) families, which are very abundant in helminth secretions (20, 21). However, molecular targets and functions remain largely elusive. The core of helminth VALs consists of CAP domains with characteristic Cysteine-rich regions (PF00188). Blast annotation and domain analysis showed that at least five proteins contained single or double CAP-domains. Interestingly, one of the proteins (ID78) with a single CAP domain is closely related to *C. elegans* Venom-Allergen-like protein 1 (vap-1).

ID72 was the most abundant protein in all NES preparations. BLAST search against the *Nb* genome on the WormBase ParaSite database revealed that this 16,696 kDa protein with 144 amino acids (aa) is encoded by the gene NBR_0001642601 with seven exons. Additionally, this gene has 5 uncharacterized paralogues in the *Nb* genome, two of which were also detected in our LC-MS/MS analysis (Figure 2A). We termed this protein family Nb-LSA1 for "*Nippostrongylus brasiliensis* larval secreted protein 1", and assigned Nb-LSA1a to ID72. Then, Clustal Omega (22) was used to align the Nb-LSA1a protein sequence with the other 5 family members (Figure 2B). Nb-LSA1a is most closely related to Nb-LSA1b (97% protein sequence identity and same size, encoded by the gene NBR_0001642701). The protein sequence identity of Nb-LSA1a to the other family members is only 19-29%. The sizes of these proteins are: 27,6 kDa (Nb-LSA1c, encoded by NBR_0001642501), 35,8 kDa (Nb-LSA1d, encoded by NBR_0001642801), 23,1 kDa (Nb-LSA1e, encoded by NBR_0002055701) and 17,2 kDa (Nb-LSA1f, encoded by NBR_0000291501). A signal peptide motif (first 17-aa) is only present in four family members and missing in Nb-LSA1c and Nb-LSA1f.

The sequence of the initially identified protein Nb-LSA1a was then used to search for homologues in other nematode species using the SWISS-MODEL database (23). A sequence similarity of 21,15% was found for the dog hookworm protein Ac-ASP-7, and 14,29% similarity for the human hookworm protein Na-ASP-1. Although the algorithm used for conserved domain search in Figure 2A did not identify a CAP motif for Nb-LSA1a, the result of the homology analysis and the known sequence diversity of the CAP domains supported the idea that Nb-LSA1a might indeed contain a CAP domain. To investigate this more closely, Nb-LSA1a was subjected to comparative analysis with ASPs of known structure, namely Ac-ASP-7 (PDB entry 3s6s), Na-ASP-1 (PDB entry 3nt8) and Na-ASP-2 (PDB entry 1u53). By comparing the sequence and structural features, conserved CAP sequence motifs could be identified in Nb-LSA1a (Figure 2C).

The CAP motifs CAP1, CAP2 and CAP3, which are relatively well conserved between the so far known CAP domain-containing proteins, are also present in Nb-LSA1a. Sequence alignment furthermore showed that Nb-LSA1a does not contain a CAP4 motif and is also missing the cysteine-rich region. This region is only weakly conserved and is not a central component for the 3D-structure of the CAP domain. Furthermore, the most likely structure for Nb-LSA1a was generated using AlphaFold prediction algorithm (24) and compared to the known crystal structure of Na-ASP-1 which is composed of two CAP domains (Figure 2D). The accuracy of our AlphaFold model was scored as highly accurate with a predicted local distance difference test (pLDDT) >90% (Supplementary Figure S4). The arrangement of α-helices and β-strands of Nb-LSA1a clearly resembles one CAP domain of Na-ASP-1. Therefore, it appears that Nb-LSA1a is a CAP domain protein.

Recombinantly expressed Nb-LSA1a is recognized by immune serum of Nb-infected mice

To further characterize the immunogenicity of Nb-LSA1a, we expressed a C-terminally His-tagged version in HEK293T cells and first performed Western blot analysis of supernatants with anti-His antibodies. Under reducing conditions (+β-ME) Nb-LSA1a appeared as a dominant band of approximately 17 kDa (Figure 3A). However, under non-reducing conditions (-β-ME), the 17 kDa band was almost gone and three other bands at around 30-40 kDa appeared (Figure 3A). This suggests that Nb-LSA1a is actually expressed as dimer/trimer.

Next, we addressed the question whether Nb-LSA1a is indeed recognized by immune serum from *Nb*-infected mice. As expected, no bands appeared when blots were hybridized with serum from naïve mice. In contrast, serum isolated from mice after secondary *Nb* infection showed basically the same staining pattern as the anti-His antibodies (Figure 3B). ELISA analysis further revealed that Nb-LSA1a-specific IgG1 and IgE is generated in *Nb*-infected mice and both antibody levels increased after secondary as compared to primary infection (Figure 3C). Based on the strong humoral immune response against Nb-LSA1a we further investigated whether immunization of mice with Nb-LSA1a could protect against *Nb* infection.

Immunization with Nb-LSA1a elicits basophil-dependent protective immunity

To determine whether immunization of mice with Nb-LSA1a is sufficient to protect against *Nb* infection we performed experiments using a standard intraperitoneal immunization protocol with alum adjuvant (Figure 4A). In brief, mice were immunized with Nb-LSA1a or NES in alum on day 0 and 7, infected with *Nb* on day 14 and analyzed 7 days

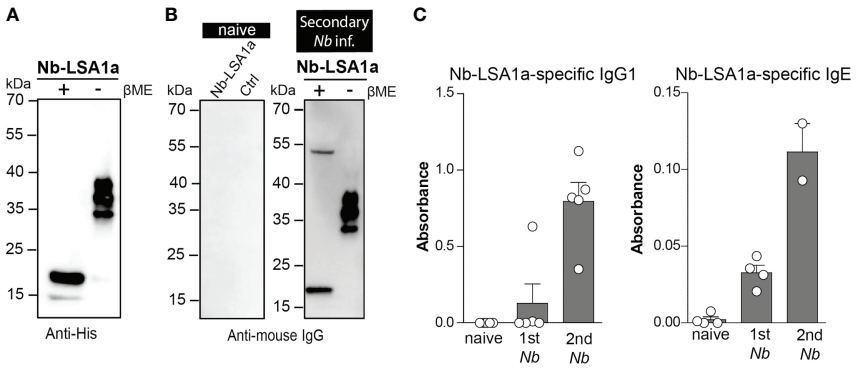

FIGURE 3
Native Nb-LSA1a forms oligomers and is detectable by serum IgG1 and IgE from secondary *Nb*-infected mice. **(A)** Detection of purified His-tagged Nb-LSA1a from supernatant of transfected HEK293T cells after SDS-PAGE under reducing (+) and non-reducing **(-)** conditions and Western blot using an anti-His-tag antibody. **(B)** Supernatant of HEK293T cells expressing Nb-LSA1-His or empty His vector (Ctrl) was analyzed by standard SDS-PAGE under reducing (+) and non-reducing **(-)** conditions and Western blotting with serum from naïve (left) or secondary *Nb*-infected mice (right), following by detection with an anti-mouse IgG antibody. **(C)** Detection of Nb-LSA1a-specific IgG1 (left) and IgE (right) in serum from naïve, primary (1st) or secondary (2nd) *Nb*-infected mice. Bars show the mean+SEM with 4-5 mice per group.

after infection. While *Nb* infection elicited similar levels of total IgG1 serum antibodies in all groups of mice, Nb-LSA1a-specific IgG1 was only present in the serum of Nb-LSA1a-immunized mice (Figure 4B, C). IgG1 in the serum of Nb-LSA1a-immunized mice also bound to NES-coated plates, which confirms that Nb-LSA1a is a prominent antigen in the whole secreted protein mixture (Figure 4D). Unexpectedly, we did not detect a significant increase of anti-Nb-LSA1a IgE (Figures 4E–G).

Importantly, Nb-LSA1a-immunized mice showed strongly reduced egg burden in fecal pellets, similar to NES-immunized mice (Figure 4H). Previous studies have shown that basophils contribute to protection against secondary *Nb* infection. Hence, we decided to compare the protective effect of Nb-LSA1a immunization in wild-type and basophil-deficient Mcpt8Cre mice (15). Egg counts in fecal pellets of Nb-LSA1a-immunized Mcpt8Cre mice were similar to egg counts from non-immunized wild-type or Mcpt8Cre mice (Figure 4I). This was not due to an impaired anti-Nb-LSA1a IgG1 response in Mcpt8Cre mice (data not shown). To further analyze whether this protective basophil-mediated effect occurs already in the skin as the first anatomical site of infection, we determined the number of larvae that reached the lung on day 2 after infection. While non-immunized wild-type or Mcpt8Cre mice contained about 200 larvae, this number was reduced to about 50 larvae only in Nb-LSA1a-immunized wild-type mice (Figure 4J).

Overall, these data indicate that immunization with Nb-LSA1a, a newly identified secreted protein of *Nb* L3 larvae, elicits a strong IgG1 response and provides basophil-mediated protective immunity against *Nb* infection mainly in the skin or before they reach the lung. This finding illustrates that secreted proteins of the free-living larval stage can have important and yet to be determined functions for migration and survival within the infected host.

Discussion

Development of efficient and safe vaccines against hookworm infections remains a major challenge. Such vaccines would reduce disease burden and ameliorate clinical conditions even if achievement of sterile immunity is probably not realistic (6, 25). Detailed understanding of the mechanisms how hookworms establish their parasitic niches and how the immune system responds to infection is detrimental to develop new vaccination strategies. Basic research using mouse models of hookworm infections such as infections of mice with *Nb* or *Heligmosomoides polygyrus (Hp)* can be helpful in this regard (26). For example, the mechanisms of worm expulsion from the intestine by IL-13-elicited and STAT6-dependent activation of goblet cells and smooth muscle cells are quite well understood (27, 28). However, details such as the role of tuft cells, ILC2s and alternatively activated macrophages are constantly emerging (12, 29, 30). In the present study, we sought to identify and characterize new *Nb*-derived antigens that elicit a humoral immune response and provide protection against *Nb* infection.

It is well established that *Nb* or *Hp* infections of mice elicit a strong germinal center response and elevations of serum IgG1 and IgE levels. However, primary infections induce an antibody response with very few somatic mutations which might explain the lack of detectable NES-specific antibodies by ELISA or Western blot (31–34). Here, we also report high levels of IgE and IgG1 in the serum of *Nb*-infected C57BL/6 mice after primary infection and a further increase after secondary infection.

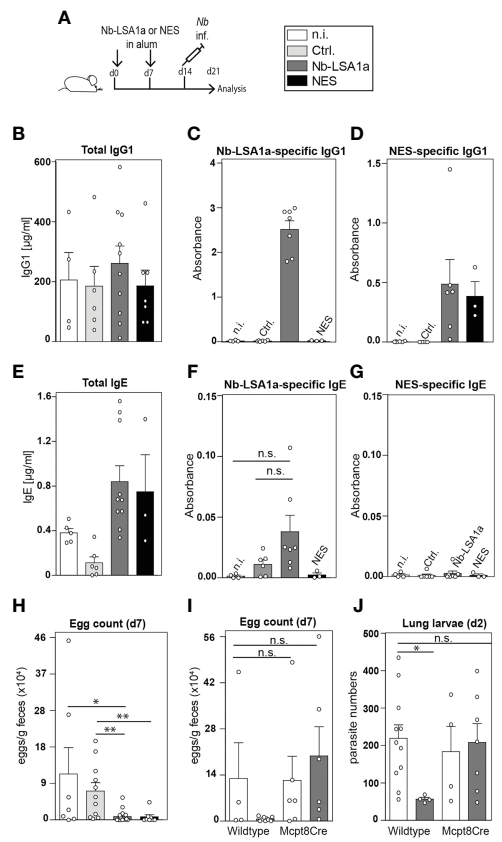

FIGURE 4
Basophil-mediated protection in Nb-LSA1a immunized mice. **(A)** Experimental setup. Mice were immunized with 1-10 µg protein (NES, Nb-LSA1a or Ctrl) in alum i.p. on day 0 and 7, infected with *Nb* on day 14 and analyzed on day 21. **(B–D)** Total **(B)**, Nb-LSA1a-specific **(C)**, and NES-specific **(D)** serum IgG1 levels of wild-type mice which had not been immunized (n.i., white bar), or immunized with supernatant of HEK293T cells transfected either with an empty vector (Ctrl., light gray bar) or Nb-LSA1a vector (dark gray bar), or immunized with NES (black bar). **(E–G)** Total **(E)**, Nb-LSA1a-specific **(F)**, and NES-specific **(G)** serum IgE levels of wild-type mice, treated as described in B-D. Calculation of mouse serum concentrations for 4 to 10 samples and absorbance calculated for 4 to 7 samples per group. **(H, I)** Fecal egg counts on day 7 post *Nb* infection. **(I)** Immunization was carried out with purification buffer (white bars) or Nb-LSA1a purified protein (dark grey bar). **(J)** Number of larvae in the lung on day 2 post *Nb* infection of wild-type or basophil-deficient Mcpt8Cre mice, immunized with purification buffer (white bars) or Nb-LSA1a purified protein (dark gray bar). Data shown are combined from four experiments with each of 3-6 individual mice per group **(H)** and two experiments with 3 **(I)** or 3-5 **(J)** mice per group. Statistical analysis was performed with Mann-Whitney U test (*P<0.05, **P<0.01). n.s. = not significant.

When analyzing the reactivity of induced antibodies towards parasite antigens (secreted as well as whole worm extracts), we only detected NES-reactive antibodies after secondary infection by ELISA.

These results indicate that *Nb*-specific IgE and IgG1 antibodies with germinal center-dependent affinity maturation are only induced after repeated infections. This assumption is also supported by a previous study which reported the identification of a *Nb*-derived antigen recognized by a monoclonal IgE antibody without somatic mutations (32). McCoy et al. further demonstrated that primary *Hp* infection is accompanied by production of antibodies with irrelevant specificities while parasite-specific antibodies only arise after multiple infections (34). One might assume that complex pathogens such as helminths express a large variety of antigens. However, we repeatedly detected a very restricted set of antigens in NES of L3 larvae that was recognized by antibodies from secondary *Nb*-infected mice at a size around 55 or 70 kDa.

When analyzing the antigen specificity of polyclonal antibody response for *Hp* infections it has been shown before that HES elicits an antibody response directed against restricted glycan and peptide epitopes (35). Interestingly, in line with our findings they also observed that this response is directed at secreted, rather than whole worm products. Immunization with three secreted SCP/TAPS proteins of adult *Hp* worms elicited protective IgG1-dependent but basophil-independent immunity by more efficient larval trapping in the submucosa of the small intestine (36). We used NES from L3 stage larvae, the infective larval stage, to screen for serum reactivity because we reasoned that humoral immunity against the first encountered antigens secreted by L3 larvae during skin invasion could lead us to identification of critical proteins required for successful parasitism.

Using LC-MS/MS analysis, we identified a new subfamily of SCP/TAPS proteins with 6 members in the *Nb* genome (Nb-LSA1a-f). Interestingly, the SCP/TAPS superfamily, members of which are also named VAL and ASP proteins, is very abundant in the human hookworm *N. americanus* and other parasitic nematodes but not in free-living nematodes (5). Previous proteomic analysis that compared the secretome of L3 larvae and adult worms from *Nb* already noticed the abundance of SCP/TAPS proteins in the secretomes (37).This suggests that SCP/TAPS proteins play a role in host infection and/or evasion from rapid elimination by the immune system. However, the biological functions and properties of these proteins remain elusive (4).

Nb-LSA1a was the most abundant protein with the highest signal intensity in all LC-MS/MS runs. Nb-LSA1a shares 21% sequence identity with *A. caninum* Ancylostoma-secreted protein (Ac-ASP-7) and 14% sequence identity with *N. americanus* Ancylostoma-secreted protein 1 (Na-ASP-1). The basis for development of vaccines was set in the field of canine hookworm infections. Here, the discovery that radiation-attenuated *A. caninum* L3 larval vaccine protected against challenge infection led to identification of the *Ancylostoma*-secreted proteins (ASPs) which belong to the SCP/TAPS superfamily (38). Such ASP proteins from *N. americanus* turned out to be a promising class of antigens from infective L3 larvae and were tested as potential human anti-hookworm vaccines (39). One potential vaccine candidate was indeed Na-ASP-2 that provided significant protection against challenge infections but at the same time data from a clinical trial in a hookworm-endemic area showed that it resulted in generalized

IgE-elicited urticarial reactions (40). In fact, Na-ASP-2-specific IgE is readily detectable in serum of people living in endemic areas. Therefore, Na-ASP-2 was not further considered and other vaccine candidates are currently under investigation, especially a combination vaccine with Na-GST-1, a glutathione-S-transferase, and Na-APR-1, a aspartic protease modified to lack protease activity (6). More recently, a phase I trial with ultraviolet C (UVC)-attenuated *N. americanus* L3 larvae was successfully completed (41). However, vaccination with defined recombinantly expressed proteins has obvious advantages with regard to vaccine production at large scales.

Our study shows that immunization of mice with Nb-LSA1a elicits a strong antigen-specific IgG1 response but no detectable antigen-specific IgE. This was surprising because anti-Nb-LSA1a IgE is clearly detectable in serum of *Nb* infected mice. One explanation would be that the quality of the humoral immune response elicited by immunization versus infection is different. Alternatively, the larger amounts of IgG1 antibodies in the serum of immunized mice may cover all epitopes on Nb-LSA1a and thereby prevent binding of IgE antibodies in ELISA and Western blot analysis. In any case, the *Nb* infection of immunized mice did not result in severe local or systemic allergic reactions. It has been shown before, that IgG1 antibodies activate macrophages during vaccination or infection of mice with the helminth *Heligmosomoides polygyrus bakeri* and these macrophages probably contribute to protection (36, 42). Therefore, we will further investigate whether Nb_LSA1a-specific IgG1 activates macrophages in the skin which could be one component of protective immunity. Importantly, the transition of L3 larvae from skin to lung was severely impaired in immunized mice and this protective effect was lost in basophil-deficient mice. Basophils have been recognized before to confer protection in the skin against secondary *Nb* infection (9). However, the critical antigens that elicit basophil-mediated protection in the skin remained unclear. We fill this gap of knowledge by showing that immunization with a single secreted protein, Nb-LSA1a, is sufficient to strongly reduce larval migration to the lung in a basophil-dependent manner. As a consequence this effect resulted in severely reduced fecal egg counts. Basophils are a major source of vasoactive substances, proteases, lipid mediators, chemokines, and Th2-associated cytokines such as IL-4 and IL-5 that promote accumulation of alternatively activated macrophages (AAM) and eosinophils in the skin (9, 43). Further studies are needed to characterize the function of basophils in human skin and to identify new secreted antigens from L3 stage larvae of human hookworms that elicit a strong IgG1 and a weak IgE response. Development of efficient hookworm vaccines that prevent larval migration from skin to lung seems possible and would provide a great benefit for millions of people living in hookworm-endemic regions.

Data availability statement

The data presented in the study have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the identifier PXD035568. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Government of Lower Franconia.

Author contributions

NT and DV designed experiments. NT, DD, LR, DM and DR performed experiments. NT, DR, LR, BW and HS analysed data. DV and BW acquired funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.979491/full#supplementary-material

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IgE receptor responsiveness of basophils in chronic inducible urticaria

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Introduction: Chronic inducible urticaria (CIndU) is a subgroup of chronic urticaria induced by a specific stimulus. We evaluated basophil characteristics in patients with CIndU and compared with those in patients with chronic spontaneous urticaria (CSU) and healthy controls (HCs).

Methods: Blood was collected from patients, and a basophil activation test (BAT) was performed. Basophil responsiveness and surface marker expression in patients with CIndU were compared with those in patients with CSU and HCs. For some patients with CIndU, blood was collected before and after wheals were induced. In these cases, we compared the responsiveness of basophils before and after the appearance of wheals.

Result: HCs (n=23) and patients with CIndU (n=24) or CSU (n=38) were enrolled in the study. The degree of basophil activation at steady state in patients with CIndU was higher than in HCs. Basophil responsiveness *via* high-affinity IgE receptor (FcεRI) stimulation with anti-IgE or anti-FcεRI antibody in patients with CIndU was equivalent to that in HCs, and higher than that in patients with CSU. No abnormalities in IgE and FcεRI expressions on the surface of basophils in patients with CIndU were observed. When we induced wheals in some patients with CIndU and performed a BAT before and after the appearance of wheals, no significant changes were found.

Conclusion: Peripheral blood basophils in CIndU were slightly activated at steady state, but no abnormalities in basophil responsiveness. In future, a higher number of cases should be enrolled to confirm the role of basophils and refine therapeutic targets for CIndU.

KEYWORDS

chronic inducible urticaria, chronic spontaneous urticaria, basophil activation test, anti-IgE-induced histamine release, responsiveness of basophils *via* high-affinity IgE receptor

Abbreviations: CIndU, Chronic inducible urticaria; CSU, Chronic spontaneous urticaria; FcεRI, High-affinity IgE receptor; HCs, Healthy controls; BAT, Basophil activation test; CholU, Cholinergic urticaria; UCT, Urticaria control test.

Introduction

Chronic urticaria is defined as the occurrence of wheals and/or angioedema for more than 6 weeks. Chronic inducible urticaria (CIndU) is a subgroup of chronic urticaria where recurrent pruritic wheals and/or angioedema are induced by a specific stimulus (1). Chronic spontaneous urticaria (CSU) is characterized by the spontaneous appearance of wheals, angioedema, or both and is associated with known (i.e., mast-cell activating autoantibodies) or unknown causes (2) (3) (4). Several studies reported that the responsiveness of basophils *via* the high-affinity IgE receptor (FcεRI) and IgE pathways was significantly lower in active CSU compared with healthy controls (HCs), with basophil hyporesponsiveness improving during disease remission (5–7). (8) In contrast, anti-IgE-induced histamine release from the basophils of five patients with cold urticaria, a subtype of CIndU, appeared to be equivalent to that of HCs (9). Basophil FcεRI expression was significantly higher in patients with CSU and CIndU compared with HCs (10, 11). However, there have been few reports on the characteristics of basophils in patients with CIndU. Here, we evaluated the characteristics of basophil in patients with CIndU, including responsiveness and surface marker expression, and compared them with those in patients with CSU.

Materials and methods

Study design

An observational study was conducted on patients with CIndU who visited the Dermatological Institute of Kobe University Hospital. Blood was collected from patients diagnosed with CIndU at the time of non-stimulation (when no wheal was present), and a basophil activation test (BAT) was performed. The basophil responsiveness and surface marker expressions of patients with CIndU were compared with those of patients with CSU and HCs. Moreover, in some patients with CIndU, urticaria was induced by a specific physical stimulus in the doctor's office, and blood was collected before and after wheals were induced. In these cases, we compared the responsiveness of basophils before and after the appearance of wheals.

Study population

Patients with CIndU and CSU who visited the Department of Dermatology, Kobe University Hospital, agreed to participate in the study, and met the inclusion criteria were enrolled. The study protocol was approved by the Kobe University Institutional Review Board (No. 180186). Inclusion criteria were to be diagnosed with CIndU or CSU by the following items and not to use omalizumab or steroids. Patient with cholinergic urticaria (CholU) are diagnosed by having wheals induced by exercise and/or passive heating (warm bath). Patients with solar urticaria are diagnosed by having wheals induced by exposure to visible and/or ultraviolet light. CSU is diagnosed as wheals that recur for more than 6 weeks without an identifiable cause. HCs were enrolled from healthy adult volunteers without urticaria symptoms and no history of urticaria. Patients treated with omalizumab and oral steroids were excluded at entry. It was set so that no patients were excluded after inclusion.

Basophil activation test

Whole blood (up to 2 mL) was taken from patients with CIndU, CSU, and HCs using ethylenediaminetetraacetic acid-containing blood collection tubes and assays were performed within 24 hours of blood sampling. An Allergenicity Kit (Beckman Coulter, Fullerton, CA, USA) was used to quantify basophil CD203c expression according to the manufacturer's instructions (12). The BAT based on CD203c expression was performed as previously described (6) (7). In addition to CD203c, CD63 (H5C6; BioLegend, San Diego, CA) was also analyzed as an activation marker that reflects histamine release (13). Basophil samples were measured by flow cytometry (FACS Verse; BD Biosciences, San Jose, CA). As previously described, the gating technique is shown in the Supplementary Material (6) (Figure S1). Basophil activation conditions were determined by the mean fluorescence intensity (MFI). CD203c or CD63 expression after anti-IgE (E124-2-8D; Beckman Coulter, Fullerton, CA, USA) or anti-FcεRI antibody (CRA1; BioAcademia, Osaka, Japan) stimulation was presented as the CD203c or CD63 response ratio, respectively, and used to calculate the responsiveness of basophils. The response ratio was calculated by dividing the stimulation MFI by the baseline MFI. In addition, the results of anti-IgE antibody stimulation were also expressed as the percentage of CD63 positive basophils. The percentage of CD63 positive basophils were determined using a threshold defined as the expression level above which only 5% of basophils in the negative control sample fluoresce, on average.

Measurement of IgE and FcεRI levels of basophils

Basophils were incubated with VioBlue-binding, anti-IgE antibody (clone: MB10-5C4) (Miltenyi Biotec, Bergisch

Gladbach, Germany), biotinylated anti-FcεRI antibody (clone: CRA1) (BioAcademia) and APC-Streptavidin (BD Biosciences, Franklin Lakes, NJ) (1.8 mg/mL) which used as a second-step reagent for the anti-FcεRI antibody and, analyzed by flow cytometry. The measurement of the IgE and FcεRI levels of basophils and FlowJo analysis were performed as for the BAT after anti-IgE and CRA1 antibody stimulation. IgE and FcεRI levels were evaluated as the MFI.

Urticaria control test

The total score for the Urticaria control test (UCT) was determined by the patient (14). The UCT is a simple, validated, four-item questionnaire that can be used for CSU and CIndU to assess the impact of urticaria symptoms on morbidity, quality of life, and quality of treatment over the past four weeks.

Autologous serum skin test

The autologous serum skin test was performed according to established methods (15). Samples of autologous serum (0.05 mL) were injected intradermally into the volar aspect of the forearm of each subject. The diameters of wheals and erythema were measured after 15 minutes. Reactions were assessed as positive if the diameter of the wheal induced by serum was equal to or larger than 6 mm.

Statistical analysis

The Kruskal-Wallis test with Dunn's multiple comparisons test was used for the statistical comparison of three groups with nonparametric variables. The Wilcoxon test was used for the statistical comparison of two groups with nonparametric variables. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Two-sided P values <0.05 were considered statistically significant.

Results

Study population

Patients with CIndU (n=24) and CSU (n=38), and HCs (n=23) who agreed to participate in this study were enrolled at the Dermatological Institute of Kobe University Hospital (Table 1). Patients with CIndU included 7 males and 17 females. The mean age was 40.2 years and the median duration of illness was 10.0 years. The median total serum IgE was 579.5 IU/mL. Patients with CSU included 24 males and 14 females. The mean age was 46.3 years. (Table 1). HCs included 7 males and 16 females. The mean age was 35.0 years. CIndU patients included 21 with CholU and 3 with solar urticaria. Five patients with CholU underwent a bathing provocation test and exercise provocation test followed by blood collection (even when wheals were induced) and a BAT. The BAT of these

TABLE 1 Clinical and laboratory characteristics of patients with chronic inducible urticaria (CIndU) and chronic spontaneous urticaria (CSU).

Demographics characteristics of patients with CIndU and CSU	CIndU (n=24)	CSU (n=38)	P values
Age, years	40.2 ± 10.3	46.3 ± 16.2	P=0.0239
Female, n (%)	17 (70.8%)	24 (63.1%)	P=0.5339
Disease duration, years	10.0 (1.0-40)	4.0 (0.2-33)	P=0.046
Total IgE (IU/mL)	579.5 (14.2-1275.3)	139.5 (3-4392)	P<0.0001
Basophil count (cell/μL)	68 (18-106)	52.5 (21.4-114)	P=0.9485
UCT	10.7 ± 3.8	7.8 ± 4.1	P=0.0253
ASST positive rate, n (%)	11/16 (68.7%)	7/17 (41.1%)	P=0.1663
Presence of angioedema at baseline, n (%)	7 (29.1%)	1 (2.6%)	P=0.0041
Treatment, n (%)			
H1 antihistamines at the conventional dosage	17 (70.8%)	19 (50%)	P=0.1886
H1 antihistamines at high dosage	5 (20.8%)	14 (36.8%)	P=0.2599
History, n (%)			
Asthma	8 (33.3%)	5 (13.1%)	P=0.1067
Allergic rhinitis	4(16.6%)	2 (5.2%)	P=0.1949
Atopic dermatitis	10 (41.6%)	1 (2.6%)	P=0.0002
Pollinosis	3 (12.5%)	2 (5.2%)	P=0.3459

ASST, Autologous serum skin test; UCT, Urticaria control test.
Data are given as the mean ± standard deviation for age, UCT; n (%) for sex, ASST positive rate, presence of angioedema, treatment, and history; and median (range) for disease duration, serum total IgE, and basophil count.

patients was compared before and after the appearance of wheals.

Measurement of CD203c, CD63, IgE and FcεRI levels on basophils at steady state in patients with CIndU, CSU, and HCs

First, we examined CD203c, CD63, FcεRI and IgE expression levels on basophils at steady state in patients with CIndU, CSU, and HCs. The expression of CD203c on basophils in patients with CIndU was significantly higher compared with CSU and HCs (Figure 1A). The expression of CD63 on basophils in patients with CIndU was significantly higher compared with HCs and was comparable with CSU (Figure 1B). The expression of FcεRI on basophils in patients with CIndU was comparable with HCs and was significantly lower than that in CSU (Figure 1C). There were no significant differences in the levels of cell-bound IgE among these three groups (Figure 1D).

Measurement of CD203c and CD63 expressions after anti-IgE or FcεRI stimulation of basophils in patients with CIndU, CSU, and HCs

Next, we analyzed the expressions of the activation markers CD203c and CD63 with anti-IgE or FcεRI stimulation in patients with CIndU, CSU, and HCs to examine basophil reactivity *via* FcεRI. When peripheral blood basophils were stimulated with anti-IgE antibody, the upregulation of CD203c expression on basophils in patients with CIndU was comparable with HCs and was significantly higher than that in CSU (Figure 2A). When peripheral blood basophils were stimulated with anti-FcεRI, the upregulation of CD203c expression on basophils in patients with CIndU was comparable with HCs and was significantly higher than that in CSU (Figure 2B). When peripheral blood basophils were stimulated with anti-IgE antibody, similar results were obtained when the detection activation marker was also CD63 (Figure 2C). The percentage of CD63 positive basophil also showed similar results when

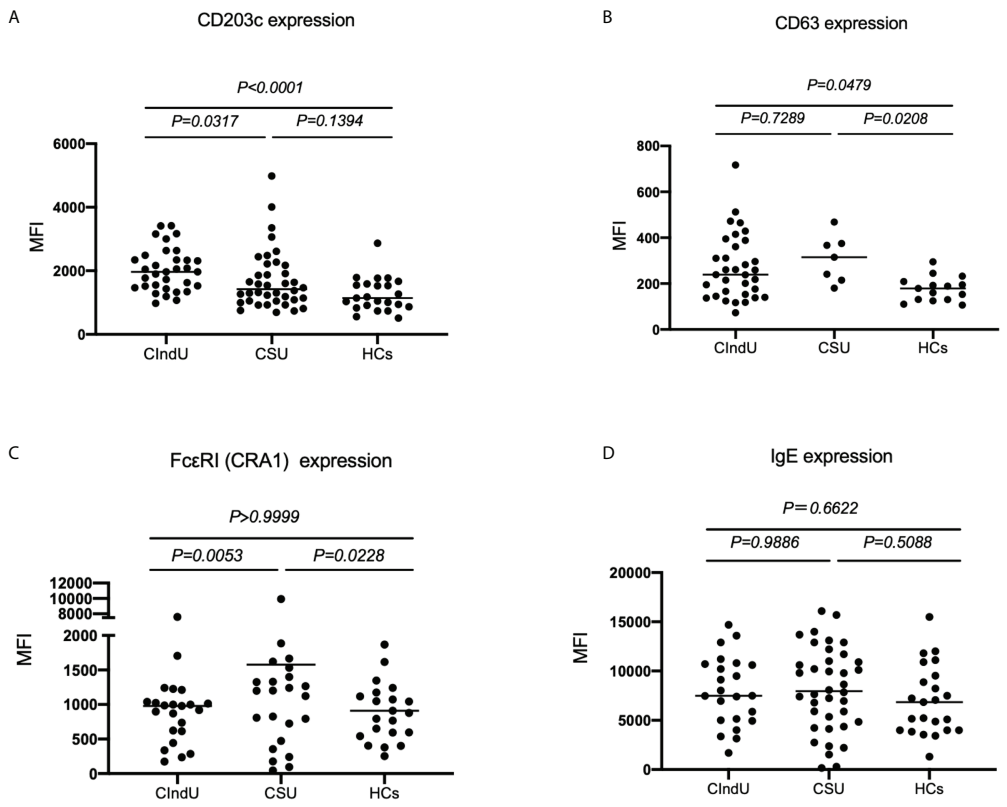

FIGURE 1
CD203c, CD63, IgE and FcεRI levels at steady state. **(A)** CD203c expression on basophils, **(B)** CD63 expression on basophils, **(C)** FcεRI expression on basophils and **(D)** IgE expression on basophils at steady state. Statistical analysis was carried out using the Kruskal-Wallis test with Dunn's multiple comparisons test.

peripheral blood basophils are stimulated with anti-IgE antibody (Figure 2D).

Measurement of CD203c, CD63, IgE and FcεRI levels on basophils at steady state in patients with CholU as a subgroup of CIndU before and after the appearance of wheals

Thirdly, we examined CD203c, CD63, FcεRI and IgE expression levels on basophils at steady state in patients with CholU before and after the appearance of wheals. There were no significant differences in the CD203 expression on basophils (Figure 3A), CD63 expression on basophils (Figure 3B), FcεRI expression on basophils (Figure 3C) and in the levels of cell-bound IgE on basophils (Figure 3D) before and after the appearance of wheals.

Measurement of CD203c and CD63 expressions after anti-IgE or FcεRI stimulation of basophils in patients with CholU as a subgroup of CIndU before and after the appearance of wheals

Finally, we analyzed the expression of the activation markers CD203c and CD63 with anti-IgE or FcεRI stimulation of basophils in patients with CholU before and after the appearance of wheals to examine basophil reactivity *via* FcεRI. When peripheral blood basophils were stimulated with anti-IgE antibodies, the upregulation of CD203c expression on basophils after wheals appeared were equivalent to that before wheals appeared (Figure 4A). When peripheral blood basophils were stimulated with anti-FcεRI, the upregulation of CD203c expression on basophils after wheals appeared was equivalent to that before wheals appeared (Figure 4B). When peripheral blood basophils were stimulated with anti-IgE antibodies,

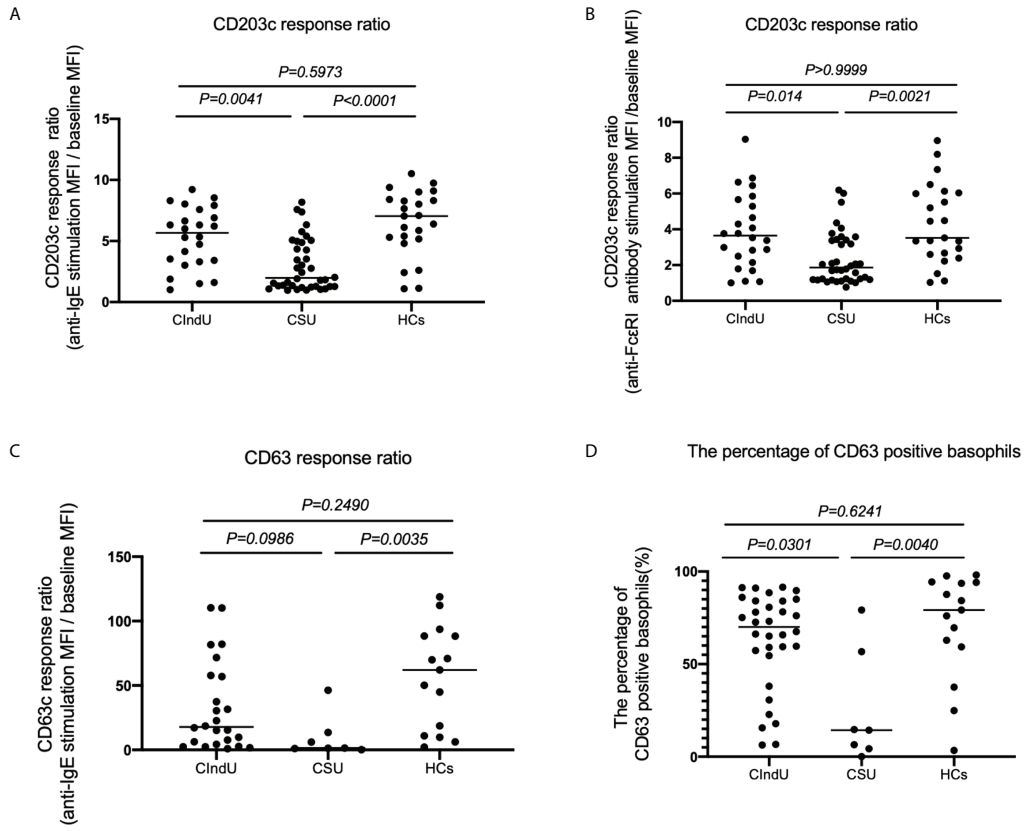

FIGURE 2
CD203c response ratio, CD63 response ratio and the percentage of CD63 positive basophils. CD203c response ratio of basophils when stimulated with **(A)** anti-IgE or **(B)** anti-FcεRI antibody. **(C)** CD63 response ratio of basophils when stimulated with anti-IgE, and **(D)** the percentage of CD63 positive basophils when stimulated with anti-IgE. Statistical analysis was carried out using the Kruskal-Wallis test with Dunn's multiple comparisons test.

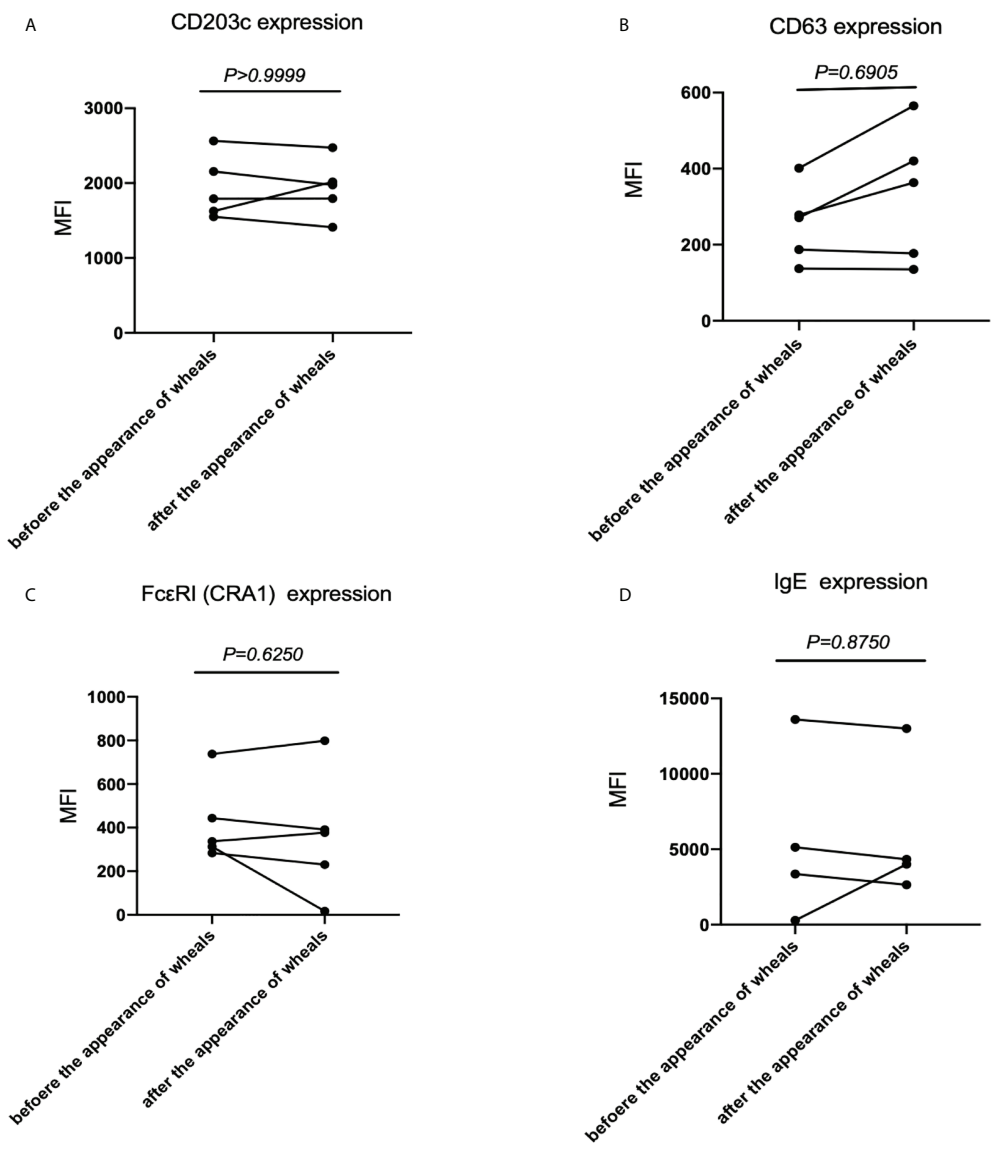

FIGURE 3
CD203c, CD63, IgE and FcεRI levels at steady state before and after the appearance of wheals in patients with CholU as a subgroup of CIndU. Comparison of **(A)** CD203c expression on basophils, **(B)** CD63 expression on basophils, **(C)** FcεRI expressions on basophils, and **(D)** IgE expressions on basophils before and after the appearance of wheals. Statistical analysis was performed by Wilcoxon test.

similar results were obtained when the detection activation marker was also CD63 (Figure 4C). The percentage of CD63 positive basophil also showed similar results when peripheral blood basophils are stimulated with anti-IgE antibody before and after the appearance of wheals. (Figure 4D).

Discussion

In this study, we focused on the characteristics related to the steady state of basophils, FcεRI-mediated responsiveness, and expression of IgE-related molecules in patients with CIndU. The degree of basophil activation at steady state in patients with CIndU was higher than in HCs. And then basophil responsiveness *via* FcεRI stimulation with anti-IgE or anti-FcεRI antibody in patients with CIndU was equivalent to that with HCs, and higher than that with CSU. In addition, no abnormalities were observed for the IgE and FcεRI expressions on the surface of basophils in patients with CIndU. In addition, When we induced wheals in patients with CholU and performed a BAT before and after the appearance of wheals, no significant changes were found.

Basophils in patients with severe CSU might be mildly activated by autoantigens or autoantibody-related IgE pathways in the blood and persistently release small amounts of histamine (16). As a result, basophils in patients with CSU were exhausted and their responsiveness *via* FcεRI was low (6) (7). In contrast, this study revealed that steady-state basophils in patients with CIndU had higher CD203c and CD63 than HCs, but there were no abnormalities in the responsiveness of basophils to stimulation with anti-IgE or FcεRI antibodies. These findings indicate that basophils at steady state in CIndU patients may be weakly self-activated by unknown mechanism, whereas the basophil responsiveness in CIndU patients is not abnormal. In CholU as a subgroup of CIndU, FcεRI-mediated responsiveness of peripheral blood basophils and expression of FcεRI and IgE did not change significantly before and after the appearance of the wheals.This FcεRI-mediated responsiveness of basophils and absence of abnormalities related to surface markers in CholU as a representative of CIndU may indicate a minor role of basophils in the pathogenesis in CIndU compared with CSU. It makes sense that basophils, which are mainly present in blood vessels, play a minor role in CIndU. This can be because sweat that leaks into the dermis from sweat ducts in CholU and serum-derived factors that are changed by sunlight reaching the dermis in solar urticaria are

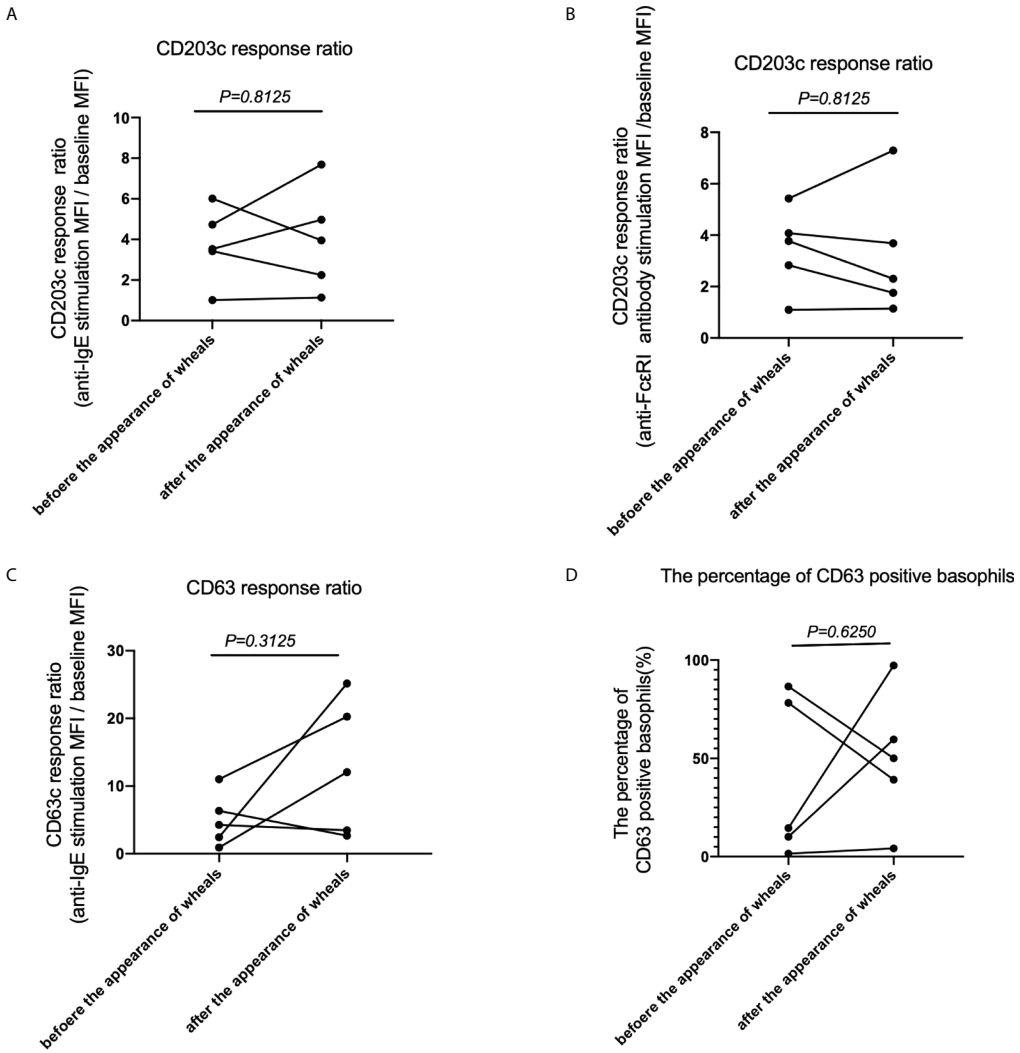

FIGURE 4
CD203c response ratio, CD63 response ratio and the percentage of CD63 positive basophils before and after the appearance of wheals in patients with CholU as a subgroup of CIndU. Comparison of CD203c response ratios of basophils when stimulated with **(A)** anti-IgE or **(B)** anti-FcεRI antibody before and after the appearance of wheals. Comparison of **(C)** CD63 response ratios of basophils when stimulated with anti-IgE and **(D)** the percentage of CD63 positive basophils when stimulated with anti-IgE before and after the appearance of wheals. Statistical analysis was performed by Wilcoxon test.

highly likely to act as allergens that induce urticaria in the dermis, respectively.

Our result regarding the expressions of FcεRI and IgE on peripheral blood basophils is different from a previously reported result (11). This difference might be related to the high proportion of patients with CholU in our study. The statistical differences between CSU and CIndU in the presence of total IgE and baseline angioedema, and the history of atopy, might be associated with our high proportion of CIndU patients with CholU. In addition, there is a significant difference in the disease duration between CIndU and CSU. Differences in disease duration affected the responsiveness of basophils in patients with CSU (6), but no abnormalities in the responsiveness of basophils were observed in patients with CIndU, regardless of the short or long disease duration. Therefore, we believe that the difference in disease duration between CSU and CIndU does not affect the difference in basophil responsiveness between the two. The significantly higher expression of CD203c and CD63 on basophils at steady state in CIndU patients compared to HCs may also be influenced to the higher proportion of CholU complicated by AD. Indeed, we previously reported higher CD203c and CD63 expression on basophils at steady state in AD patients (17). Therefore, a population that does not differ statistically should be analyzed. There were several study limitations including the small number of cases and low diversity of disease subtypes in CIndU. In future studies, a higher number of cases should be enrolled to confirm the role of basophils and refine therapeutic targets for CIndU.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board of Kobe University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MM and AF conceived the idea of the study. MM, YO, and SI developed the statistical analysis plan and conducted statistical analyses. MM and AF contributed to the interpretation of the results. AF, KW, and TF supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically for intellectual content. All authors approved the final version of the manuscript to be published.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.995596/full#supplementary-material

FIGURE S1
Flow cytometry data analysis. On the FSC/SSC plot **(A)**, the basophil scatter gate and leukocyte gate are defined. On the CD3-PE-Cy7/SSC plot **(B)**, the CD3 negative population is defined. On the CRTH2-FITC/CD203c-PE plot **(C)**, both CRTH and CD203c positive groups are defined as basophils. The gating basophils on the CRTH2-FITC/CD203c-PE plot are non-activated basophils **(C)** and the gating basophils on the CRTH2-FITC/CD203c-PE plot are activated basophils with anti-IgE **(D)** The gating basophils on the CRTH2-FITC/CD63-Pacific Blue are non-activated basophils **(E)** and the gating basophils on the CRTH2-FITC/CD63-Pacific Blue are activated basophils with anti-IgE **(F)** FITC, Fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; PE-Cy7, PE-cyanine 7; SSC, side scatter.

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Basophils activation of patients with chronic spontaneous urticaria in response to C5a despite failure to respond to IgE-mediated stimuli

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Urticaria is characterized by the occurrence of wheals and flares in response to vasoactive mediators, such as histamine. Various studies have suggested the involvement of basophils in the pathogenesis of chronic spontaneous urticaria (CSU). However, histamine release from peripheral basophils in response to stimuli acting on the high affinity IgE receptor (FcεRI) is impaired in many patients with CSU (non/low responders). We previously demonstrated that tissue factor (TF)s expressed on vascular endothelial cells in response to a combination of various stimuli, such as that of histamine and lipopolysaccharide (LPS), activates the extrinsic coagulation pathway and produces anaphylatoxin, complement 5a (C5a), which then activates basophils and mast cells *via* the C5a receptor (C5aR). We have revealed that histamine release was induced in response to C5a and formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP), regardless of the response to anti-IgE antibody, the reduced numbers of basophils and severity of urticaria. Moreover, we found that spontaneous release of histamine *ex vivo* from basophils of patients with CSU is higher than that from healthy individuals. These results suggest that basophils and the complement system, which could be activated by coagulation factors, may play a critical role in the pathogenesis of CSU, especially in cases refractory to treatment involving the IgE/FcεRI pathway.

KEYWORDS

peripheral basophils, IgE, complement, chronic spontaneous urticaria (CSU), histamine

Introduction

Chronic spontaneous urticaria (CSU), also called as chronic idiopathic urticaria (CIU), is a skin disorder characterized by daily or almost daily recurring wheals and flares, with itch occurring anywhere on the body for more than 6 weeks. The formation of wheals and flares are induced by chemical mediators, especially histamine, which may be released from mast cells and basophils (1). Generally, basophils and mast cells express high affinity IgE receptors (FcεRIs) on the plasma membrane surface and bind antigen-specific IgEs to FcεRIs. When specific antigen binds to IgEs on the surface of cells, basophils and mast cells are activated and release inflammatory mediators, such as histamine, followed by an increase of vascular permeability and edema formation. Several reports suggest that 30-50% of patients with CSU have IgG autoantibodies against IgE antibody and/or FcεRI (2, 3). Moreover, IgE autoantibodies against endogenous molecules, such as dsDNA, interleukin (IL)-24, tissue factor (TF) and thyroid peroxidase (TPO), have also been detected in a certain population of patients with CSU (3). Furthermore, basophils migrate from blood vessels into the skin during wheal formation, and are suggested to contribute to the persistence of wheals in CSU (4). Rapid effect of omalizumab, an anti-IgE monoclonal antibody for the treatment of CSU also shows the importance of FcεRI-dependent activation of basophils rather than mast cells (5). However, the number of peripheral blood basophils and the histamine releasing activities of peripheral basophils of healthy donors and patients with CSU in response to anti-IgE antibody (anti-IgE), an activator of the IgE-FcεRI pathway, are significantly decreased (non- or low-responder) (6) (7). These features of basophils could be explained by the activation of FcεRI on basophils either spontaneously or by endogenous stimuli. However, histamine releasing activities of IgG antibodies against IgE and/or FcεRI or IgE antibodies against autoantigens in patients with CSU shown *in vitro* have not been demonstrated to fully activate basophils by themselves in patients with CSU *in vivo*, even in severe cases (2, 8). Moreover, the presence of such autoantibodies is not detected in more than a half of patients with CSU, and the expression of FcεRI on basophils may be scant especially in patients with CSU refractory to omalizumab treatment (9). Therefore, how peripheral basophils are activated and release histamine in patients with CSU, especially in non- or low-responders, has been largely unclear. To date, we have revealed a relationship between TF expression, activated coagulation factors and complement factors. Treatment of vascular endothelial cells with histamine released from human peripheral basophils or VEGF together with several proinflammatory molecules, such as lipopolysaccharide (LPS), tumor necrosis factor (TNF)α, IL-1β or IL-33, synergistically increase TF expression on endothelial cells (10). High expression of TFs on the cell surface then activates the extrinsic coagulation pathway and produces active forms of coagulation factors, such as factor (F)Xa and FIIa (thrombin), resulting in inter-cellular gap formation of vascular endothelial cells *via* protease activated receptor 1 (PAR1). Moreover, Asero and our group reported that plasma levels of prothrombin fragment 1 + 2 (PF_{1+2}) and D-dimer in patients with CSU are higher compared to normal controls, and correlate with disease severities (11, 12). Furthermore, the extrinsic coagulation potential is elevated in patients with CSU (13). Of note, we revealed that an active form of complement 5 (C5a), produced by the activated coagulation factors or plasmin, induces histamine release from basophils of healthy donors and skin mast cells *via* the C5a receptor (C5aR) (3) (14). In fact, the increase of plasma C5a concentration was reported in patients with CSU (15). Moreover, several reports suggest that anticoagulant drugs, such as heparin or warfarin are effective for the treatment of CSU (16, 17). These reports imply that basophils trigger and/or promote the activation of the coagulation pathway and subsequently-produced C5a plays a major role in the pathogenesis of CSU. Previously, Zuberbier, et al. revealed that basophils of patients with CSU pre-treated with interleukin-3 (IL-3) release histamine in response to C5a, but not to anti-IgE (18). On the other hand, Luquin et al. reported that histamine release from basophils of patients who suffered from CSU without basopenia, was less than that from basophils of healthy controls (19). Moreover, Vasagar, et al. reported that basophils isolated from patients with CSU in base line conditions by density fractionation express higher in CD63 but normal in CD203c as compared with healthy controls (20). In this study, we obtained basophil-enriched leukocytes fractions from patients with CSU with different disease severity and assessed their histamine release in response to anti-IgE, C5a and formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP) stimulation in a non-IL-3 treated condition. The levels of spontaneous release of histamine from leukocyte fractions, plasma histamine, and basophil activation markers, CD203c and CD63 in patients with CSU were also compared with those of healthy controls.

Abbreviations: CSU, chronic spontaneous urticaria; FcεRI, the high affinity IgE receptor; TF, tissue factor; LPS, Lipopolysaccharide; C5a, complement 5a; C5aR, C5a receptor;; fMLP, formyl-l-methionyl-l-leucyl-l-phenylalanine; CIU, chronic idiopathic urticaria; IL, interleukin; TPO, thyroid peroxidase; TNF, tumor necrosis factor; F, factor; PAR1, protease activated receptor 1; $PF_{1+2,}$ prothrombin fragment 1 + 2; HSA, human serum albumin; CD, cluster of differentiation; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; AD, atopic dermatitis; 7-day Urticaria Activity Score, UAS7; urticaria control test, UCT; NS, not significant.

Methods

Reagents and instrument

The chemicals used in this study were obtained from the following sources: human serum albumin (HSA) and fMLP from

Sigma–Aldrich Japan (Tokyo, Japan). Anti-human IgE antibody from BETYL (Montgomery, TX). Ficoll-Paque Plus was from GE Healthcare Japan Corporation (Tokyo, Japan). Reverse-phase HPLC was from Shimadzu (Kyoto, Japan). Fluorescence labeled anti-cluster of differentiation (CD)63 antibody was from Biolegend (San Diego, CA). C5a were from R&D Systems Inc. (Minneapolis, MN). Allergenicity® kit was from Beckman Coulter, Inc. (Brea, CA). Single cell fluorescence levels were analyzed by fluorescence-activated cell sorting by Attune™ Acoustic Focusing Cytometer (Life technologies, Carlsbad, CA, USA). Basophil number in the blood was counted by automated complete blood cell counter (Sysmex Japan, Tokyo, Japan)

Measurement of histamine

Leukocytes including basophils were obtained from peripheral blood as described in our previous paper (14). Briefly, fresh blood was obtained with ethylenediaminetetraacetic acid (EDTA) from each donor by venipuncture. The whole blood was mixed with the same volume of 1% methylcellulose in saline and then allowed to stand at room temperature for 30 min. A supernatant with abundant leukocytes was collected, leaving red blood cells. The amount of histamine in or out of cells was measured by means of reverse-phase high performance liquid chromatography (HPLC) (14). Histamine release tests were performed as described previously (21) with a goat anti-IgE (670 ng/ml), C5a and fMLP at the indicated concentrations. Cells were not pretreated with IL-3 prior to the stimuli in experiments. Spontaneous release from basophils of each patient was calculated by the amount of histamine in buffer and basophils after incubation for 45 min.

Analysis by flow cytometry

Expression levels of CD203c and CD63 on the surface of basophils were detected using Allergenicity® kit according to the manufacturer's instructions adding the anti-CD63 antibody. Briefly, whole blood cells were stained with anti-CD203c-PE and anti-CD63-APC in the presence or absence of anti-IgE or C5a at indicated concentrations for 15 min at 37°C. After incubation, red blood cells were ruptured by osmotic pressure. Fluorescence level of anti-CD203c and anti-CD63 was measured using Attune™ Acoustic Focusing Cytometer (Life technologies).

Subjects

Blood samples were collected from patients with CSU, who visited the Department of Dermatology in Hiroshima University. Healthy volunteers were recruited with written consents to participate in this study. The study protocol was approved by the institutional ethics committee (E-1716). Demographic characteristics of donors whose samples were analyzed in Figures 1, 2B, and Supplementary Figure 2 are described in Figure 1A, Table 2, and Supplementary Figure 2A, respectively. Demographic characteristics of donors whose samples were analyzed in Figures 2A, 3–6, Supplementary Figures 1, 3, 4 are described in Table 1.

Statistical analysis

Difference among each group was tested by the *t*-test or Tukey's test using GraphPad PRISM ver.6 (GraphPad Software, San Diego, CA).

Results

To determine the status of peripheral blood basophils in patients with CSU, we first measured the ratio of basophils in peripheral blood leukocytes and the expression levels of cell activation markers on basophil surface by means of flow cytometry. In line with previous reports, the ratio of basophils in peripheral blood leukocytes of patients with CSU was significantly low compared to that of healthy donors. (Figures 1A, B). However, the expression levels of CD63, a degranulation marker, and CD203c, an activation marker, on the surface of peripheral basophils of patients with CSU were not significantly increased (Figures 1A, C, D). We then analyzed spontaneous release of histamine from basophils of healthy and CSU donors, whose information is summarized in Table 1. As shown in Figure 2A, spontaneous release of histamine from basophils of patients with CSU was slightly, but significantly higher than that from basophils of healthy donors. The degree of spontaneous release of histamine was not correlated with total amount of histamine in whole blood of patients with CSU (Supplementary Figure 1). The measurement of plasma histamine in another set of healthy controls and patients with CSU (Table 2) showed that the amount of histamine in plasma of patients with CSU was significantly higher than that of healthy controls (Figure 2B). We then investigated histamine release activity of basophils of healthy donors and patients with CSU in response to anti-IgE (670 ng/ml) and an anaphylatoxin, C5a, at indicated concentrations (Figure 3). Although peripheral basophils express both C3a receptor (C3aR) and C5aR, we previously reported that C3a induces only marginal release of histamine from peripheral basophils (14). Moreover, we have confirmed that C5aR is expressed in basophils from patients with CSU and healthy donors (Supplementary Figure 2). Therefore, we focused on the effect of C5a as an anaphylatoxin in comparison with anti-IgE. As shown in Figure 3, basophils of most healthy donors released a large amount of histamine in response to anti-IgE and C5a. However, histamine release from basophils induced by C5a is weaker than that induced by anti-IgE. Basophils of a certain population of healthy donors and

A

	Number			Age (mean)
	Male	Female	Total	
Healthy	8	7	15	33.7
CSU	14	29	43	45.9

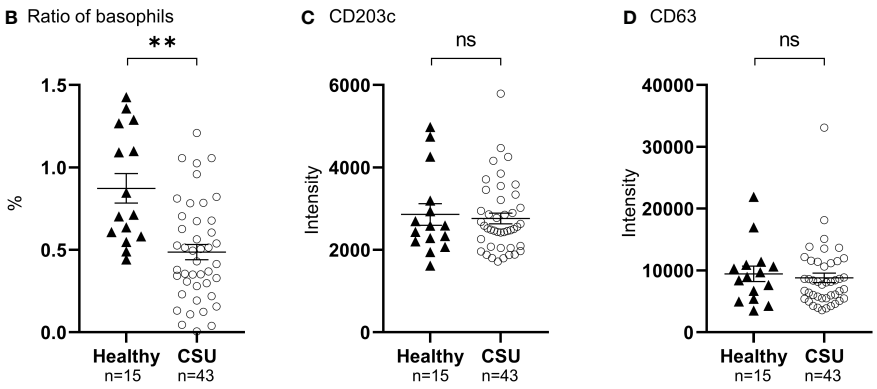

FIGURE 1
Analysis of peripheral basophil conditions by flow cytometry. **(A)** Characteristics of patients with CSU, and healthy controls. **(B)** Ratio of peripheral basophils to total white blood cells in patients with CSU and healthy donors. Five hundred basophils were detected in each measurement. Mean ± SEM of the number of basophils and white blood cells counted in the blood of 27 patients with CSU were 23.8 ± 3.00/μL, and 6911± 393, respectively. **(C, D)** Detection of basophil activation markers, CD203c and CD63, by flow cytometry analysis. The statistical difference was determined by the t-test. Differences between two groups was considered significant at **P < 0.01. ns, not significant.

patients with CSU released no or only slight amount of histamine. For convenience, we defined these patients as non- or low-responder when their histamine release from basophils was less than 5% or 20% in response to anti-IgE, respectively. The population of non- or low-responders in patients with CSU tend to be higher than that in healthy donors. However, no apparent difference in severity of urticaria was found between non-/low-responders and responders (Table 1). On the other hand, C5a induced histamine release from basophils of most subjects in the groups in a dose-dependent manner. Moreover, histamine release in response to C5a from basophils of patients with CSU was similar to or even higher than that of healthy

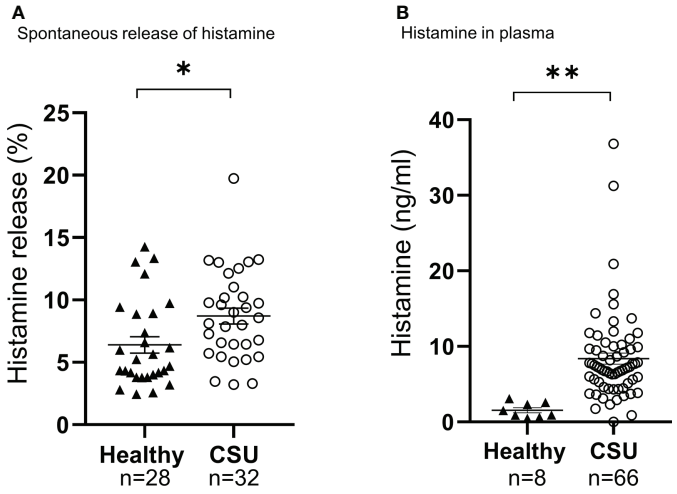

FIGURE 2
Analysis of spontaneous histamine release of basophils **(A)**, and amount of histamine in plasma **(B)**. Data represent mean ± standard error of the mean. The statistical difference was determined by the *t*-test. Differences between two groups was considered significant at *P <0.05 **P < 0.01.

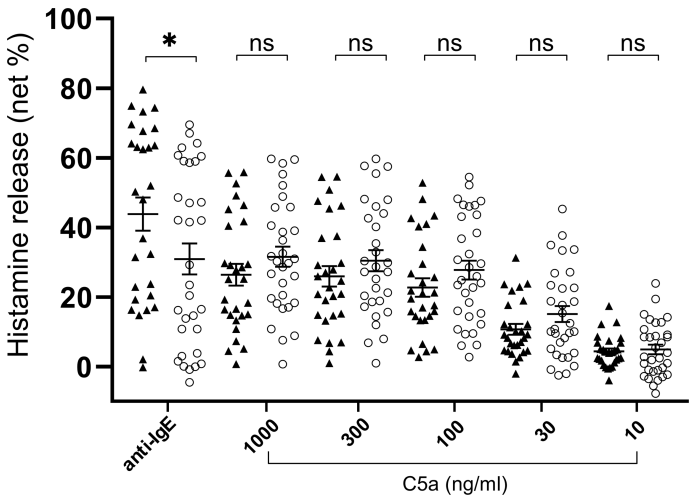

FIGURE 3
Histamine release from basophils of healthy donors (closed triangle, n=28) and patients with CSU (open circle, n=32. Only C5a 1000ng/ml, n=31). Data represent mean ± standard error of the mean. The statistical difference was determined by the T test. Difference between two groups was considered significant at *P <0.05. ns, not significant.

donors (Figures 3, 4). Of note, histamine release from basophils in response to anti-IgE and that to C5a in CSU responders tended to be negatively correlated, whereas those in healthy donors showed a tendency of positive correlation (Figure 5). Basophils of both responders and non-/low-responders were also activated by fMLP which activates basophils by an FcεRI-independent pathway (Supplementary Figure 3). To further confirm the potential of histamine release by basophils in the blood, we analyzed the amount of total histamine and the number of basophils in the blood of patients with CSU. As shown in Figure 6, the level of whole blood histamine was positively correlated with the number of basophils, but it was not totally reduced even in blood with low numbers of basophils (Figure 6). Unexpectedly, the amount of histamine per basophil tended to be inversely correlated with the number of basophils in the blood of patients with CSU (Supplementary Figure 4).

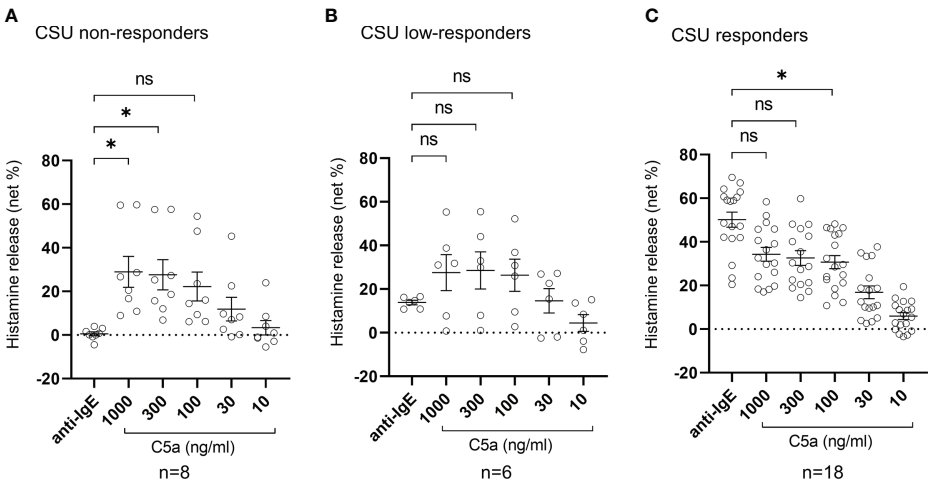

FIGURE 4
Histamine release from basophils of non-responder, low-responder, responder patients with CSU in response to anti-IgE or C5a. **(A)** CSU non-responders, **(B)** CSU low-responders, and **(C)** CSU responders. Data represent mean ± standard error of the mean. The statistical difference was determined by Tukey's test. Difference between each group was considered significant at *P <0.05. ns, not significant.

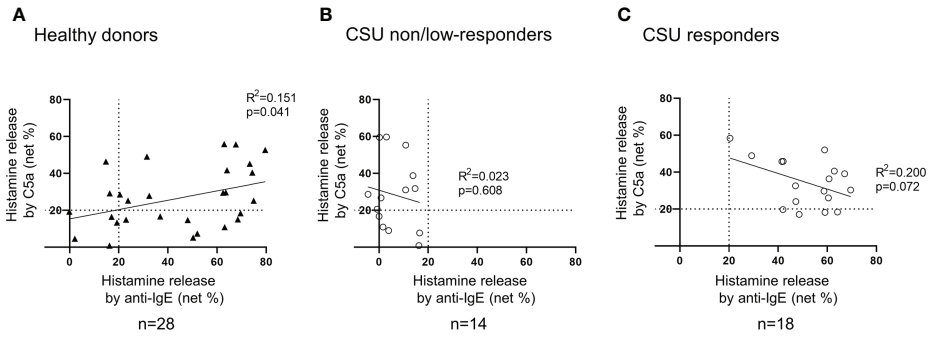

FIGURE 5
Correlation between histamine release from basophils in response to anti-IgE and that to C5a. **(A)** Healthy donors. Histamine release in response to anti-IgE and that to C5a tend to be positively correlated. **(B)** CSU non/low responders. Not more than 20% of histamine release was observed in response to anti-IgE, but various degrees of histamine release were evoked by C5a. **(C)** CSU responders. A weak tendency of a negative correlation was observed.

Discussion

In this study, we demonstrated that basophils of low- and non-responders of CSU patients, whose basophils release low or no amount of histamine in response to anti-IgE, maintain the capacity to release histamine in response to stimuli, that is independent of the IgE-FcεRI pathway, such as C5a and fMLP. Antigen-IgE activates basophils *via* several tyrosine kinases, such as syk, and thus, non- or low-release of histamine from basophils is considered to represent a loss of function of tyrosine kinases (20). We also confirmed that the number of basophils was significantly decreased in the peripheral blood of patients with CSU.

Vasagar et al. also reported the elevation of CD63, but not of CD69 and CD203c, on the surface of basophils of patients with CSU (22). However, we found that the expression levels of both degranulation/activation markers, CD63 and CD203c, were not increased in basophils of patients with CSU. Expression levels of degranulation/activation markers of basophils may change during cell preparation. Vasagar, et al. labelled basophils isolated by double gradient fractionation, whereas we employed heparinized whole blood samples without particular cell isolation procedure. Moreover, the level of basophil activation in peripheral blood may change at the timing of blood collection due to the migration of activated basophils to dermis, and diurnal fluctuation in new basophil differentiation in the bone marrow and their emergence

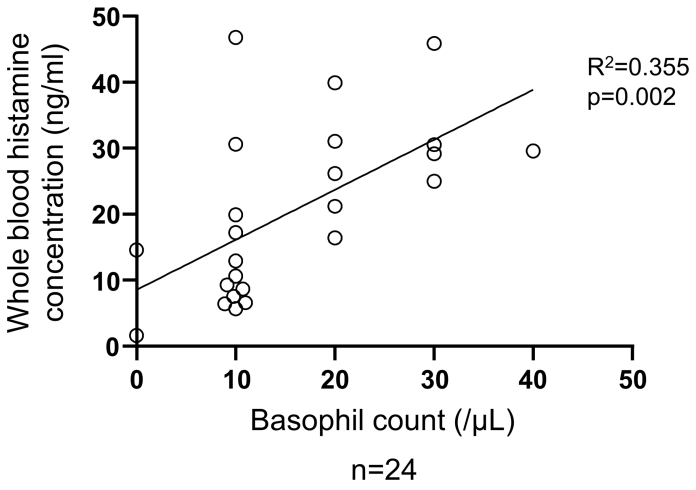

FIGURE 6
Correlation between basophil count and total histamine level in the blood of patients with CSU. Simple regression analysis revealed positive correlation between basophil count and whole blood histamine concentration (R^2 = 0.355, P=0.002).

TABLE 1 Demographic characteristics of patients with CSU and healthy donors in Figures 2A, 3–6, Supplementary Figures 1, 3, and 4.

	Healthy	CSU
Subject number	n=28	n=32
Gender (male/female)	14/14	10/22
Age (years); mean ± SEM (range)	40.7 ± 2.46 (21-64)	45.8 ± 8.10 (13-76)
Non-responder UCT UAS7	n=2	n=8 9.00 ± 0.94 (n=5) 22 ± 0 (n=2)
Low-responder UCT UAS7	n=5	n=6 4.60 ± 1.25 (n=5) 22.75 ± 4.55 (n=4)
Responder UCT UAS7	n=21	n=18 8.07 ± 1.04 (n=14) 19.6 ± 3.94 (n=9)

into the peripheral blood circulation with a lifetime of only 3-4 days (23) (24). Therefore, further studies are necessary to characterize the detailed behaviors of activation markers on peripheral basophils. Nevertheless, this study demonstrated that basophils of patients with CSU are not constitutively activated in the blood. On the other hand, both spontaneous histamine release from basophils isolated *ex vivo*, and plasma concentration of histamine of patients with CSU were higher than those of healthy donors, suggesting a certain difference of function between basophils of patients with CSU and those of healthy individuals (Figure 2).

Previous reports of spontaneous histamine release from basophils of patients with CSU are not consistent. Luquin, et al. reported a significant increase in patients with CSU (19), but Wahn, et al. found no difference between patients with CSU and healthy controls (25). It should be noted that both Luquin, et al. and Wahn, et al. studied pretreated basophils with IL-3 after the isolation by dextran sedimentation from the blood, whereas we isolated basophils by methyl-cellulose sedimentation and did not treat with IL-3. The real spontaneous release of histamine from basophils into the blood circulation should be a subject of future studies. Nevertheless, our results suggested that basophils of patients with CSU are susceptible to non-IgE stimuli or even to non-specific conditions to promote histamine release. Interestingly, spontaneous release of histamine (%) was not correlated to the whole blood histamine concentration (ng/ml) (Supplementary Figure 1), which mostly reflects the sum of the intracellular amounts of histamine in basophils (Figure 6) (26), but the amounts of histamine of individual basophils of patients were maintained or even higher in many patients with basopenia (Supplementary Figure 4). In fact, capacity to release histamine of basophils to either C5a or anti-IgE was maintained in almost all patients with basopenia as well (Supplementary Figure 5).

When histamine was released from basophils in the vicinity of vascular endothelial cells together with certain inflammatory substances, such as LPS, TNFα, IL-1β or IL-33, they synergistically induce TF expression on the vascular endothelial cells in a local area of the blood vessel. Highly-expressed TF then activates the extrinsic coagulation pathway, followed by the increase of vascular permeability induced by active forms of coagulation factors *via* PAR-1, C5a production, basophils/mast cells activation *via* C5aR and edema formation (Figure 7). As described above, Luquin et al., also reported the increase of spontaneous release and impairment of anti-IgE induced release of histamine from basophils of patients with CSU compared to those of healthy donors (19). However, they reported that the histamine release of basophils of patients with CSU in response to C5a was significantly lower than that of healthy donors (19). Considering possible difference of backgrounds among patients, we divided patients with CSU into 3 groups, namely, responders, low-responders, and non-responders, and activated their basophils in. the whole leukocyte fraction without separation to minimize possible mechanical and chemical damages by isolation procedures. The results of this study revealed that basophils circulating in the blood of patients with CSU may be largely impaired in the IgE-mediated pathway, and decreased in number, but preserve a high reactivity to C5a and histamine in individual cells. The underlying mechanism of loss of function in the IgE-FcεRI-syk pathway without an increase of cell activation markers, and decrease of basophils in the blood circulation remains unclear. However, the presence of patients with active CSU despite an impaired

TABLE 2 Characteristics of patients with CSU and healthy controls in Figure 2B.

	Healthy	CSU
Subject number	n=8	n=66
Gender (male/female)	4/4	25/41
Age (years); mean ± SEM (range)	31.9 ± 2.87(25-50)	37.5± 18.8 (5-90)

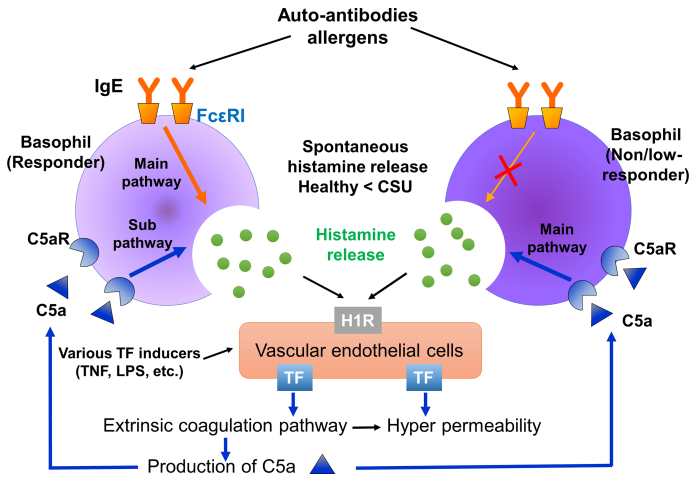

FIGURE 7
Summarized image of the role of basophils of responders and non-responders in CSU. Basophils of responders are activated *via* both the IgE-FcεRI and C5a-C5aR pathways. On the other hand, basophils of non/low-responders are activated *via* the C5a-C5aR, but not the IgE-FcεRI pathway. Therefore, C5a-C5aR stimulation may be a main activation pathway of non/low-responders with CSU. Histamine released from basophils may synergistically induce TF expression on vascular endothelial cells together with certain proinflammatory substances, such as LPS, TNFα. Spontaneous histamine released from basophils and other TF inducers may contribute to the synergistic expression of TF on vascular endothelial cells, followed by C5a production through the extrinsic coagulation pathway.

reaction to IgE-mediated stimuli suggests causative involvement of a non-IgE-FcεRI pathway in wheal formation of CSU. Thus, the basophil-C5a axis may play critical roles in the pathogenesis of CSU, especially that refractory to IgE-targeting medications, such as omalizumab.

Conclusion

We demonstrated that basophils of patients with CSU, that release no or only little amount of histamine in response to anti-IgE, release substantial amounts of histamine in response to C5a as basophils with normal reactivity to anti-IgE. C5a produced by activated coagulation/fibrinolysis factors, such as FXa, FIIa, and plasmin may contribute to the pathogenesis of CSU, especially in patients whose basophils are impaired in the IgE-FcεRI pathway. C5a and its related molecules, including C5aR might be an effective therapeutic target for patients with CSU including those that are refractory to IgE-targeting medications.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the institutional review board of Hiroshima University Hospital approved the study protocol (approval number: E-1716). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

DM, YY, KI, ST, AT, KO and MH designed the study and wrote the manuscript. DM, YY, KI and MH contributed to data collection. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MH received research grants from Eisai, GlaxoSmithKline, Kaken Pharmaceutical, Kyowa-Kirin, Mitsubishi Tanabe, Novartis, Sanofi,

and Taiho Pharmaceutical, honoraria from Kaken Pharmaceutical, Kyorin Pharmaceutical, Kyowa-Kirin, Mitsubishi Tanabe, MSD, Novartis, Sanofi, Taiho Pharmaceutical, Teikoku Seiyaku, and Uriach.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.994823/full#supplementary-material

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Decreased peripheral basophil counts in urticaria and mouse model of oxazolone-induced hypersensitivity, the latter suggesting basopenia reflecting migration to skin

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A decrease in the number of basophils in the peripheral blood, or basopenia, has been noted, reflecting the activity of chronic spontaneous urticaria (CSU). Infiltration of basophils into the skin has also been reported, but the mechanism of basopenia in CSU has not been clarified. The phenomenon of basopenia during the active phase of urticaria was confirmed, and basophil numbers increased following symptom improvement in 15 out of 17 patients treated with omalizumab and in 13 of 15 patients treated with antihistamines. Our examination by immunostaining also revealed basophil infiltration of the CSU lesions, as in previous reports, but since most of our patients were already taking oral steroids, it was not considered appropriate to examine the relationship between basophil numbers in tissue and peripheral blood. Then, we used mouse model of contact hypersensitivity with a single application of oxazolone, which is known to stimulate basophil infiltration, and investigated basophil counts in the skin, peripheral blood, and bone marrow. In this model, a decrease in peripheral blood basophil numbers was observed one day after challenge, but not after 2 days, reflecting supplementation from the bone marrow. Indeed, when cultured basophils expressing GFP were transplanted into the peripheral blood, GFP-positive basophil numbers in the peripheral blood remained low even after 2 days of challenge. Despite differences among species and models, these results suggest that one reason for the decrease of basophils in the peripheral blood in CSU may involve migration of circulating basophils into the skin.

KEYWORDS

Basophils, Urticaria, Basopenia, *in vitro* IgE, ovarian response, Oxazolone

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Introduction

Basophils are the least numerous types of granulocytes in the peripheral blood (PB), generally representing less than 1% of leukocytes. They differentiate from bone marrow (BM) and enter the circulation. Basophils have basophilic granules in their cytoplasm, high-affinity IgE receptors on their surface and as is the case with mast cells, release chemical mediators such as histamine (1–3). In mice, a member of the mouse mast cell protease (mMCP) family termed mMCP-8 (with coding gene *Mcpt8*) is unique to basophils and has served as a highly specific differentiation marker for this cell type (4, 5). However, studies of the roles of basophils have been neglected in immunological research due to their presence in relatively small numbers, and they have sometimes been confused with mast cells, which reside in tissues (6).

A decrease in the number of basophils in the PB, or basopenia, has long been reported in urticaria (7). Some reports suggested that basophils in urticaria patients are impaired in IgE-mediated histamine release, but this study reported that the peripheral basophil counts in the patients with chronic spontaneous urticaria (CSU) were slightly, but not significantly, lower than in healthy subjects (8). A study in 2008 (9) examined whether the presence of autoantibodies in CSU affects the impaired histamine release from basophils, but this study did not focus on the number of basophils. In another report, however, Oliver et al. (10) evaluated observations at two time points and showed that leukocyte histamine levels, reflecting the number and presence of basophils in the PB, vary inversely with skin rash and itch scores. A systematic search of 73 CSU studies reported in 2021 (11) did not list basophil count as a predictor of the efficacy with treatment, while it has been reported that PB basophil counts are inversely correlated with CSU activity, and that antihistamine treatment increases the number of basophils in the PB (10, 12). In particular, omalizumab, a monoclonal anti-IgE antibody, was shown to be effective in the treatment of CSU (13), and in the course of validating the efficacy of omalizumab, basopenia came to the attention again (14–16), along with various functional abnormalities of basophils shown by urticaria patients (17, 18).

In addition, infiltration of basophils has been reported in the skin tissue of CSU (19–21), but its relationship to basopenia has not been investigated. Thus, we have not had direct evidence that decreasing the number of circulating peripheral basophils reflected basophil migration into tissues. In this study, we confirmed that the PB basophil count, which was decreased during the active phase of CSU, increased with successful treatment with omalizumab and antihistamines. We also confirmed that basophils were present at the lesion site of CSU by immunostaining. However, we could not directly verify whether the basopenia reflected local basophil infiltration. Therefore, the relationship between changes in basophil counts in PB and infiltration of basophils into local skin tissues with inflammation was examined using an oxazolone (OX)-induced contact hypersensitivity model, in which basophils are known to migrate to lesion sites, due to the lack of an appropriate mouse model for CSU.

Materials and methods

CSU and peripheral basophil counts

The patients suffering from CSU were recruited in Kansai Medical University Hospital (Hirakata, Japan). PB samples were collected before and after treatment with antihistamines or omalizumab (300 mg, every 4 weeks), along with other laboratory tests. The severity of urticaria was evaluated by urticaria activity score over 7 days (UAS7) at the outpatient examination. Patients were reevaluated 4-8 weeks after the start of omalizumab use and those who had symptom resolution or USA7 improvement were collected. For patients treated with antihistamines, blood was also collected when symptoms relieved, but the timing varied from 1 to 9 weeks, depending on the case. The basophil counts in PB were calculated from leukocyte counts and leukocyte fractions in the clinical laboratory at the hospital, and in some cases were confirmed to be CD3-/CRTH2+/CD203c+ cells by flow cytometry (FACS) using the Allergenicity Kit (Beckman Coulter, Brea, CA). Total IgE levels were also measured by electro-chemiluminescence immunoassay in the hospital's clinical laboratory.

All human materials were approved by the Institutional Review Board of Kansai Medical University (2018199) and the study was conducted in accordance with the Declaration of Helsinki.

Mice

C57BL/6JJmsSlc mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). *Mcpt8*^{GFP} mice on the C57BL/6J background (22) were kindly provided by Drs. Miyake and Karasuyama (Tokyo Medical and Dental University). All mice were maintained under specific pathogen-free conditions in the animal facilities with the guidelines of Kansai Medical University for animal care, and all animal studies were approved by the Institution Annimal Care and Use Committee of Kansai Medical University (22–046, 22–047).

BM-derived cultured basophils

BM-derived cultured basophils (BMBa) were prepared as described previously (23). Briefly, BMBa were prepared by

culturing BM cells in the presence of 0.3 ng/mL recombinant murine IL-3 (BioLegend, San Diego, CA) in RPMI1640 with 10% FCS for 1 week.

OX-induced contact hypersensitivity model

OX (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich, St. Louis, MO) was dissolved in ethanol. Female C57BL/6J mice at the age of 8 weeks old were percutaneously sensitized with 100 µL of 3% OX on their shaved back skin and challenged 5 days later with topical applications of either 30 µL of 1% OX on both the dorsal and ventral surfaces of their ears for challenge. Ear thickness of sedated mice was measured with a caliper (PEACOCK Dial Thickness Gauge 0.01mm type G, Ozaki MFG, Tokyo, Japan).

In some experiments, $1x10^6$ BMBa were intravenously injected through the tail vein one day before OX-challenge.

FACS analysis

The ear skin was treated with 200 µL of Liberase solution by mixture of 10 mg of Liberase I (Roche, Basel, Switzerland) dissolve in 26 mL of RPMI-1640 medium with 1% FCS at 37°C for 1 hour, then added 20 µL of 0.5 M EDTA and incubate at 37°C for 5 min with shaking to stop the collagenase reaction. BM was harvested from the one side of femur and pressed out with 1 mL of PBS. In PB, flow-count fluorospheres (Beckman Coulter) were added to each 100 µL of samples. Then, cells from BM and PB was lysed in lysis buffer (BD Biosciences, Franklin Lakes, NJ). Single cell suspensions were obtained by FACS buffer (PBS containing 2% FCS, 0.1% sodium azide, and 1 mM EDTA) from the treated skin, BM and PB. All the samples were stained with an indicated combination of monoclonal antibody (mAb) for 30 min and analyzed by FACS Canto II (BD Biosciences). Following antibodies for flow cytometry were all purchased from BioLegend: PE/Cy7-conjugated CD45 (30F11); PE-conjugated CD49b (DX5); Pacific blue-conjugated CD117 (c-kit, 2B8); and APC-conjugated CD200R3 (Ba13). Dead cells were excluded by staining with propidium iodide (PI, Immunostep, Salamanca, Spain) or 7-AAD (BD Biosciences). Cells were analysed with FlowJo (BD Biosciences). Each cell lineages were defined in the CD45+ hematopoietic lineage cells as follows: basophils (c-kit–/CD49b+/CD200R3+), and mast cells (c-kit+/CD200R3+). The number of basophils in PB was identified by calculating the total number of CD45+ cells from the number of flow-count fluorospheres, and skin and BM basophils were accessed as a percentage of live cells evaluated for PI or 7-AAD negative.

Histopathological analysis

Formalin-fixed, paraffin-embedded human skin samples, biopsied from the urticaria lesion, were stained with hematoxylin and eosin (HE) or a human basophil-specific mAb, BB1 (20), and mast cell tryptase-specific mAb, G3 (24), in combination with the alkaline phosphatase (AP)-conjugated secondary antibody (Vector Labs, Burlingame, CA) and color was developed with Fast-Red substrate, followed by hematoxylin counterstaining. Digital images of each slide were acquired by NanoZoomer 2.0 HT (Hamamatsu, Shizuoka) and the number of cells was counted by the related image viewing software NDP view 2 by selecting 3 parts of areas randomly at 100 µm view.

Mouse ear specimens were fixed with 4% paraformaldehyde and embedded in paraffin, and sections were stained with HE or with a basophil-specific anti-mMCP-8 (TUG8, BioLegend) with donkey anti-rat IgG (Alexa Fluor 594-conjugated, Thermo Fisher, Waltham, MA) and anti-GFP (B-2, Abcam, Cambridge, UK) with donkey anti-goat IgG (Alexa Fluor 488-conjugated, Abcam).

Quantitative PCR

Total RNA was extracted from tissues or isolated cells by RNeasy Mini Kit (QIAGEN, Germantown, MD), followed by cDNA synthesis with SuperScript III First-Strand Synthesis System (Thermo Fisher). Q-PCR of the cDNA was performed with a Fast SYBR Green Master Mix (Thermo Fisher) by using following primer sets: Mm_Mcpt8_1_SG QuantiTect Primer Assay (Qiagen, GeneGlobe Id: QT00131565) and GAPDH as housekeeping gene: 5'-CATCACTGCCACCCAGAAGACTG and 5'-ATGCCAGTGAGCTTCCCGTTCAG. Relative expression value of *Mcpt8* was calculated by 2ΔCT for the housekeeping gene.

Statistical analysis

Statistical differences were determined by the statistical tests stated in each figure legend using GraphPad Prism (San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Peripheral basophil count in CSU was recovered after treatment

Of the 17 CSU patients recruited who were treated with omalizumab, we observed 15 cases in which there was a recovery

in basophil counts (mean 30.4/μL rising to 50.0/μL, $p = 0.003$) (Figure 1A) that was accompanied with an improvement in rash as assessed by UAS7.

Successful omalizumab treatment is associated with neutralization of serum IgE and increased serum IgE levels. In patients whose serum IgE levels could be evaluated before and after treatment with omalizumab (n = 13), 11 patients had an increase in serum IgE levels as the skin rash improved (mean 437/μL rising to 730/μL, $p = 0.014$) (Figure 1B). There was no association between pre- and post-treatment IgE levels ($r = 0.08$, $p = 0.37$). Two cases with no increase in basophil numbers after initiation of omalizumab (indicated by red triangles in Figure 1A and 1B), had no increase in IgE levels (Figure 1C with red triangles). When an increase in serum IgE levels was used as an indicator that omalizumab was sufficiently neutralizing IgE, the PB basophil counts increased associated with improvement in the skin rash.

Of the 15 retrospectively collected CSU patients who had been treated with antihistamines, three received concomitant oral corticosteroids and one cyclosporine. We compared the basophil count in PB before treatment and at the time the rash disappeared or was relieved by antihistamine treatment. Of the 15 cases, we observed that basophil numbers increased in 13 cases and decreased in two cases. Mean basophil counts of 14.9/μL rose to 43.1/μL after the treatment ($p = 0.012$) (Figure 1D).

Basophil detection in the affected skin of CSU patients

Although skin biopsies were not usually performed in CSU cases, some retrospectively collected antihistamine-refractory cases underwent skin biopsy to differentiate them from other conditions such as collagen diseases or vasculitis. Immunostaining of biopsied tissue with basophil-specific antibody, BB1, showed basophil infiltration in the lesioned skin (Figure 2). As shown in Table 1, of the 22 CSU patients, we identified basophils in 12 samples. The number of basophils averaged 2.4 ± 5.4 in the field of observation, which was approximately 1/10 of the number of mast cells (24.5 ± 11.9) identified as tryptase-positive cells in the same field. In the present study, there was no trend toward a decrease in the number of basophils in the PB in patients with skin infiltration of basophils (Table 1). However, most of the patients who had skin biopsies performed were resistant to antihistamine treatment, and most of them were receiving oral steroids at the time they were referred to our hospital for skin biopsies, so we did not consider it appropriate to examine the correlation between the number of PB basophils and the number of basophils infiltrating the tissues.

Basophil changes in tissue and blood in mice OX model

A decrease in the number of basophils in PB may reflect migration of basophils into the tissues, but direct evidence of such migration is difficult to obtain in humans. Therefore, we decided to investigate the relationship between the decrease in basophil count in PB and basophil migration to the skin, using a mouse contact dermatitis model induced by OX, which is known to cause basophil migration to the local skin lesions (25).

Redness of the ears was observed only in the groups both treated with sensitization with 3% OX on their back skin and challenged 5 days later with 1% OX on both the dorsal and ventral surfaces of their ears (Figure 3A). Swelling of the ear reflecting inflammation was similarly observed only in the sensitized and elicited groups, and swelling became noticeable the day after the challenge, and was further enhanced two days later (Figure 3B). Two days after challenge, mice were sacrificed and skin samples were taken for immunostaining, and a large number of mMCP8-positive basophils were observed infiltrating the local skin area in the sensitized and challenged groups (Figure 3C), reflecting the swelling of the skin.

We further identified basophils using FACS analysis, by first gating lymphocyte fractions with forward side scatter (FSC) and side scatter (SSC), then narrowing down to single cells with basophils identified as CD45+/CD117-/CD49b+/CD200R3+ cells (Figure 3D). The results confirmed the presence of large numbers of basophils in the tissue of the sensitized and challenged groups, consistent with the results of immunostaining (Figure 3E). However, when basophils in PB were examined at this time, there was no decrease in numbers of basophils in PB similar to that in human CSU patients. Although there was a trend for a slight decrease in the number of basophils in the group challenged with OX, this did not reach significance (Figure 3F). Interestingly, at this time we found that the BM basophil counts were increased only in the sensitized and challenged group (Figure 3G).

Based on these results, we hypothesized that failure to observe a decrease in basophil numbers in the PB at 2 days post-challenge, reflecting migration of basophils to the skin at the site of inflammation, was a result of mobilization of new basophils from BM into the PB. Therefore, we decided to reexamine the basophil kinetics over time, although the skin swelling was still slight at 1 day after sensitization (Figure 3B). The number of infiltrating cells increased on the second day from that on the first day, reflecting the swelling of the ear (Figure 3H). On the other hand, when the basophil count in the PB was examined, a decrease in the PB basophil count was observed on one day after OX challenge, but it recovered to the original level two days later (Figure 3I). When the basophil count in the BM of the mice was examined at this time, an increase in

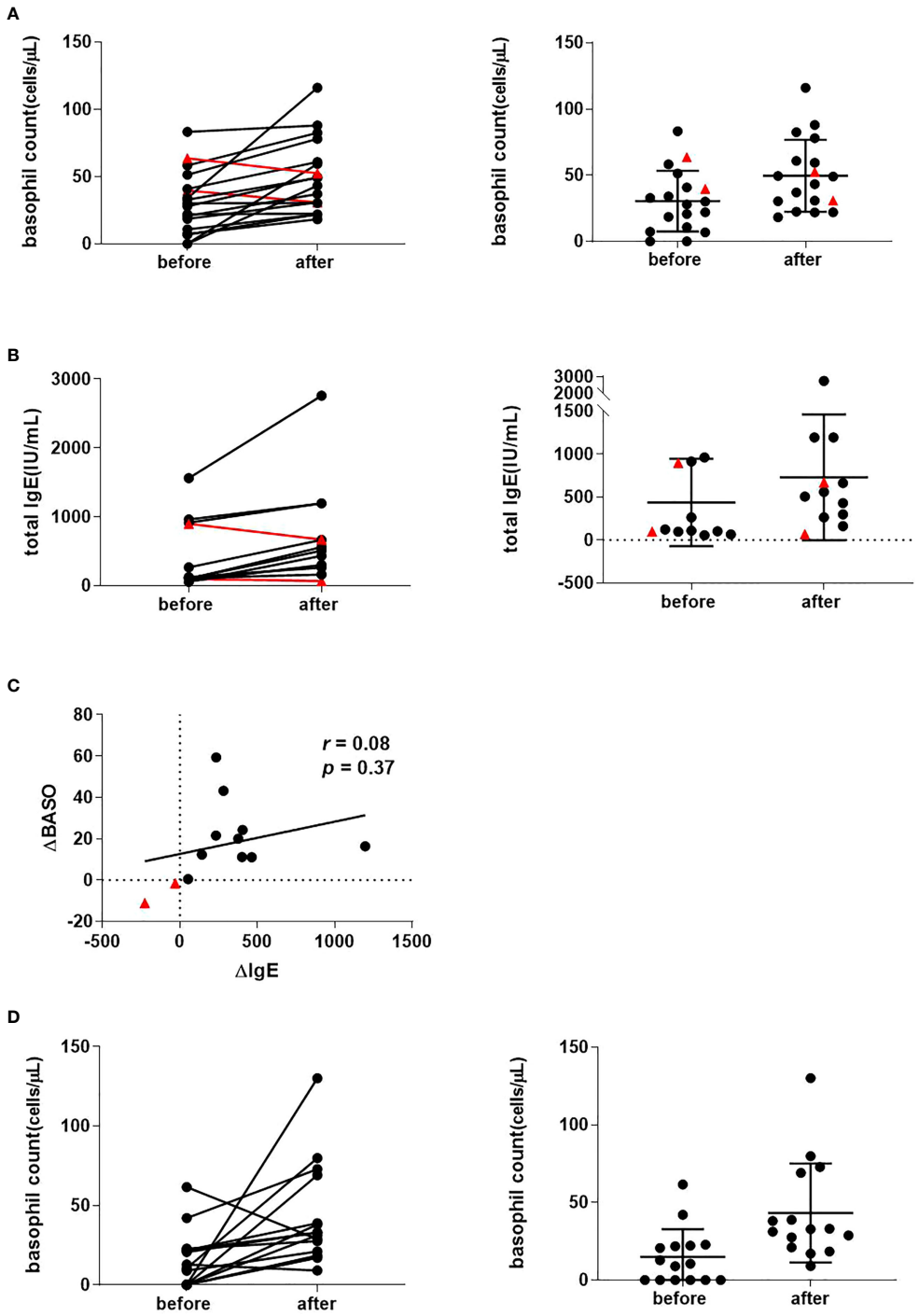

FIGURE 1
Peripheral basophil counts in 17 CSU patients before and following recovery with omalizumab treatment. **(A)** Peripheral basophil counts before and after omalizumab treatment. Left panel: Changes before and after treatment in individual cases. Right panel: Mean values before and after treatment. Data show individual values as means and SD. **(B)** Serum IgE levels before and after omalizumab treatment (n = 13). Left panel: Changes before and after treatment in individual cases. Right panel: Mean value before and after treatment. Data show individual values as mean and SD. **(C)** Correlation between changes in peripheral blood basophils and serum IgE before and after treatment (n = 13). ΔBASO = (after – before) basophil count, ΔIgE = (after – before) total IgE levels. Red triangles show a decrease in basophils after symptom improvement compared to pre-treatment. **(D)** Peripheral basophil count of CSU patients was recovered with antihistamine treatment. **(A)** Peripheral basophil count before and after antihistamine treatment (n=15) combination with corticosteroid (n = 3) and cyclosporine (n = 1). left panel: Changes before and after treatment in individual cases. right panel: Average value before and after treatment. Data show individual values as means and SD.

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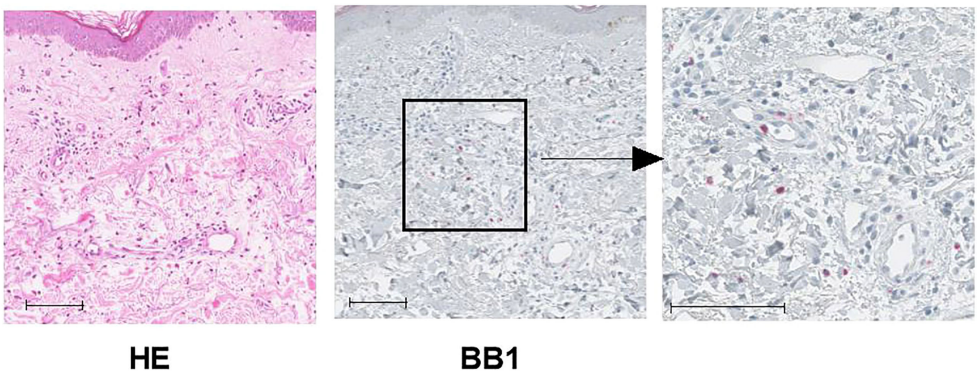

FIGURE 2
Representative image of histological findings of biopsy in urticaria lesions. This tissue is from case 4 in Table 1. Left panel: HE stains, Middle panel: Immunochemical stain with BB1, anti-human basophil specific mAb, scale bar = 100 μm. Right panel: Higher magnification for BB1 stain, scale bar = 100 μm.

TABLE 1 Mast cell and basophil counts in urticarial skin lesions and blood basophil counts.

Case	G3[†]	BB1[‡]	Blood Basophil[§]	Treatment[¶]
1*	28	0	0	AH
2	26	3	30.9	mPSL 125 mg + BMZ 0.25 mg + AH
3*	47	0	0	AH
4	51	26	10.6	AH
5*	31	7	9.9	none
6	12	2	38.8	BMZ 0.5 mg + AH
7	20	0	0	BMZ 0.75 mg + AH
8	34	0	13.2	PSL 5 mg + AH
9	6	0	10.2	AH
10**	12	2	40	AH
11	17	0	19.8	none
12*	29	2	26.8	none
13	8	0	6.9	AH
14	15	3	50.4	AH
15	20	1	0	PSL 20 mg + AH
16	46	0	7.8	AH
17	24	0	9.7	BMZ 0.75 mg + AH
18*	26	2	14.8	PSL 10 mg + AH
19	27	1	0	PSL 10 mg + AH
20	28	0	15	mPSL 125 mg + AH
21	15	1	0	PSL 8 mg + AH
22	18	2	11.4	PSL 10 mg + AH
Mean ± SD	24.5 ± 11.9	2.4 ± 5.4	14.4 ± 14.2	

[†]Tryptase+ mast cells in the CSU lesion skin (/field).
[‡]Basophils in the CSU lesion skin (/field).
[§]Basophil counts in the peripheral blood (/μL).
[¶]Treatment of urticaria at the time of biopsy. Those who had received any oral corticosteroid or salazosulfapyridine, which could affect the basophil count in the peripheral blood, were marked with *, and those whose treatment before biopsy was unknown were marked with ** after the case number.
AH, antihistamine; BMZ, betamethasone; mPSL, methylprednisolone; PSL, prednisolone.

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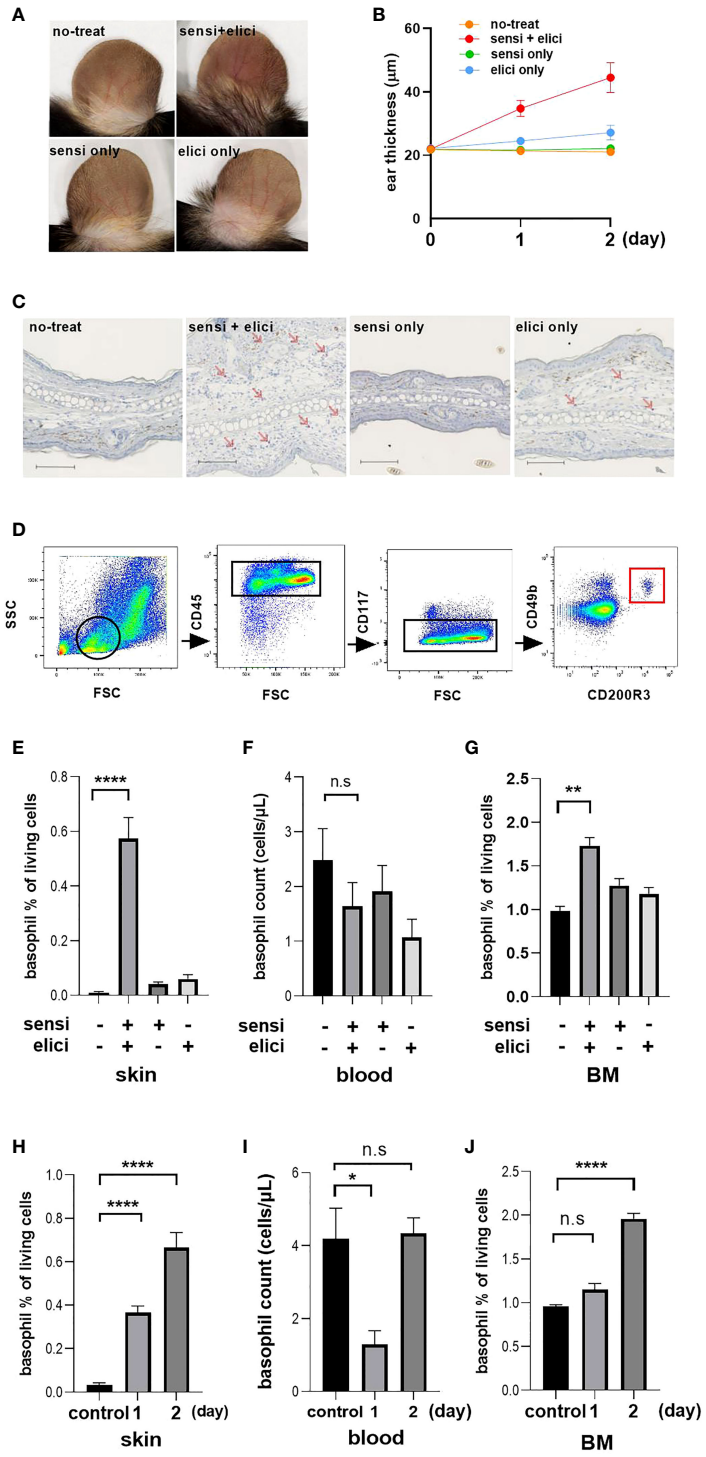

FIGURE 3
Basophil changes in tissue and blood in mouse OX model. Each experiment was conducted at least 3 times individually (each group, n = 3), and data from one of these experiments are shown as representative. **(A)** Representative image of photo taken two days after OX challenge. **(B)** Thickness of the ear. **(C)** Representative image of immunohistochemical staining of mouse ear tissue with TUG8, anti-basophil specific mAb that recognizes mMCP-8, scale bar = 100 μm. **(D)** Gating for the identification of activated basophil. From the lymphocyte and granulocyte population gated by FSC and SSC, we identified activated basophils as CD45-positive and CD117-negative and CD49b-positive and CD200R3-positive. **(E–G)** Basophil counts in skin, blood and BM two days after sensitization and challenge. **(H-J)** Basophil count at 1 day after sensitization. Controls were mice without either sensitization or challenge. *p < 0.05, **p < 0.001, ****p < 0.0001 ns. no significance. "sensi" is sensitization and "elicit" is elicitation.

the basophil count in the BM was observed two days after OX challenge (Figure 3J).

Restoration of peripheral basophil numbers two days after challenge reflects mobilization from BM

Based on the results of the mouse OX model, it was suggested that reduction in the number of basophils in the PB one day after challenge is due to migration to the skin, but two days after challenge, the number of basophils in the PB is restored due to the mobilization of new basophils from BM. To evaluate the supplementation of basophils from BM to PB, we generated BMBa from *Mcpt8*^{GFP} mice (22), which express GFP specifically in basophils, and transferred these cells into PB.

Following BMBa transplantation, there were higher numbers of GFP-positive cells in skin tissue on the first day after challenge, but not on the second day (Figure 4A). When mRNA was extracted from skin tissue and examined using quantitative PCR, the expression of *Mcpt8* was higher in the tissue collected one day after challenge (Figure 4B). One day after the challenge, there was an infiltration of cells in the dermis for which the cytoplasm had granular GFP positive staining (Figure 4C). Finally, the number of GFP-expressing basophils in the PB was observed over time, and we can confirm that GFP-positive cells in the PB were lower after 1 day of challenge and there was no apparent increase after two days (Figure 4D), suggesting that the recovery of the number of basophils in the PB observed on the second day of challenge reflected the mobilization of new basophils from the BM.

Discussion

The impetus for this study came from our experience with one CSU patient, who had had severe CSU for 6 years and had persistently low, almost undetectable peripheral basophil counts for at least 1 year (26). When urticaria was improved by treatment with omalizumab, we noticed that his peripheral basophil count recovered. When the patient discontinued omalizumab treatment, the PB basophil counts again dropped to zero and urticaria recurred. Re-administration of omalizumab improved the skin rash and rescued the peripheral basophil count.

Our observation of an apparent basopenia associated with CSU is consistent with other reports (14–16). Of particular interest has been suggestions that a reduced basophil count may predict omalizumab efficacy (11, 15–18). Johal et al. (17) reported that those with decreased PB basophils had higher symptom scores and slower symptom improvement with omalizumab treatment than those without. Rijavec et al. (15) reported very low absolute basophil counts in circulating blood (1.7 basophils/μL) was reported to be a predictor of poor response to omalizumab. However, the mechanism of basopenia during the active phase of urticaria has not been clarified (27).

In a report examining various inflammatory diseases by immunostaining with basophil-specific antibody BB1, basophils were detected in 6 out of 10 cases of urticaria examined (20). In fact, in our CSU patients who had previously undergone skin biopsies, we found that while basophils were rarely seen in the skin of non-inflamed healthy controls, basophils were seen to varying degrees in 12 out of 22 CSU cases in urticarial lesions, as shown in Table 1. Based on these observations, we hypothesized that the decrease in basophils in the PB during the active phase of urticaria reflects the migration of basophils to the cutaneous region. However, skin biopsies are not usually performed for urticaria. In addition, many refractory cases that have undergone biopsy have already been treated with oral steroids at the time of skin biopsy. Therefore, it is difficult to correlate the migration of basophils to skin tissue with PB.

Although there is no suitable mouse model that reproduces the pathogenesis of urticaria, repeated application of OX has been reported to shift the immune response toward Th2 and induce migration of basophils to the lesion site (25). In this report, basophils were reported to infiltrate the skin even two days after a single application of OX. In addition, very interestingly, the latest single cell RNA sequencing analysis shows that basophils, which are newly migrated to the skin by OX treatment, are the source of IL-4 and IL-13 and tilt the immune response toward Th2, rather than mast cells residing in the tissues (28). In our present study, 5 days after sensitization, a single challenge of OX resulted in migration of a sufficient number of basophils to the skin, though there was not a concomitant decrease in basophils in the PB. However, as the number of basophils in BM increased two days after challenge, prompting us to consider the possibility that the lack of a decrease in basophils in the PB may be a consequence of new basophils being supplied from BM to the peripheral circulation.

In our investigation of the kinetics of basophil migration in tissue, PB and BM in the mouse OX model, the number of basophils migrating to the tissues one day after challenge was not as high as at two days after. However, at this time point the number of basophils in the PB was clearly decreased, while that in the BM had not yet increased. Since urticaria generally resolves within 24 hours, the reaction after 1 day of challenge in the mouse model may reproduce features of human urticaria. In CSU, however, the migration of basophils is not necessarily a transient phenomenon, since even after one skin rash disappears, another rash may appear at a different site. As a result, new basophils are recruited from the BM to the peripheral circulation to compensate for the shortage of basophils migrating to the skin, and the decrease of basophils in the PB

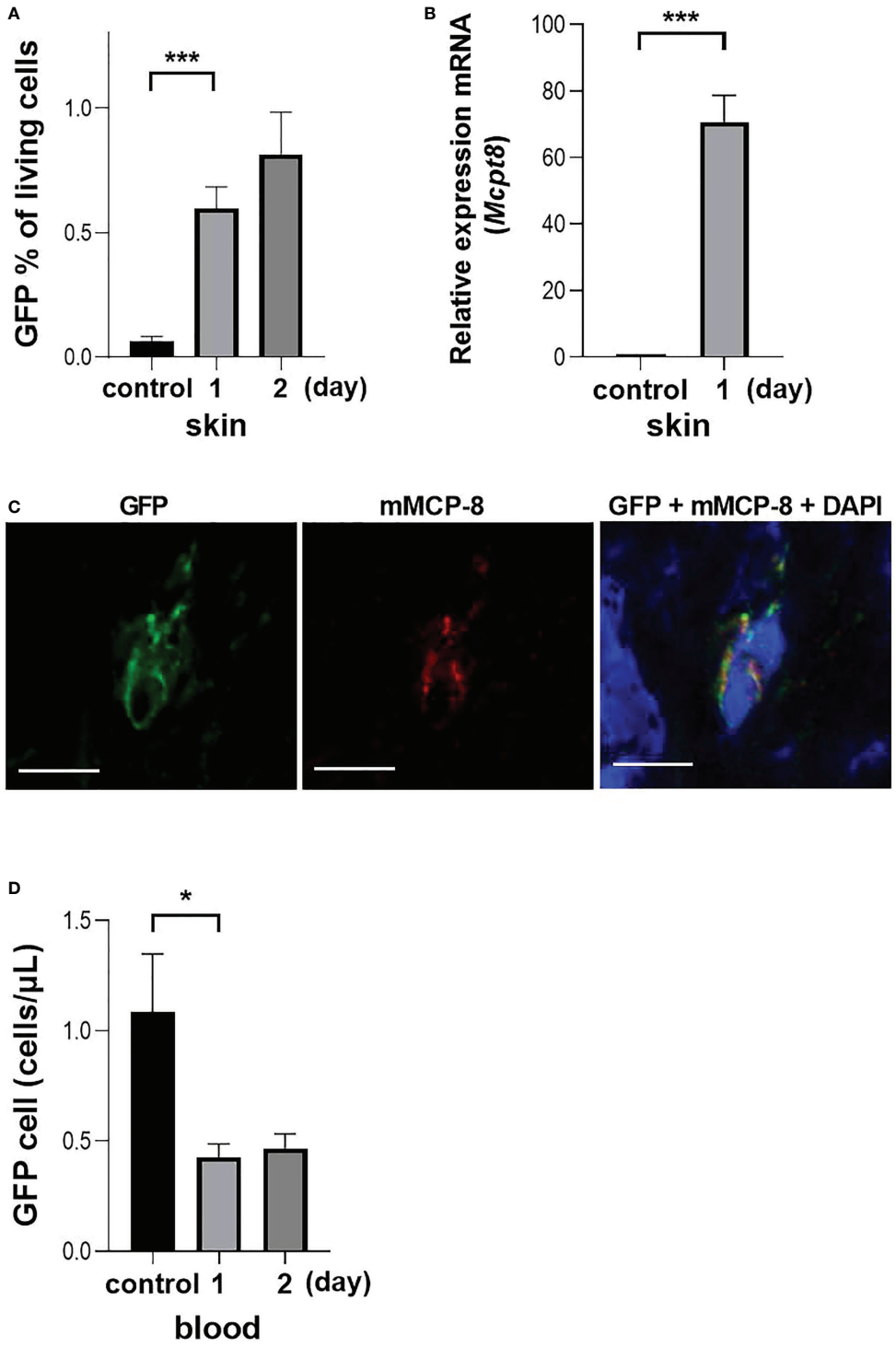

FIGURE 4
Basophil migration in an OX model with GFP-expressing BMBa transplantation. Each experiment was conducted at least 3 times individually (each group, n = 3), and data from one of these experiments are shown as representative. **(A)** GFP positive basophil count in skin at one and two days after challenge. **(B)** Q-PCR of *Mcpt8* in skin tissue. **(C)** Representative image of staining of skin tissue one day after challenge. Left panel: GFP-positive cells. Middle panel: anti-mMCP-8 staining with TUG8. Right panel: overlay with GFP, mMCP-8 and DAPI, scale bar = 5 μm. **(D)** GFP positive basophil count in blood at 1 and 2 days after challenge. Control mice were treated with BMBa but neither sensitized nor challenged, and samples were taken on day 2 after *i.v.* injection of BMBa. *$p < 0.05$ and ***$p < 0.005$.

should not be observed after the second day. However, the discrepancy observed in basophil kinetics between the mouse OX model and human urticaria may reflect differences between the mouse and human. In parasite infestation, basophilia in the PB has been observed in mice, whereas in humans, an increase in basophil numbers was reported to be exceedingly rare (29). In addition, in clinical practice, while neutrophilia or eosinophilia are often encountered, there are few diseases associated with increased basophil numbers other than rare basophilic leukemias. Thus, human BM production of basophils may be severely restricted. To exclude the influence of new basophils emerging from BM, in the present study BMBa expressing GFP were transferred into the PB and their contribution could be distinguished from that of cells mobilized from BM. As expected, we observed a decrease in the number of basophils in the PB, reflecting the migration of basophils to the inflamed skin, regardless of mobilization from BM.

In this study, we used a single application of OX to study the migration of basophils to the skin. Given the success of omalizumab targeting IgE in CSU, we may consideration should be given to a model in which there is cell migration or activation *via* IgE. In that case, however, the influence of not only basophils but also other IgE-mediated activated cells, especially mast cells pre-localized in the skin, would need to be considered. In addition, it has been reported that mast cells are also required for contact hypersensitivity, since symptoms are reduced in mast cell-deficient mice (30–32). On the other hand, studies in models of atopic dermatitis with repeated applications of OX have shown that skin thickness is reduced, even in the absence of basophils (25). We believe that further investigation is needed to determine whether IgE-mediated stimulation also reduces the number of basophils in the PB, reflecting the migration of basophils to the skin area, and whether there is any interrelationship between the roles of basophils and mast cells.

The number of basophils in PB may be useful as an index of urticarial activity. Even though the studies we report here have the limitation that they are experiments with different species and models and that it is impossible to explain their results in a unified manner, we believe that one of the mechanisms by which the number of basophils in PB decreases at the onset of urticaria is that basophils migrate from the circulation into the skin. A focus on basophils should lead to new understanding of the pathogenesis of CSU.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Kansai Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Ethics Committee of Kansai Medical University

Author contributions

IK, HT, and NK designed the experiments. NM, RT-I, CN, AO, and AW supported the planning and validated the strategy. IK, NK, RT-I, HT, and CN performed experiments. IK, NM, and NK wrote the manuscript. IK, NM, CN, HT, and NK performed data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Basophils from allergy to cancer

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Human basophils, first identified over 140 years ago, account for just 0.5-1% of circulating leukocytes. While this scarcity long hampered basophil studies, innovations during the past 30 years, beginning with their isolation and more recently in the development of mouse models, have markedly advanced our understanding of these cells. Although dissimilarities between human and mouse basophils persist, the overall findings highlight the growing importance of these cells in health and disease. Indeed, studies continue to support basophils as key participants in IgE-mediated reactions, where they infiltrate inflammatory lesions, release pro-inflammatory mediators (histamine, leukotriene C_4: LTC_4) and regulatory cytokines (IL-4, IL-13) central to the pathogenesis of allergic diseases. Studies now report basophils infiltrating various human cancers where they play diverse roles, either promoting or hampering tumorigenesis. Likewise, this activity bears remarkable similarity to the mounting evidence that basophils facilitate wound healing. In fact, both activities appear linked to the capacity of basophils to secrete IL-4/IL-13, with these cytokines polarizing macrophages toward the M2 phenotype. Basophils also secrete several angiogenic factors (vascular endothelial growth factor: VEGF-A, amphiregulin) consistent with these activities. In this review, we feature these newfound properties with the goal of unraveling the increasing importance of basophils in these diverse pathobiological processes.

KEYWORDS

allergy, angiogenesis, angiopoietins, basophil, cancer, cysteinyl leukotrienes, cytokines, vascular endothelial growth factors

Introduction

Paul Ehrlich discovered, over 140 years ago, peripheral blood basophils and tissue mast cells using novel hematological techniques that combined the use of alkaline dyes and conventional light microscopy (1, 2). Unlike mast cells, which are only found as mature cells in tissues, basophils represent just 0.5-1% of all leukocytes in the bone marrow and peripheral blood (3, 4). Basophils and mast cells are long recognized as being morphologically similar in appearance and for sharing several unique features (5, 6). For example, they are the only two cells that express the full tetrameric ($\alpha\beta\gamma2$) form of the high-affinity receptor for IgE (FcεRI). They both also uniquely store histamine in cytoplasmic granules (7), releasing it and other proinflammatory mediators (e.g., cysteinyl leukotrienes) when appropriately activated (5, 8). In fact, these shared characteristics continue to cause misperceptions, leading some to believe that basophils and mast cells are one and the same. However, compelling evidence over the last decades now supports that human basophils possess morphological, immunological, biochemical, and pharmacological characteristics quite different from those of human mast cells (5–7, 9).

Until recently, there was some dispute as to whether mice have basophils. However, the work of Ann M. Dvorak using electron microscopy, clearly identified basophils in mice as a rare population of bone marrow cells, with some ultrastructural characteristics like those observed in human basophils (7, 10, 11). As discussed below, there remains considerable debate as to whether mouse basophils are truly representative of human cells, particularly with regard to function (11–17). Of course, much of this debate often defaults to issues pertaining to the disparities expected with *in vitro vs. in vivo* experiments (18). Nonetheless, the one newest function perhaps most shared by basophils from both species is their capacity to secrete large quantities of IL-4, even though debate persists about the stimuli most responsible for this response.

Many fundamentals of basophil biology have been extensively reviewed elsewhere, especially regarding their role in allergic diseases (5, 19–24). In this review, we briefly touch on this research field but will additionally focus on the concept of basophils participating in tumorigenesis and wound-healing and how these processes are seemingly linked and driven by the capacity of these cells to secrete IL-4, IL-13, angiogenic factors and pro-fibrotic cytokines.

Abbreviations: ANGPT, angiopoietin; AREG: amphiregulin; BCG, bacillus Calmette-Guérin; BEC, blood endothelial cells; BET, basophil extracellular traps; CAF, cancer-associated fibroblast; CML, chronic myeloid leukemia; CRC, colon carcinoma; CSF1, colony-stimulating factor 1; CSU, chronic spontaneous urticaria; cys-LT, cysteinyl leukotriene; cys-LTR, cysteinyl leukotriene receptor; CyTOF, cytometry by Time-Of-Flight; DC, dendritic cell; DMBA, 7,12- dimethylbenz[a] amthracene; ET, extracellular trap; Flt3L, l, FMS-like tyrosine kinase 3 ligand; HGF, hepatocyte growth factor; ILC2, group-2 innate lymphoid cell; JAK2, janus kinase 2; LTC_4, leukotriene C_4; MI, myocardial infarction; NET, neutrophil extracellular trap; NGF, nerve growth factor; nLung, non-involved lung tissue; NSCLC, non-small cell lung cancer; PC, pancreatic cancer; PDAC, pancreatic ductal adenocarcinoma; PV, polycythemia vera; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; SCC, squamous-cell carcinoma; TAM, tumor-associated macrophage; TDLN, tumor-draining lymph nodes; Tfh, T follicular helper cell; TME, tumor microenvironment; TPA, 12-0-tetradecanoylphorbol-13-acetate; Treg cell, T regulatory cell; TrkA, tropomyosin receptor kinase A; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor; VEGF, vascular endothelial growth factor; VISTA, V-domain immunoglobulin suppressor of T-cell activation; WT, wild type.

Basophil development

Basophils originate from stem cell progenitors in the bone marrow (25–27). Both in humans and mice, IL-3 is the most important growth factor for basophil development (12, 17, 28, 29). In fact, basophils from both species can be developed *in vitro by* simply culturing bone marrow cells (or $CD34^+$ precursors in humans) in the presence of IL-3 for 10-14 days (12, 30–32).

While IL-3 is clearly most important for basophil development from precursors, other growth factors are reported to facilitate expansion/function. For example, the FMS-like tyrosine kinase 3 ligand (Flt3L) has been combined with IL-3 to expand the number of culture-derived basophils (33). Siracusa and co-workers reported that mouse basophils can be generated by thymic stromal lymphopoietin (TSLP) through the engagement of the heterodimeric TSLP receptor (TSLPR/IL-7Rα) (34). These authors demonstrated that IL-3 and TSLP induced the differentiation of two types of murine basophils displaying different gene expression and functions (35). In humans, it has been suggested that about 10% of basophils from asthmatics express the TSLP receptor and release histamine and cytokines in response to TSLP (15). In contrast, more recent studies have shown that human basophils do not express the IL-7Rα subunit of the heterodimeric TSLP receptor (14) and do not respond to *in vitro* TSLP stimulation (12, 14, 16). By contrast, TSLP induces the release of IL-4, IL-13, CXCL1, and CXCL2 from mouse basophils (12).

Heterogeneity of basophils: In species and tissue *versus* peripheral blood

Human and mouse basophils express FcεRI (36, 37) and will up-regulate the degranulation markers, CD63 (38–40) and CD203c when activated appropriately (9, 39, 41–43). Basophils from humans and mice express the IL-3 (IL-3Rα/CD123) (34,

44), GM-CSF (CD116) (45, 46), and IL-33 (ST2/IL1RL1) receptors (47–50). The heterodimeric TSLP receptor, TSLPR/IL-7Rα, is expressed by mouse basophils (12, 34), but the presence of this receptor on basophils from allergic donors and healthy subjects remains controversial (12, 14–16). Human basophils reportedly express receptors for IL-5 (CD125) (51) and for Nerve Growth Factor (NGF) (tropomyosin receptor kinase A: TrkA) (52–54). Both human and mouse basophils display a variety of chemokine receptors (5, 55–60). The IgG receptors FcγRIIA, FcγRIIB, and small amounts of FcγRIIIB are expressed by human basophils, whereas mouse basophils express FcγRIIB and FcγRIIIA (61, 62).

Preformed mediators, such as histamine (≃ 1 pg/cell), basogranulin (63, 64) and very low concentrations of tryptase (65) are present in human basophils. Human (66) and mouse basophils release granzyme B (67), that reportedly exerts cytotoxic effects on tumor cells (68, 69). Basophils from both species can synthesize cysteinyl leukotriene C_4 (LTC_4) through the 5-lipoxygenase pathway (70). Mouse basophils additionally produce prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) through the cyclooxygenase pathway (71, 72). Human basophils do not synthesize detectable levels of PGD_2 or other mediators requiring cyclooxygenase activity (12, 73).

Substantial evidence now shows that human (12, 24, 74–82) and mouse (12, 47, 80) basophils secrete IL-4. Both human (12, 75, 76, 78, 81–85) and mouse basophils (12, 47) also generate and release IL-13, yet the evidence for this response is far more prevalent in the former species. Mouse basophils can release IL-6 (47, 86, 87) and TNF-α (47, 86). Two reports indicate that these cytokines are secreted from human basophils (88, 89), even though they do not appear to be products commonly released by these cells. Human and mouse basophils release granzyme B (66, 67) that exerts a cytotoxic effect on tumor cells.

Human basophils secrete several angiogenic factors such as vascular endothelial growth factor-A (VEGF-A) (64), angiopoietin-1 (ANGPT1) (90), hepatocyte growth factor (HGF) (47, 91), and amphiregulin (AREG) (92–94). Mouse (47) and human basophils (91) express *Hgf* and release, under certain conditions, AREG (94) and VEGF-A (Gambardella et al., unpublished).

The life-span of circulating basophils is relatively short (≃ 2.5 days in mice) (95) and therefore newly generated basophils are constantly supplied from the bone marrow to the blood (25). Basophils physiologically circulate in peripheral blood and migrate within tissues mainly during certain types of inflammation in mice (86, 95–98) and humans (99–104). Basophils, present during mouse lung development, exhibit a phenotype different from circulating blood basophils (47). In the lung, specific gene signature of lung-resident basophils is modulated by IL-33 and GM-CSF (47). These cells play a prominent role in the development and polarization toward the M2 state of alveolar macrophages, raising the possibility that in tumors associated with M2 macrophages (105–107), basophils contribute the polarization of tumor-associated macrophages.

Basophils derived from murine bone marrow cells are often used as a model system for studies of the immunological functions of these cells (86, 97, 108–111). It should be pointed out that these cells, developed by murine bone marrow cells in the presence of IL-3, have an activated phenotype (82, 112). Recently, Pellefigues et al. carefully demonstrated functional heterogeneity between naïve murine basophils obtained from spleen and bone marrow-derived basophils (108). In humans, functional heterogeneity of peripheral blood basophils has been demonstrated by applying mass cytometry (CyTOF) to simultaneously assess several proteins and functions of basophils (113).

Angiogenic factors released by basophils

Angiogenesis occurs physiologically during embryonic development, pathologically in inflammation and cancer (114, 115). Both cancer and immune cells (116, 117) produce several proangiogenic factors (118, 119). The vascular endothelial growth factor (VEGF) family includes VEGF-A, VEGF-B, VEGF-C, and VEGF-D. VEGFs activate specific receptors (VEGFR1, VEGFR2, and VEGFR3) on blood endothelial cells (BECs). VEGF/VEGFR axis plays pivotal roles in tumor and inflammatory angiogenesis (118). VEGF-A is released by human basophils (64). All members of the VEGF family are chemotactic for human basophils through the engagement of VEGFR2 on their surface (64, 120). Therefore, VEGFs released by cancer cells and immune cells in the tumor microenvironment (TME) (118, 120–123) can favor basophil infiltration in TME.

Angiopoietins (ANGPTs) are other players of inflammatory and tumor angiogenesis (124, 125). ANGPT1, released by perivascular mural cells, binds to the Tie2 receptor on endothelial cells and promotes endothelial stabilization (126). ANGPT2, secreted by activated endothelial cells, induces vascular permeability (127). ANGPT1 and ANGPT2 mRNAs are expressed by human basophils (90), and their activation induces ANGPT1 release. Mouse lung-resident basophils express mRNA for HGF, a potent angiogenic factor (47, 91, 128).

Cysteinyl leukotrienes (cys-LTs) are powerful proinflammatory mediators (129). The cys-LTs include leukotriene C_4 (LTC_4), the main lipid mediator synthesized by human and mouse basophils (54, 70). ϒ-glutamyl transpeptidases metabolize LTC_4 to LTD_4 and to LTE_4 by the membrane-bound enzymes (129). Cys-LTs are potent agonists of three different receptors (CysLTRs) $CysLT_1R$,

$CysLT_2R$, and $CysLT_3R$ (130–132). LTC_4 and LTD_4 induced the formation of angiogenesis (133). The angiogenic properties of LTC_4 and LTD_4 were mediated *in vivo* by the activation of $CysLT_2R$ on BECs. In mouse models, pharmacologic antagonism of $CysLT_2R$ inhibited tumor growth and metastasis formation (133). These results illustrate the relevance of cys-LTs as non-canonical angiogenic factors in cancer. Moreover, these findings suggest that $CysLT_2R$ might be a target in cancer (133). LTC_4 is released by activated human (70, 134) and mouse (54) basophils and future studies should investigate whether basophil-derived LTC_4 might contribute to angiogenesis in human cancer.

Formation of extracellular DNA traps by basophils

Activated neutrophils (135–137), eosinophils (138, 139), mast cells (140–143), macrophages (144–148), and basophils (149, 150) can release extracellular traps (ETs), which are DNA structures decorated with a variety of proteins [e.g., myeloperoxidase and elastase) (151), lactoferrin and pentraxin 3) (151, 152), and matrix metalloproteinase 9) (151)]. ETs released by human neutrophils (neutrophils extracellular traps: NETs) were initially characterized by their antibacterial activity (138, 151, 153, 154). Increasing evidences demonstrate that ETs, particularly NETs, play a role in asthma (137) and in fundamental aspects of tumorigenesis (155). NETs favor the formation of metastasis in mice and in humans (156–159) and awaken dormant cancer cells (160). An increase of NET release occurs when neutrophils from myeloproliferative neoplasms are associated with $JAK2^{V617F}$ mutations and mice with knock-in of $JAK2^{V617F}$ (161). We have provided evidence that anaplastic thyroid cancer cells can induce NET formation (162). Collectively, these findings demonstrate that NETs can promote tumor growth and metastasis formation. Basophils from humans and mouse can release extracellular DNA traps (BETs) *in vitro* and *in vivo* (149, 150, 163). The translational relevance of these findings should be explored in experimental models and human cancers.

Basophils in allergic disorders

Basophils play a major role in a variety of allergic disorders (8, 164–166). Anaphylaxis is a rapid-onset, potentially life-threatening allergic reaction caused by the release of vasoactive mediators from mast cells and basophils after allergen exposure (167). Mouse models of anaphylaxis suggest that basophils play a major role in the IgG-, but not IgE-mediated anaphylaxis (168). In these studies, the depletion of basophils by anti-CD200R3 mAb inhibited IgG-mediated anaphylaxis, whereas it had minor effect on IgE-mediated anaphylaxis. By contrast, mast cells are central for IgE-mediated mouse models of anaphylaxis (168, 169).

Several lines of indirect evidence suggest that basophils participate in human anaphylaxis (24). For example, the number of circulating basophils was significantly lower in subjects undergoing anaphylactic reactions compared to healthy controls (170). Peanut-induced allergic reactions also resulted in a significant decrease in circulating basophil counts and an increase in CCL2 levels compared with those in pre-challenge samples.

While there is a plethora of information from murine models regarding the role of basophils in allergic/asthma-like inflammation, the involvement of basophils in human asthma again derives mainly from indirect evidence (164). Most compelling, basophils have been found in the airways of asthmatics (171, 172), in post-mortem cases of fatal asthma (173) and after antigen challenge of airway mucosa (174). Basophil releasability (i.e., the ability of a basophil to release a given percentage of histamine in response to a given immunological stimulus) is long reported to be increased in asthma and more recently subject to circadian changes (175). Moreover, allergen-induced asthmatic responses are accompanied by infiltration of basophils expressing IL-4 mRNA (103). The *in vitro* secretion of both IL-4 and IL-13 has been shown to track with the basophil-enriched fractions of cells recovered after infiltrating the lung following segmental allergen challenge (176, 177). Moreover, these so-called basophil cytokine responses also correlated with the frequency of eosinophils recovered from the lung. Thus, basophils might represent an important source of Th2-like cytokines (IL-4 and IL-13) in the lung microenvironment, particularly that associated with human allergic disease.

Brooks and collaborators reported that basophils are increased in the sputum of patients with eosinophilic asthma compared to those with non-eosinophilic asthma (178). In asthmatics, basophils were positively correlated with sputum eosinophils and inversely with sputum neutrophils, but not with FEV_1, FEV_1/FVC or bronchodilator reversibility. Sputum basophils positively correlated with sputum eosinophils (179). In comparison with blood basophils, sputum basophils have a higher expression of activation markers (e.g., CD203c) (179). These findings indicate that basophils may be involved in eosinophilic asthma and that sputum basophil assessment could be a useful additional indicator of "Th2-high" asthma. Basophil counts in peripheral blood during childhood asthma are associated with exacerbations (180). The proportion of degranulated basophils can also be associated with recurrent exacerbations.

Hill et al. reported that omalizumab, a mAb that targets IgE and neutralizes it from binding to FcεRIα on basophils, reduces blood basophil frequencies in asthmatic children (181). Furthermore, treatment of severe asthma patients with benralizumab, a mAb against IL-5Rα, markedly decreased the number of both eosinophils and basophils (182–184). These findings suggest that benralizumab may have a positive effect on severe asthmatics by reducing not only eosinophils but also basophils.

A number of mouse studies indicate that basophils are involved in the development of asthma-like pathology. In an ovalbumin-induced asthma model, basophils recruited to the lungs, amplify the Th2 cell differentiation (185). In a papain-induced asthma model, basophil-derived IL-4 induces the IL-5 and CCL11 expression in ILC2 cells, causing eosinophil infiltration (68). Indeed, in a model of IgE-dependent dermatitis, the production of IL-4 from basophils was shown to directly condition endothelium for increased VCAM-1 expression, which facilitated the *in vivo* entry of eosinophils into lesion sites (186). This mechanistic observation may help elucidate the eosinophil/basophil IL-4 associations commonly seen in human disease.

Chronic spontaneous urticaria (CSU) is a common skin disease, characterized by spontaneous appearance of wheals, angioedema or both, for more than 6 weeks due to known or unknown causes (187, 188). A role for basophils in the pathophysiology of CSU is suggested by a number of findings (189, 190). CSU subjects have been shown to have significant increases in the numbers of intradermal basophils compared with non-atopic control subjects (191). Basopenia has long been reported in patients with CSU (192) and more recently postulated as the result of basophil migration from the circulation into the skin (104, 191, 193). The degree of basopenia often correlates with disease severity (194) and improves during times of remission (195). CSU subjects exhibit enhanced expression of the activation markers CD63 and CD69 on basophils compared to non-allergic subjects (196).

Rauber et al. identified three distinct immunologic phenotypes of CSU (197). One group of patients' basophils reacted to FcεRI stimulation, whereas the others had anti-FcεRI nonreactive basophils. Among the latter, it was found a subgroup with basopenia. This subgroup had augmented serum-induced basophil activation, increased levels of autoantibodies against thyroid peroxidase, and worse quality of life. These phenotypes were associated with different clinical characteristics, pointing to basophils as important players in CSU pathophysiology (197). Oda et al. demonstrated that basophils from CSU patients had higher FcεRI expression compared to healthy controls. The proportion of CD203c^{high} basophils after anti-IgE or anti-FcεRI stimulation was lower in CSU patients compared to controls and characteristics of more severe patients (198).

Omalizumab is a mAb anti-IgE often used in treating severe allergic asthma (199, 200). More recently, it has also proved highly effective in patients with CSU (201). Surprisingly, this treatment, regardless of the disease being treated, is associated with increased expression of Syk, which is often also manifested by basophils showing greater histamine release *in vitro* when undergoing IgE/FcεRIα-dependent stimulation. This enhanced responsiveness is seen even through cell-surface FcεRIα/IgE levels are reduced with this treatment (196, 202). These observations have since prompted the same group of authors to suggest that Syk expression and IgE-mediated histamine release in basophils could function as biomarkers for predicting the clinical efficacy of omalizumab in patients receiving this therapy (203).

In following CSU subjects treated with omalizumab, MacGlashan and collaborators have also identified three basophil phenotypes in CSU patients: 1) subjects with basopenia, 2) normal basophil numbers with normal IgE-mediated histamine release, and 3) normal basophil numbers with poor histamine release. Basopenia was associated with the presence of autoantibodies to unoccupied FcεRI and basophil numbers did not change during omalizumab treatment. Omalizumab resulted in similar kinetics for decreases in surface FcεRI and IgE in all three groups of CSU patients (204).

Atopic dermatitis is a common inflammatory skin disorder characterized by chronic eczema and severe itching (205). Th2 cells mediate inflammation in atopic dermatitis with the release of IL-4 and IL-13, which contribute to clinical manifestations (206, 207). Keratinocyte-derived alarmins, such as IL-33, TSLP, and IL-25 (IL-17E) that elicit Th2 cytokines responses by activating group-2 innate lymphoid cells (ILC2s) play an upstream pathogenic role in atopic dermatitis (208, 209). Recent evidence indicates that LTC_4 also plays a role in mouse models of atopic dermatitis (210). IgG autoantibodies against IgE from atopic dermatitis can induce the release of IL-4/IL-13 and LTC_4 from human basophils (134, 211), indicating that these cells contribute to this allergic disorder.

Early studies reported that up to 80% of food-allergic children exhibit high spontaneous basophil histamine release (212). Moreover, food-allergic children release histamine in response to an IgE-dependent histamine-releasing factor (213). Schroeder and collaborators demonstrated that basophils from food-allergic children also spontaneously release IL-4 and overexpress CD203c (214). Interestingly, spontaneous basophil histamine release and IL-4 secretion decreased in children undergoing sublingual immunotherapy (215). *In vitro* studies show that this enhanced releasability of histamine and IL-4 from basophils of food-allergic children is transferred to basophils of normal subjects by sensitizing normal cells with plasma from the former group. However, the addition of omalizumab during this passive sensitization completely

abated the responses, thus pointing to the involvement of IgE in transferring hyperresponsiveness (214).

Figure 1 schematically illustrates the versatile contribution of basophils and their mediators to the development of allergic disorders.

Peripheral blood basophils in human hematological tumors

Polycythemia vera (PV) is a clonal proliferation of erythroid, megakaryocytic, and myeloid cell lines (232, 233). More than 90% of patients with JAK2-STAT activating mutations (JAK2V617F or exon 12 mutations) are characterized by an overactive JAK-STAT pathway (234, 235). Pruritus and increased basophil-derived mediators (e.g., histamine) are common in PV patients (233, 236, 237). Peripheral blood basophils (238) and CD63 expression are increased in PV patients and are hyperresponsive to IL-3. Increased releasability of histamine from PV basophils can contribute to pruritus in these patients.

Basophilia can develop during the advanced phase of chronic myeloid leukemia (CML) (239) and the transcription factor IKAROS is reduced in the bone marrow from these patients (240). Basophils from CML patients express HGF, promoting CML cell expansion

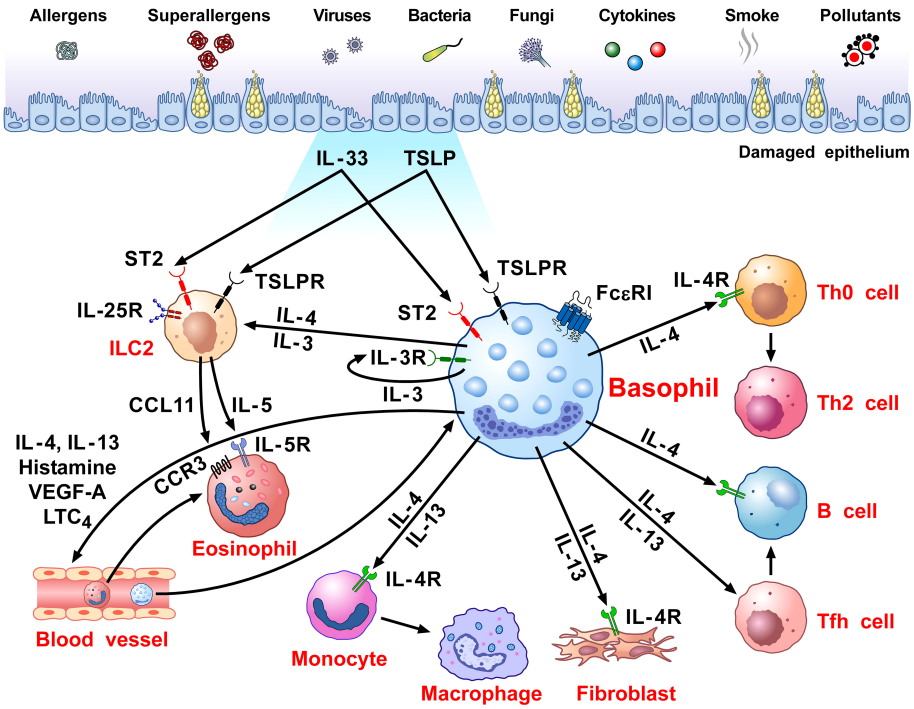

FIGURE 1
Schematic representation of the versatile role of basophils in the pathobiology of allergic disorders. Several immunological (i.e., allergens, superallergens, viral, bacterial and fungal proteins, cytokines) and non-immunological stimuli (e.g., pollutants, diesel exhaust particles) activate mucosal (i.e., lung and gut) and skin barriers to release different alarmins (i.e., TSLP, IL-33, IL-25) (130, 216, 217). Alarmins activate group 2 innate lymphoid cells (ILC2s) through the engagement of specific receptors (TSLPR, ST2, IL-25R, respectively) (218, 219) to release IL-5 and CCL11 that promote eosinophil infiltration into inflamed tissue (220, 221). Human and mouse basophils express the high-affinity receptor for IgE (FcεRI) (36, 37) and the receptors for IL-3 (IL-3Rα/CD123) (34, 44), GM-CSF (CD116) (45, 46), IL-33 (ST2/IL1RL1) (47–50), IL-5 (CD125) (51) and a variety of chemokine receptors (5, 55–60). The TSLP receptor (TSLPR/IL-7Rα) is expressed by mouse basophils (12, 34), but its presence on basophils from allergic and healthy donors remains controversial (12, 14–16). TSLP activates mouse but not human basophils (12, 17). IL-3 plays a key role in the development, survival and activation of human and mouse basophils (17). IL-3 activates human and mouse basophils to release cytokines and chemokines (12, 17, 46, 222). IgE-FcεRI crosslinking by antigens, superallergens and functional anti-IgE autoantibodies activates basophils to release a wide spectrum of inflammatory and immunomodulatory factors (24, 70, 75, 76, 78, 79, 134, 211, 223). IL-33 activates human and mouse basophils to release several cytokines and chemokines (12). Activated human (12, 24, 74–82) and mouse (12, 47, 80) basophils secrete large amounts of IL-4. Both human (12, 75, 76, 78, 81–85) and mouse basophils (12, 47) also release IL-13. Human basophils secrete several angiogenic factors such as vascular endothelial growth factor-A (VEGF-A) (64). Basophil-derived IL-4 activates ILC2s to enhance the release of IL-5 and CCL11, leading to eosinophil infiltration (68). IL-4 promotes Th2 cell differentiation and enhances humoral immune responses (224). IL-4, together with IL-13, induces T follicular helper cells (Tfh) to promote IgE responses (225, 226). Basophil-derived IL-4 and IL-13 act on inflammatory monocytes inducing their differentiation into M2 macrophages (227). IL-4 and IL-13 activate fibroblasts to promote the production of chemokines (CCL5 and CCL11) (228) and collagen (229). IL-4 and IL-13 and vasoactive mediators (histamine, LTC_4, VEGF-A) act on blood endothelial cells (64, 230) to upregulate the expression of vascular cell adhesion molecule-1 (VCAM-1) (231), leading to enhanced trans-endothelial migration of eosinophils and basophils (186).

(91). In a mouse model of CML, basophil-derived CCL3 promotes CML development (241, 242). The presence of basophilia is considered an independent risk factor for the progression from myelodysplastic syndrome to acute myeloid leukemia (243, 244).

Basophils in solid cancers

Basophils are physiologically present in low numbers in peripheral blood. Under certain inflammatory circumstances, the number of circulating basophils can be altered, activated, or migrate from the bloodstream to the sites of inflammation (23, 245). Increased and decreased peripheral blood basophils can be associated with the progression of certain human solid cancers (Table 1) (256, 257). Basophilia positively correlates with improved outcomes in melanoma (246, 247), ovarian cancer (248), non-small cell lung cancer (NSCLC) (251), and glioblastoma (252), while basopenia is associated with a poor prognosis for colorectal cancer (245, 249, 250). Basophilia is also linked to improved outcomes in melanoma patients receiving immunotherapy (247). By contrast, in other solid tumors, such as prostate (253) and gastric cancers (250), a detrimental role of circulating or tissue-infiltrating basophils has been reported. Moreover, baseline basophil count predicts recurrence in bladder cancer patients receiving bacillus Calmette-Guérin (BCG) following resection (254). Interestingly, in a mouse model of breast cancer, basopenia correlated with an increased number of pulmonary metastasis (258). However, basophils are not associated with prognosis in breast cancer patients (259). Basophils may support humoral immunity by secreting several B-cell modulating molecules. Once activated, basophils may express CD40L, IL-4, and IL-6 to sustain B-cell proliferation and empower the production of IgM and IgG1. Gomez and colleagues demonstrated that, *in vitro* and *in vivo*, basophils sustain plasma cell survival (245, 260). Histamine is released from basophils and it has been suggested that it can be involved in colon carcinoma (CRC) (245).

Bax and coworkers have investigated the presence and functions of basophils from peripheral blood and in ovarian cancer (248). The same group reported that basophilia and basophils who possess greater ability for *ex vivo* stimulation are associated with improved outcomes (261). Additionally, a positive correlation between improved progression-free survival of patients and activated basophil markers ($CD63^+$, $CD203c^+$, CD123, CCR3, FcεRI) was observed in the TME of ovarian cancer (Bax, Chauhan et al., 2020). These results indicate that activated peripheral blood and intratumoral basophils correlate with a survival benefit in ovarian cancer patients (261). Nevertheless, these favorable effects that basophils might mediate in targeting the tumor for destruction can potentially result in unfavorable outcomes. For example, basophils have been found in ascitic fluid from ovarian cancer patients and it has been suggested that their release of vasoactive mediators (e.g., histamine) may exacerbate fluid accumulation in the peritoneal cavity (58).

It has been reported that the expression of cytokines by lung-resident basophils can be induced by local signals (e.g., IL-33, GM-CSF) (47, 102), emphasizing the plasticity of these cells. Hence, the lung microenvironment might influence the transcriptional and functional development of basophils. Likewise, these resident basophils seemingly play an important role in lung development and function by forming cellular networks and facilitating so-called macrophage imprinting. Low percentages of basophils (0.4%) were located in the immune infiltrate of human non-small cell lung cancer (NSCLC) tumors (262). Basophils have been identified in the

TABLE 1 Role of peripheral blood basophils in human solid cancers.

Tumor type	Prognostic/ predictive role	Reported observation	References
Melanoma	Favourable	Basophilia is associated with improved outcome in melanoma patients receiving immunotherapy with nivolumab plus ipilimumab and in newly diagnosed stage I-II melanoma patients	(246, 247)
Ovarian cancer	Favourable	A higher frequency of circulating basophils and the presence of activated basophil signature are associated with improved overall survival in ovarian cancer patients	(248)
Colorectal cancer	Favourable	Low pretreatment basophil counts are associated with worse prognosis and higher tumor aggressiveness in colorectal cancer patients	(245, 249, 250)
NSCLC	Favourable	Higher basophil counts are associated with increased probability of responding to ICI therapy in two cohorts of stage-IV NSCLC patients	(251)
Glioblastoma	Favourable	Increased pre-operation circulating basophils predict better progression free survival in patients	(252)
Prostate cancer	Unfavourable	Elevated baseline basophils and basophil-to-lymphocyte ratio are associated with worse clinical outcomes in metastatic hormone sensitive prostate cancer patients	(253)
Bladder cancer	Unfavourable	Baseline basophil count may predict recurrence in BCG-treated primary bladder cancer patients	(254)
Gastric cancer	Unfavourable	Elevated baseline basophil counts are prognostic for unfavorable clinical outcomes in gastric cancer patients treated with ICI plus chemotherapy,	(255)

BCG, Bacillus Calmette-Guérin; ICI, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer.

immune landscape in early (stage I) lung adenocarcinoma and in non-involved lung tissue (nLung) (102). It remains unclear what the exact function basophils mediate in the TME, yet emerging evidence points to their capacity to secrete IL-4 and IL-13 as playing a potential role. For example, mouse and human studies have shown that basophils, by secreting these cytokines, facilitate the development/expansion of M2-like monocytes/macrophages (227, 263–265), which are often a prominent part of the immune cell landscape of the TME. However, in chronic inflammation, the exposure of basophils to certain cytokines, such as IL-33, may induce the polarization of lung macrophages to M2-like phenotype characterized by the expression of anti-inflammatory genes *Clec7a, Arg1, Itgax*. In this context, basophils are participants in the inflammatory entourage in lung cancer (261). It seems equally possible that basophil-derived IL-4/IL-13 also favor tumorigenesis by diminishing Th1-like immunity that is better suited to contest the cancer (80). Should these hypotheses prove correct, then another important question that arises pertains to the endogenous stimulus responsible for inducing these cytokines. In this regard, Schroeder et al. demonstrated that purified human basophils release histamine, IL-4 and IL-13 when co-cultured with the lung adenocarcinoma cell line A549 (16). Unexpectedly, these effects required IgE-expressing basophils and were suppressed by specific inhibitors of FcεRI signaling. A subsequent study revealed that the IgE-binding lectin, galectin-3, expressed on the A549 cells, was responsible for this model of basophil activation (223). In fact, galectin-3 is a biomarker and/or factor implicated in many kinds of cancer, chronic inflammation, cardiovascular disease, autoimmunity, and also beneficially in wound healing (266). These results thus reveal an innovative mechanism by which galectin-3 expressed by human lung carcinoma cells are able to activate basophils [and likely other cell types, namely dendritic cells (DCs) and monocytes] (267) to release cytokines and pro-inflammatory mediators. Further studies are necessary to understand the role of galectin-3 in activating basophils, and how IL-4/IL-13 and other mediators could contribute to human and experimental lung cancer.

Interestingly, many immune cells and markers that have a mounting prominence in cancer/tumorigenesis are also observed in experimental models of wound healing. For example, scaffolds that promote wound-healing often induce Th2 immune responses, whereby IL-4 and IL-13 are recognized as critical cytokines that help initiate the process (268). M2 cells, whose development is often dependent on the actions of IL-4/IL-13, are also widely implicated in wound healing. Not surprisingly, much emphasis is placed on the role of Th2 cells in being the source of IL-4/IL-13. However, in a recent publication that explored the mechanisms associated with wound healing following experimental myocardial infarction (MI), basophils were identified as a critical source of IL-4/IL-13 required for the healing process. Specifically targeting basophils using conditional knockouts or by antibody-mediated depletion, significantly impaired this wound healing (269). Moreover, the administration of IPSE-α1, an IgE-binding glycoprotein isolated from helminth eggs and well known for activating basophils for IL-4/IL-13, greatly augmented healing following the MI. While the endogenous ligand for stimulating IL-4/IL-13 from basophils in this model was not reported, it is intriguing to speculate that galectin-3 is involved. Indeed, galectin-3 is often a prominent marker in wound healing, both at the transcriptional and protein levels (266).

Investigation on the role of basophils in models of melanoma has provided interesting results in Foxp3^{DTR} mice, in which these cells caused melanoma rejection (270). CCL3 and CCL4 produced by intratumoral basophils induced CD8^{+} lymphocyte recruitment in TME. The administration of FcεRI (MAR-1) mAb in Foxp3^{DTR} melanoma-bearing mice depleted basophils and abrogated the recruitment of CD8^{+} T cells thus preventing the rejection of melanoma. Furthermore, the IL-3/anti-IL-3 antibody complexes combined with adoptive T cell transfer induced basophilia and consequent T cell infiltration, which positively correlated with melanoma rejection. Unfortunately, the MAR-1 antibody can also deplete/activate other immune cells (e.g., mast cells, DCs, monocytes) which express FcεRI (271, 272). Thus, studies in newer genetically engineered basophil-deficient mouse models (80, 97) appear necessary to establish the role of basophils in melanoma.

IL-33 is a cytokine that induces tumoricidal functions in eosinophils (273, 274) and upregulates granzyme B mRNA and the surface expression of CD63 (67), suggesting phenotypic and functional activation. Moreover, IL-33-activated basophils co-cultured with B16.F10 melanoma cells, inhibited tumor cell-growth compared to melanoma cells co-cultured with unstimulated basophils (67).

In a pioneering observation, Ann M. Dvorak first demonstrated piecemeal degranulation of basophils in human pancreatic cancer (PC) (7). Elegant studies evaluated the role of basophils in experimental and human ductal adenocarcinoma (PDAC) (80). In PDAC patients, they identified *IL4* expressing basophils in tumor-draining lymph nodes (TDLNs). Basophils in TDLNs were an independent negative prognostic biomarker of patient survival. They also evaluated basophil role in PC using the *Mcpt8*-Cre basophil deficient (275) and wild-type (WT) mice. After PC implant, cancer was detected in 80% WT, but not in basophil-deficient mice. Basophils were found in TDLNs and cancer-associated fibroblasts (CAFs) released TSLP, which activated DCs to produce IL-3 from CD4^{+} T cells. CCL7, produced by DCs and CD14^{+} monocytes, induced basophil migration into TDLNs. Basophils activated by IL-3 played a pro-tumorigenic role through the production of IL-4, which favored Th2 and M2 polarization. These findings are consistent with our results indicating that basophil-derived IL-4 (and IL-13) promote M2-like cells (263).

Topical exposure of the skin of mice to an environmental DNA-damaging xenobiotic [i.e., 7,12-dimethylbenz [a] anthracene (DMBA)] caused the development of squamous-cell carcinomas (SCCs), high serum levels of IgE and tumor infiltration of IgE-bearing basophils (276). In this model, FcεRI$^+$ basophils mediated the DMBA-induced IgE protection against carcinogenesis. In contrast, topical exposure of the skin of mice to the proinflammatory agent 12-0-tetradecanoylphorbol-13-acetate (TPA) increased serum IgE and IgE-bearing basophils in the skin that promoted carcinogenesis (97). In a two-stage model of epithelial carcinogenesis (DMBA and subsequent exposure to TPA), Hayes and coworkers also discovered that mice lacking IgE (*lgh7*$^{-/-}$) were less responsive to tumor development compared to WT mice (97). IgE-signaling was crucial for mediator release from basophils and infiltrating tissue basophils showed expression of *Cxcr2, Cxcr4*, and *Ptgdr2* (CRTH2, the PGD_2 receptor). Basophil infiltration into the inflamed skin was mediated by TSLP/IL-3-mediated upregulation of CXCR4 on basophils. The *Mcpt8*$^{Cre/+}$ mice, presenting normal mast cell numbers but strongly reduced basophils (275), were less responsive to tumor growth. Table 2 summarizes the role of basophils in the TME of different solid cancers.

Colony-stimulating factor 1 (CSF1) is a primary regulator of monocytes/macrophage that sustains macrophage polarization towards an M2-like phenotype (278). Mouse basophils resident in the lung express high levels of *Csf1* and contribute to M2 polarization of lung macrophage (47). The functional relevance of basophil-derived CSF1 was also underlined *in vivo* in a murine model of atopic dermatitis, where it promoted M2-like macrophage polarization (279). Interestingly, an inhibitor of CSF1/CSF1 receptor signaling reduced tumor-associated macrophage (TAM) infiltration in the TME of sarcoma models (278). These experimental findings may have translational relevance in cancer: there is the possibility that CSF1, in conjunction with basophil-derived IL-4/IL-13, might enhance the M2-like/TAM polarization of macrophage in TME (280).

A synopsis of the above findings signifies some conflicting views of the role that basophils potentially mediate in tumorigenesis. A more classical interpretation (from the ovarian, lung, colorectal, and melanoma data) suggests basophils mediate anti-tumor effects (248, 261, 270, 276). While the mechanisms underlying the beneficial outcomes are poorly defined, it has been proposed that some basophil-derived mediators (e.g., granzyme B and TNF-α) exert tumoricidal activity while others (e.g., CCL3 and CCL4) facilitate the recruitment of cytotoxic CD8$^+$ T cells (Figure 2). In contrast, there is growing evidence that basophils, under certain circumstances, can promote tumorigenesis (Figure 3). In this instance, the tumor cell itself seemingly modulates basophil responses, causing a release of cytokines that favor the development of protumorigenic TME. Interestingly, this latter scenario shares many similarities with that seen in wound healing.

Conclusions

Basophils were initially considered as effector cells of allergic diseases (166, 230). The discovery that murine (290) and human

TABLE 2 Role of basophils in tumor microenvironment.

Tumor type	Effect on cancer	Observed role	Mechanism	References
Melanoma	Anti-tumoral	Treg depletion results in infiltration of basophils and CD8$^+$ T cells in the TME that promote tumor rejection in mice IL-33-activated mouse basophils induce melanoma cell death *in vitro*	CCL3/CCL4 secretion by intratumoral basophils induces CD8$^+$ T cell recruitment in TME Release of Granzyme-B	(270) (67)
Ovarian cancer	Anti-tumoral	Activated signature (CD123, CCR3, FcεRI, CD63, CD203c gene expression) in tumor-resident basophils is associated with improved outcomes in these patients	NA	(248, 261)
Lung cancer	Anti-tumoral	Higher expression of basophil markers (CD123, CCR3, and FcεRI) in tumors is associated with improved overall survival in lung cancer patients	NA	(261)
	Pro-tumoral	Lung inflammatory cytokines trigger basophil-induced M2 polarization	Basophil secretion of IL-4/IL-13	(47)
Skin cancer	Anti-tumoral	Topic exposure to DNA-damaging carcinogen DMBA promotes tumor-protective IgE response through skin infiltrating basophils	Possible release of cytotoxic soluble mediators	(276)
	Pro-tumoral	Skin inflammation by TPA, MC903 or R848 induced IgE/FcεRI-signalling in basophils promote epithelial carcinogenesis	TSLP/IL-3-mediated upregulation of CXCR4 on basophils	(97)
Gastric cancer	Pro-tumoral	Increased tumor-infiltrating basophils in tissues from gastric cancer patients are negatively associated with therapy response	Increased tumor M2 macrophage infiltration	(255, 277)
Pancreatic cancer	Pro-tumoral	IL-4-secreting basophils are significantly increased in TDLNs of PDAC patients, correlate with predominant Th2 inflammation and represent an independent prognostic factor of poorer survival after surgery	Recruitment in TDLN mediated by alternatively activated monocyte-secreted CCL7/MCP3	(80)

DMBA, 7,12-dimethylbenz [a] anthracene; MC903, vitamin D3 analogue; NA, not assessed; PDAC, pancreatic ductal adenocarcinoma;R848, resiquimod; TPA, 12-O-tetradecanoylphorbol-13-acetate.

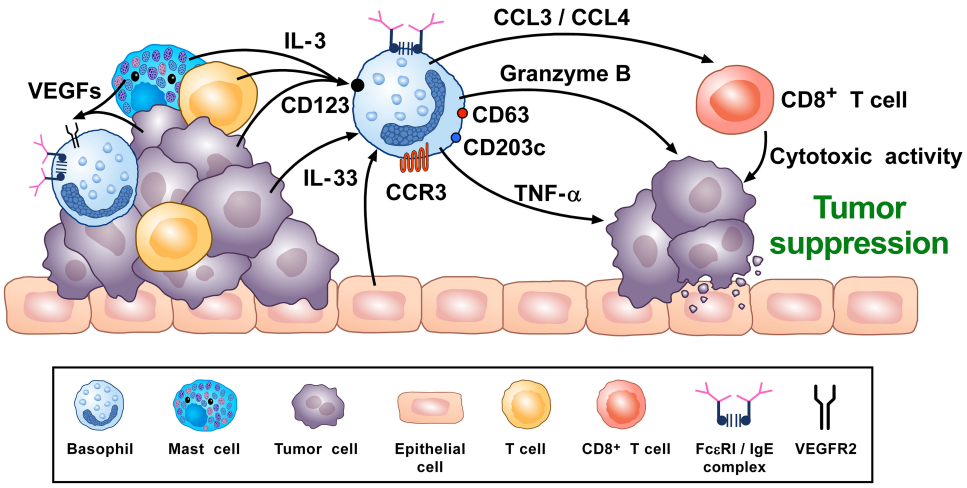

FIGURE 2
Theoretical representation of how basophils can promote tumor suppression. Basophils have been located in the immune infiltrate of several human (80, 102, 261, 262) and experimental tumors (80, 97, 102, 276). Vascular endothelial growth factors released by cancer cells and immune cells in tumor microenvironment (TME) (e.g., mast cells, macrophages) (118, 120–123) can favor basophil infiltration in TME through the engagement of VEGFR2 on these cells (120). IL-3, produced by intratumoral lymphocytes, mast cells and cancer cells (17, 82, 281), is the most important growth and activating factor for human and mouse basophils, through the engagement of the IL-3 receptor (IL-3Rα/CD123) (17). CCL3/CCL4 secreted by intratumoral basophils induces $CD8^+$ T cell recruitment in TME, promoting melanoma rejection in mice (270). IL-33, a pleiotropic cytokine produced by epithelial and tumor cells (282), plays a central role in tumorigenesis (282). IL-33 upregulates granzyme B mRNA and the surface expression of CD63, suggesting functional and phenotypic basophil activation. IL-33-activated mouse basophils induce melanoma cell death *in vitro* (67). Mouse (47, 86) and, under specific circumstances, human basophils (88, 89) release TNF-α. Human and mouse basophils release granzyme B (66, 67). Both TNF-α and granzyme B exert cytotoxic effects on tumor cells (68, 69). Activated signature (CD123, CCR3, CD63, CD203c gene expression) in tumor resident basophils is associated with improved outcome in ovarian cancer patients (248, 261). Topical exposure to a DNA-damaging carcinogen promotes tumor-protective IgE response through skin infiltrating basophils (276). Taken together, these results suggest that, in certain experimental and clinical conditions, basophils and their mediators may play an anti-tumorigenic role.

basophils produce immunomodulatory cytokines (e.g., IL-4, IL-3, and IL-13) (28, 76–78, 81, 85, 291) changed dramatically this erroneous concept. In addition, human and murine basophils release several canonical (24, 64, 90, 91) and non-canonical angiogenic factors (133) that play a pivotal role in inflammatory and tumor angiogenesis. Further *in vitro* and *in vivo* studies are needed to investigate the contribution of angiogenic factors released by mouse and human basophils in experimental and human tumors.

Basophils have been identified in human lung (102), gastric (99, 100), pancreatic (7, 80) and ovarian cancer (248). Lung-resident basophils (47) can provoke M2 polarization of lung macrophages, as occurs in several tumors (105, 106). The presence of basophils and their activation signatures appear to be linked with more favorable patient outcomes in certain tumors (melanoma, lung cancer, ovarian cancer) (248, 261, 270). Otherwise, with particular reference to gastric and pancreatic cancers, increased tumor-infiltrating basophils are negatively associated with less favorable overall survival (80, 255, 277).

Basophil functions *in vivo* have been evaluated through several models of basophil-deficient mice (275, 292–294). It should be remembered that, in some instances, studies using antibody-depleted basophils have produced erroneous findings due to lack of antibody specificity (271, 272) and even new mouse basophil-targeted mutants have some off-target hematological alterations (295). Therefore, the evaluation of basophil functions in complex and heterogeneous disorders, such as cancer and allergic diseases using multiple genetically engineered models of basophil deficiency, demands caution in data interpretation.

Collectively, recent findings highlight the critical contributions of basophils during homeostatic conditions and beyond their ability to promote allergic inflammation. Further studies are needed to understand the mechanisms and environmental factors driving basophils to play a pro- or anti-tumorigenic role in experimental and human cancers. A better knowledge of the involvement and functions of basophils in human immunity appears necessary considering the participation of these cells in immune and cancer cell crosstalk and in priming of several immune cell types. Single-cell RNA-seq of the immune landscape of tumor cells will be of paramount importance to characterize the role of basophils in different types of human and experimental cancer. Understanding of the molecular mechanisms

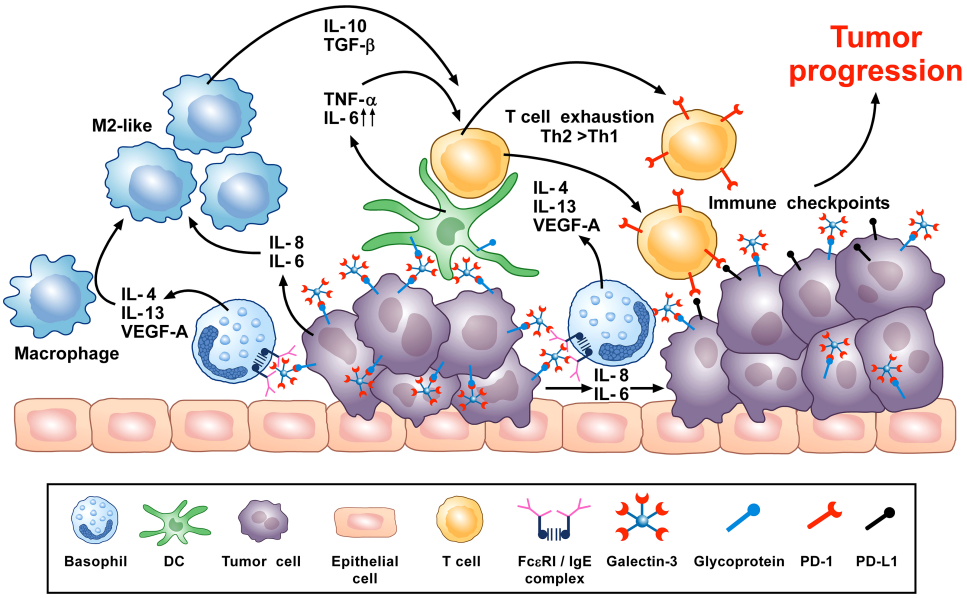

FIGURE 3
Theoretical representation of how basophils can promote tumor progression. Galectin-3 (Gal-3) is a lectin highly expressed by many types of cancer cells, frequently manifesting as a marker of poor prognosis with capacity to mediate immunosuppression within the tumor microenvironment (TME) (266). Recent *in vitro* studies show that Gal-3, expressed by the A549 adenocarcinoma cell line (or EC-Gal-3), has the capacity to activate basophils to secrete copious amounts of IL-4/IL-13 (16, 223). Both cytokines are known to promote M2-like macrophages, which are major players in the TME (227, 263–265). IL-4-producing basophils have been identified in the TME of human pancreatic cancer, with mouse models indicating that this IL-4 promotes a Th2>Th1 response that is more conducive to tumorigenesis (80). Additionally, basophils are long known to secrete VEGF-A (64) that promotes angiogenesis. Other studies show that basophils can induce IL-6/IL-8 secretion from cell lines through a mechanism requiring cell-to-cell contact (283) (JTS, unpublished). This tumor cell-derived IL-6/IL-8 is implicated in playing a critical role in metastasis formation (284). Likewise, dendritic cells and monocytes activated by EC-Gal-3 are shown to produce high levels of TNF-α/IL-6 *in vitro* (285). Chronic production of these cytokines, when combined with M2 cell-derived IL-10/TGF-β, are implicated in promoting T-cell exhaustion by up-regulating checkpoint inhibitors (e.g., PD-1) that interact with tumor cell-associated markers (PD-L1) to suppress cytotoxic T cell activity (286). V-domain immunoglobulin suppressor of T-cell activation (VISTA) is another immune checkpoint receptor which plays a role in cancer progression (287, 288) and regulates allergen-specific Th2-mediated immune responses (289). Overall, it is proposed that the combined actions of these dysregulated innate immune responses synergize to promote tumorigenesis.

orchestrated by basophils in the TME of several cancer types could allow to develop novel pharmacological/immunological strategies to modulate basophil functions and perhaps to prevent tumor progression.

Author contributions

RP and ARG are co-first authors of this manuscript. All authors contributed to the article and approved the submitted version. GS and GV are co-senior authors of this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Skin-homing basophils and beyond

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Basophils have been implicated in type 2 inflammation and numerous disorders in the skin such as helminth infection, atopic dermatitis, and urticaria. Although similar in form and function to tissue-resident mast cells, classical studies on basophils have centered on those from the hematopoietic compartment. However, increasing studies in tissues like the skin demonstrate that basophils may take on particular characteristics by responding to unique developmental, chemotactic, and activation cues. Herein, we highlight how recent studies in barrier immunology suggest the presence of skin-homing basophils that harbor a unique identity in terms of phenotype, function, and motility. These concepts may uniquely inform how basophils contribute to diseases at multiple epithelial surfaces and our ability to therapeutically target the innate immune system in disease.

KEYWORDS

basophil, CysLTR2, histamine, IL-33 and ST2, leukotriene C4, mast cell, MRGPRX2

Introduction

Basophils are rare granulocytes, accounting for <1% of leukocytes in the peripheral blood, spleen, and bone marrow. Basophils were first described by Paul Ehrlich in 1879. Subsequently, several groups have discovered that basophils in the blood are a source of histamine in the 1950s (1–3). However, it was not until 1972 that basophils were shown to be activated by allergens in an IgE-mediated fashion (4). Given their similarity in form and function to tissue-resident mast cells, basophils have long been considered "circulating mast cells", although their differences and similarities are often debated. Thus, they have long been studied as a surrogate for mast cells due to their accessibility *via* the peripheral blood.

Monitoring of human basophils by flow cytometry has revealed changes in cell surface markers and activation of basophils (5). Moreover, a recent study on human basophils by Blom et al. reported unique chemokine receptor expression patterns upon

IgE-mediated or non-IgE-mediated activation, strongly suggesting heterogeneous activation manners in human basophils (6). In contrast to human basophils, the characteristics and functions of murine basophils *in vivo* have come to light with the advent of antibody-mediated cell depletion methods (e.g., anti-FcεRIa, CD200R3, or Thy1 antibodies) (7–9). However, such methods were not sufficient to distinguish the unique role of murine basophils from mast cells *in vivo* due to the bystander effects on mast cells. This problem was overcome with the development of unique transgenic mouse technologies and the identification of basophil-specific genes and markers (e.g., Mcpt8-DTR, Mcpt8-Cre, Bas-TRECK Tg, and Basoph8 mice) (10–13). Indeed, these advances have made it possible to directly compare basophils with mast cells, revealing that these two myeloid cell populations differ in surface marker expression, factors required for terminal differentiation, signaling pathways, release of inflammatory mediators, and impact on disease.

Furthermore, it is generally accepted that basophils are effector cells of the innate immune system that promote type 2 immunity and inflammation through the release of a variety of mediators including the type 2 cytokines IL-4 and IL-13. Although residing in the circulation, basophils are rapidly recruited into the tissues such as the intestine, lung, and skin upon inflammation (14). Thus, they have been implicated in promoting the expulsion of helminth parasites from mucosal barriers and in the pathophysiology of a variety of allergic disorders such as asthma, atopic dermatitis (AD), food allergy, and chronic spontaneous urticaria (CSU) (15–19). Further, recent studies have shed light on novel functions of basophils which may even reside in peripheral organs (20–22). However, how basophils are recruited to the tissues upon stimulation and the manner in which they are activated or survive in tissues remain poorly understood. Moreover, the precise contribution of basophils to various allergic disorders such as AD continues to be debated even though many studies have implicated basophils as putative drivers in AD pathogenesis based on both mouse and human studies (17, 23–27).

Herein, we highlight recent advances in basophil biology in peripheral organs such as the skin and how they provoke new hypotheses and theories about basophil function more broadly. We propose revisiting a number of assumptions about the properties of basophils in tissues using new approaches, technology, and therapeutics.

Developmental, maturation, and activation cues from the tissue

Both basophils and mast cells differentiate from hematopoietic stem cells *via* common myeloid progenitors and granulocyte monocyte progenitors (GMPs). Although similar in terms of granularity, expression of the high affinity IgE receptor (FcεRI), and shared effector molecules, basophils largely reside in the circulation while mast cells reside in other tissues. Recent studies demonstrate that mast cells arise from the yolk sac and aorta-gonad-mesonephros, and the degree to which they are replenished by bone marrow precursors is variable depending on the organ (28). Skin-resident mast cells, in particular, are devoid of bone marrow-derived mast cells and are mostly seeded in the early phase of embryonic development (29, 30). These findings help to explain, at least in part, why the majority of allergic disorders involving mast cells develop early in life. Furthermore, these findings provoke the hypothesis that dysregulated mast cell development could be one explanation for the heterogeneity of allergic pathologies and therapeutic responses. Notwithstanding these insights into the diversity of mast cell subpopulations, it is largely unknown whether related developmental sophistication underlies basophil heterogeneity.

IL-3 is an important growth factor for both basophils and mast cells (31). For example, IL-3 deficient mice exhibited impaired expansion of basophils and mast cells in a setting of nematode infection despite no obvious abnormality in their number at steady state (32). IL-3 is also capable of promoting basophil differentiation from bone marrow cells and survival *in vitro* (33, 34). Moreover, IL-3 augments cytokine production from basophils after IgE crosslinking, a canonical activation mechanism in basophils (35). Collectively, many of these early studies established IL-3 as a key regulatory cytokine for basophils as well as mast cell proliferation and function. However, most of these studies centered on studying basophils within the hematopoietic compartment. The precise properties of basophils within barrier tissues have been traditionally poorly understood.

In addition to IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), Toll-like receptors (TLRs), and thymic stromal lymphopoietin (TSLP) are also known to regulate basophil development (36–38). Among them, TSLP has been shown to act directly on bone marrow and extramedullary progenitors to promote basophil hematopoiesis independently of IL-3 in mice (20, 36). Furthermore, murine basophils differentiated by TSLP have unique transcriptional profiles and activation states compared to those developed under IL-3-enriched conditions (20). In contrast to murine basophils, human basophils from healthy donors do not respond to TSLP without IL-3 priming (39). However, disease-associated human basophils from patients with asthma were responsive to TSLP alone (40). These findings suggest that inflammatory conditions could affect the responsiveness of the human basophil. In the skin, TSLP is consistently upregulated during AD-associated skin inflammation and has long been pursued as a therapeutic target in humans (41, 42). However, the efficacy of targeting TSLP as a therapy in AD has been brought into question, as the anti-TSLP monoclonal antibody (mAb) tezepelumab has not been successful in treating AD (43).

Several studies have implicated TSLP-elicited basophils in murine models of allergic diseases such as AD, food allergy, and eosinophilic esophagitis (15, 16, 20). In addition, TSLP-elicited murine basophils exhibit a highly activated phenotype as evidenced by upregulation of key activating cytokine receptors including those for IL-18 (IL-18R) and IL-33 (ST2, IL-33R) in comparison to IL-3-elicited basophils (20). Both IL-18 and IL-33 are now considered canonical activating cytokines for basophils and strongly implicated in AD-associated inflammation in both mice and humans (44–48). These findings suggest that skin inflammation in AD may skew basophil development *via* epithelial cell-derived TSLP, creating a reservoir of basophils that can be rapidly activated by skin-associated IL-18 and IL-33. We refer to these basophils as uniquely skin-homing (Figure 1). Similar to IL-33, it is now appreciated that IL-18, in contrast to other organs, acts as an alarmin in the skin to potently promote type 2 immune responses (49). These findings may explain, in part, the failure of tezepelumab in phase 2 clinical trials for AD, as transient blockade of TSLP may not be sufficient to reset the population of basophils that are hyperresponsive to other alarmins in the skin (43). In other words, a typical 12-week clinical trial would likely not be able to capture clinical responses related to such biological effects. Notwithstanding the duration, another possibility is that TSLP blockade alone is no longer sufficient to suppress basophil-mediated skin inflammation after the accumulation of basophils in the skin that exhibit a unique transcriptional signature; such basophils may require simultaneous blockade of IL-18 and/or IL-33 for synergistic therapeutic efficacy. Future studies are warranted to determine the precise array of regulatory cytokines that need to be disrupted to suppress basophils and AD-associated inflammation.

The emergence of skin-homing basophils

Classically considered short-lived, both murine and human basophils have been shown to rapidly lose their viability in a matter of a few days *in vivo* and *in vitro*, respectively (9, 50). However, these survival assays were performed on bulk populations of basophils from the bone marrow, blood, or spleen. It is increasingly appreciated that when basophils traffick into the skin (or possibly the lung), they can acquire distinct transcriptional and functional properties (21, 22). We have long observed that while basophils are generally absent in healthy skin, upon the induction of AD-like inflammation, they traffick into the skin as early as day 4 and persist stably through day 12, and likely well beyond (17, 51). Further, it has recently been shown that basophils in AD-like skin are distinct from splenic basophils and persist in the skin beyond the acute inflammatory phase to aid in the resolution of inflammation. Strikingly, these late-phase basophils promote the expansion of M2-like macrophages *via* cooperative production of IL-4 and monocyte colony-stimulating factor (M-CSF) (21). It has recently been shown that basophils which reside in the lung at early developmental stages imprint a unique developmental program in alveolar macrophages. Indeed, these lung-

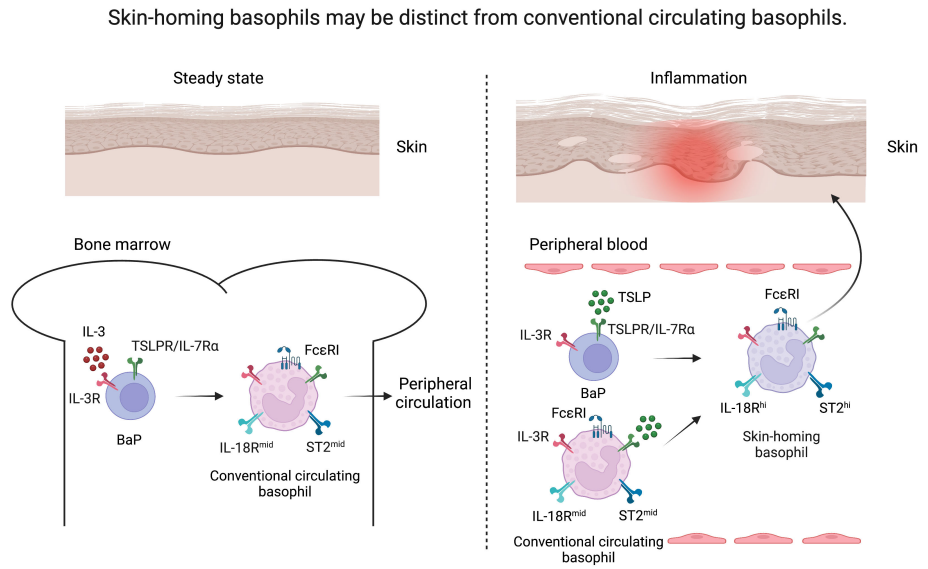

FIGURE 1
Skin-homing basophils may be distinct from conventional circulating basophils. Under steady state, basophil progenitors (BaP) develop into FcεRI^{hi} basophils under the influence of IL-3 in the bone marrow (conventional circulating basophils, left). Upon inflammation, TSLP released from the skin drives the maturation of BaPs to exhibit a highly activated phenotype (skin-homing basophils, right) as evidenced by upregulation of IL-18R and ST2 in comparison to IL-3-elicited basophils.

associated basophils demonstrated a distinct transcriptional profile from those in circulation (22). In addition to transcriptional differences, basophils in the skin could show morphologically distinct characteristics compared to circulating basophils. For example, Cheng et al. found that basophils accumulated in antigen-sensitized skin close to blood vessels, while those in non-sensitized skin were more widely distributed upon antigen challenge (52). Similarly, basophils have been shown to exhibit unique motility and apparent contacts with sensory neurons upon the antigen challenge as well in the setting of AD-like disease (53). Taken together, these studies provoke the hypothesis that basophils, upon entry into the skin, acquire a distinct transcriptional program leading to the distinct morphological changes and unique survival and effector programs not observed from traditional studies in the hematopoietic compartments, which likely focused more on conventional circulating basophils. However, it is important to note that these studies on basophil heterogeneity used *Mcpt8* as a basophil-specific marker for transcriptional studies and cell-depletion. Recent studies have suggested that integrin$\beta7^+$ mast cells also express Mcpt8 both in the skin and the lung under allergic inflammation (54, 55). Nevertheless, how basophils could acquire distinct identities in peripheral organs remains to be fully clarified and addressed.

While it is increasingly appreciated that there is developmental and functional heterogeneity of basophils, it has only recently come to light how diversity of basophil function can influence different aspects of a single disease (56, 57). For example, it is well-recognized that basophils are associated with human AD and promote the pathogenesis of AD-like disease in mice (17, 21, 23, 24, 27). By contrast, as described above, it has been observed that in the resolution phase of AD-like disease in mice, basophils in the skin also promote restoration of barrier function and disease resolution (21). In a context of itch sensation, basophils appear to be dispensable for chronic itch, while they are known to be essential for allergen-mediated acute itch (25, 27, 53). Indeed, it has been shown that TSLP promotes a program that is also highly enriched for the arachidonic acid pathway which leads to the production of leukotrienes and other bioactive lipids that serve as key effector molecules of murine basophils (20). One such leukotriene, namely LTC4 is now recognized as a very potent pruritogen (53, 58, 59). Taken together, these findings demonstrate the sophisticated array of effector functions orchestrated by basophils.

CSU exemplifies how skin-homing basophils can help to explain disease pathogenesis. CSU is an itchy, immune-mediated skin disorder that afflicts 1% of the population and has a profoundly negative impact on quality of life. It is defined by both hives and itch; these processes are mediated, in part, by activation of IgE and release of histamine from mast cells. Notwithstanding the role of mast cells, it is also appreciated that basophils accumulate in the lesions of urticaria, and that blood basophil deficiency is a feature of CSU (60). Thus, it is hypothesized that basophils recruited to the skin could contribute to the pathogenesis of CSU. The role of basophils in CSU is further suggested by a report that the number of basophils in the blood of CSU patients increases after anti-IgE mAb (omalizumab) treatment (60). Another study has revealed that the surface expression of FcεRI on basophils was lower in CSU patients who showed better response to omalizumab (61). In addition, it is known that IgE and FcεRI trigger the migration of murine mast cells toward antigens and that IgE and FcεRI also mediate human basophil migration *in vitro* (62–64). Thus, these studies suggest that omalizumab may inhibit IgE-mediated activation in basophils, resulting in decreased motility into the skin. Future studies will be required to clarify this possibility.

However, the activation of basophils in CSU does not seem to be exclusively explained by IgE- and FcεRI-mediated pathways. Antihistamines are the first-line therapy for CSU; however, even high doses are insufficient in 54% of patients (65). Anti-IgE therapy is the second-line strategy to which 40% of patients with CSU are refractory as well (66). These therapeutic gaps strongly suggest that other histamine- and IgE-independent pathways are operative. In 2015, a seminal paper by McNeil et al. identified that Mas-related G protein-coupled receptor B2 (MrgprB2), and its human ortholog, MRGPRX2 are key receptors that respond to a host of cationic neuropeptides and drugs that induce IgE-independent mast cell activation or allergic-like reactions (67). Indeed, MRGPRX2 has been identified as a possible biomarker in CSU (68). Although the expression and function of MRGPRX2 were mainly studied in mast cells, it has recently been reported that human basophils also express MRGPRX2 (69, 70). Given the potential role of MRGPRX2 on both mast cells and basophils, the heterogeneity of the therapeutic response in CSU may be explained, in part, by the overall composition of IgE-reactive vs. MRGPRX2-reactive mast cells and basophils, respectively, in CSU. This remains a major area of investigation to inform new pathways for treatment.

MRGPRX2 is now emerging as a therapeutic target in the field of allergy. However, MRGPRX2 expression on basophils either at steady state or upon activation remains a major area of controversy (71). It is hypothesized that MRGPRX2 is often internalized in basophils but could be exposed upon activation (71). In support of this, it has been shown that MRGPRX2 expression on human basophils was upregulated by cross-linking of IgE, complement component 5a (C5a), natural N-formyl peptide (fMLP) or IL-3 stimulation *in vitro* (69, 70). In relation to mast cells, MRGPRX2 function was promoted by TSLP but was dampened by SCF or IL-4 (72–74). Therefore, we speculate that maturation and/or activating factors for basophils including IL-18, IL-33, or TSLP could modulate MRGPRX2 expression on human basophils, contributing to their functional heterogeneity (Figure 2). To this end, future studies are required to determine the precise ligands and their effects on non-

canonical basophil activation and function. We hypothesize that, given MRGPRX2's close association with skin-resident mast cells, its expression on basophils likely marks their identity as also being more skin-associated or homing.

Trafficking of basophils into the skin

Although we have discussed how immune dysregulation may promote the emergence of a unique population of basophils that is capable of responding to skin-derived signals, how basophils are recruited into the skin remains a mystery. It is well-known that basophils rapidly accumulate into peripheral tissues including the skin in a variety of settings such as helminth infection, tick bite, or allergic inflammation (11, 19, 75–77). However, there is still a paucity of evidence on the specific chemokines or cellular processes involved in basophil trafficking. Human studies *ex vivo* have revealed that basophils can migrate toward numerous chemokines (e.g., CCL2/3/5/7/11/13, and CXCL12/13), C5a, Prostaglandin D2 (PGD2), Thromboxane B2, urokinase-type plasminogen activator, and bacterial/viral peptides (fMLP and gp41) (78–87). Notably, serum levels of CCL2 were found to be elevated in a setting of venom- or food-induced anaphylaxis, which correlated a decrease in circulating basophil numbers (88). In addition, basophil accumulation in human skin or xenografted skin was observed after intradermal injection of CCL2 or CCL17, respectively (89, 90). Another study revealed increased migration of basophils in patients with systemic lupus erythematosus toward CXCL12 compared to those from healthy controls (91). A recent study by Blom et al. revealed that human basophils activated by IgE, C5a, or fMLP express various types of chemokine receptors including CCR4, CCR10, CCR6, CCR8, XCR1 and CCX-CKR, some of which are known as skin-homing receptors (6). In this study, they also found a bimodal expression of certain chemokine receptors such as XCR1, cutaneous lymphocyte antigen (CLA), or CXCR4 even among the $CD63^+$ activated subset, further supporting phenotypic heterogeneity of human basophils upon activation. Puan et al. revealed that FUT6 is essential to sialyl-Lewis x (CD15s) expression on human basophils and its deficiency severely reduces their rolling capacity on E-selectins and cutaneous allergic symptoms (92). In mice, both PGD2 and CXCL12 have also been shown to be important in basophil trafficking to secondary lymphoid organs in a murine lupus model, while other studies showed CCL7-dependent migration to the draining lymph nodes in a context of pancreatic tumor or type 2 skin inflammation (91, 93–95). The accumulation of basophils in the lymph nodes after helminth infection depends on IL-3 from $CD4^+$ T cells (96), while IL-3 supplied by skin-resident $CD4^+$ memory T cells is essential for their recruitment to the skin in the setting of tick bite (97). In the setting of AD-associated inflammation, basophil recruitment to the skin is uniquely dependent on TSLP (20); similar dependence on TSLP has also been observed with intradermal injection of lipoteichoic acid (LTA), a cell wall component of bacteria (98). Moreover, under TPA-induced chronic skin inflammation, TSLP and IL-3 externalize CXCR4 expression on basophils and their recruitment to the skin depends on CXCL12-CXCR4 axis and IgE (99). Taken together, these studies demonstrate that a number of factors have been implicated in basophil trafficking in the past (Table 1). However, future studies will have to be aimed at understanding the tissue-specific signals that drive basophil migration into various organs and their unique interactions in the context of disease.

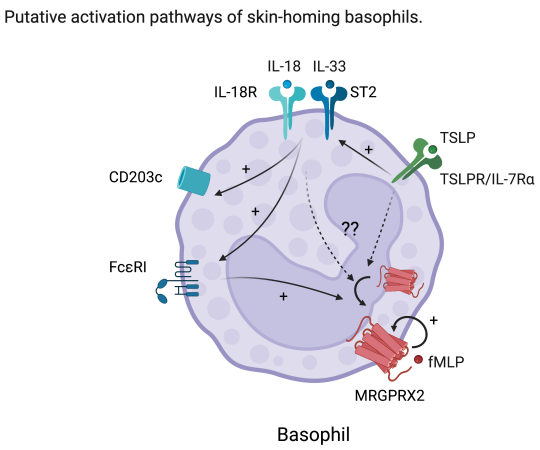

FIGURE 2
Hypothetic characteristic activation of skin-homing basophils. TSLP enhances the response to IL-18 and IL-33 in basophils. IL-18 and IL-33 further activate basophils resulting in upregulation of conventional activation markers such as CD203c and enhancement of FcεRI expression. However, the effects of these skin-derived cues on MRGPRX2 expression in basophils are still unknown. We hypothesize that skin-derived cues also upregulate or externalize MRGPRX2, which is considered to be internalized at steady state on human basophils.

Additionally, it appears that basophils can exhibit heterogeneous behavior even within the same tissue under the same inflammatory condition. We have found that *in vivo* stimulation with allergen in the skin results in the emergence of two distinct populations of basophils - one that is enlarged and immotile and another that is small and highly motile in the setting of AD-associated acute itch flares (53). Although why such heterogeneity of motility exists within the skin remains unknown, these findings support the hypothesis that there are likely numerous different subsets of basophils across tissues that respond differentially to even the same signals. Thus, basophil trafficking could be regulated in a subset-dependent manner, indicating increasing complexity in terms of their regulation.

As noted above, there is significant evidence that basophils imprint unique transcriptional and functional programs onto macrophages in the skin and lung (21, 22). However, whether

TABLE 1 *In vivo* or *ex vivo* evidence for basophil trafficking to tissues.

Species	Trafficking sites	Factor	Tentative Source	Experimental or disease condition	Experiment type	Ref
Human	N/A	CCL2, CCL5, CCL7	N/A	Transwell migiration	*Ex vivo*	(78)
		CCL5, CCL7, CCL11, CCL13			*Ex vivo*	(79)
		CCL2, CCL3, CCL5, CCL11, CXCL12			*Ex vivo*	(80)
		CCL2, CCL11, CXCL12, IL-8			*Ex vivo*	(81)
		CCL2, CCL11		Transendothelial migration	*Ex vivo*	(82)
		C5a		Transwell migiration	*Ex vivo*	(83)
		Prostaglandin D2			*Ex vivo*	(84)
		Thromboxane B2			*Ex vivo*	(85)
		Urokinase-type plasminogen activator			*Ex vivo*	(86)
		fMLP or gp41			*Ex vivo*	(87)
		CD15s		Rolling assay	*Ex vivo*	(92)
Human	N/A	CCL2	Stromal cells?	Anaphylaxis	*In vivo*	(88)
	Skin	CCL2	N/A	Intradermal injection into human skin	*In vivo*	(89)
	Skin	CCL17	Endothelial cells, Keratinocytes?	Intradermal injection into skin xenograft of humanized mice	*In vivo*	(90)
	Secondary lymphoid organs	CXCL12	N/A	Systemic lupus erythematosus	*In vivo*	(91)
	Skin?	CD15s	Basphils	Mosquito-bite or skin prick test to house dust mite	*In vivo*	(92)
Murine	Skin	IL-3	CD4+ T cells	Tick-bite	*In vivo*	(97)
	Skin	TSLP	N/A	MC903-induced skin inflammation	*In vivo*	(20)
	Skin	TSLP	Keratinocytes?	Lipoteichoic acid injection	*In vivo*	(98)
	Skin	TSLP/IL-3, CXCR4 and IgE	N/A	TPA-induced skin inflammation	*In vivo*	(99)
	Lymph nodes	IL-3	CD4+ T cells	Helminth infection	*In vivo*	(96)
	Lymph nodes	PGD2	N/A	Lupus nephritis	*In vivo*	(91)
	Lymph nodes	CXCL12	N/A	Lupus nephritis	*In vivo*	(93)
	Lymph nodes	CCL7	Monocytes	Pancreatic cancer	*Ex vivo*	(94)
	Lymph nodes	CCL7	Dendritic cells	Papain-induced type 2 skin inflammation	*In vivo*	(95)

N/A, not applicable.

skin-resident macrophages recruit basophils into the skin *via* reciprocal interactions remain to be shown. There is a large body of work that suggests that other circulating granulocytes like neutrophils are heavily influenced by tissue-resident macrophage-derived signals upon tissue damage or pathogen entry (100–102). Indeed, macrophages are capable of producing various types of mediators which have been implicated in basophil chemotaxis *in vitro* (e.g., CCL2, CXCL1, CXCL2, C5a) (103, 104). Thus, we speculate that homologous mechanisms likely underlie basophil recruitment as well in the context of helminth parasite invasion or allergic barrier disruption. Future studies will be required to determine the full range of cellular and molecular cues that aid in the homing of basophils into the skin.

Finally, whether specific populations of basophils go back into the circulation and travel distally also remains poorly understood. In the setting of helminth infection, it is reported that group 2 innate lymphoid cells (ILC2s) in the tissue are extruded to the circulation to disseminate type 2 inflammation (105). Both skin-homing basophils and ILC2s receive similar activation cues from the skin (e.g., IL-18 or IL-33) to critically mediate type 2 inflammation, despite being rare populations. In light of our speculation that skin-homing basophils acquire the ability to survive much longer, it is possible that basophils could also move from the skin into the circulation and on to other distal sites. However, future studies will be required to fully understand the importance of basophil movement into and out of the skin.

Conclusion

The unique characteristics of basophils have been greatly informed in the last decade due the development of unique tools. Studies using animal models have revealed their critical involvement in a number of disease states in the skin including helminth infection, tick bites, and AD (15–17, 106). However, in addition to their ability to promote allergy, basophils are increasingly appreciated for their dynamic ability to respond to allergen, cytokines, and exhibit both proinflammatory and restorative properties. By understanding how specific subsets of basophils may have unique proinflammatory, survival, and survival properties, we speculate that selectively targeting such basophils may represent a highly effective therapeutic strategy for a variety of skin diseases such as AD, or CSU.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

RS contributed to writing manuscript and crating Figures under supervision of BK. All authors contributed to the article and approved the submitted version.

Conflict of interest

BK has served as a consultant for 23andMe, AbbVie, ABRAX Japan, Almirall, Amagma Therapeutics, Arcutis Biotherapetuics, Arena Pharmaceuticals, argenx BV, AstraZeneca, Bellus Health, Blueprint Medicines, Boehringer Ingelheim, Bristol Myers Squib, Cara Therapeutics, Clexio Biosciences, Cymabay Therapeutics, Daewoong Pharmaceutical Company, Eli Lilly and Company, Escient Pharmaceuticals, Evommune, Galderma, Genentech, GlaxoSmithKline, Granular Therapeutics, Incyte Corporation, Janssen, Kiniksa, LEO Pharma, Maruho Co., Medicxi, Menlo Therapeutics, Novartis, OM Pharma, Pfizer, Recens Medical, Regeneron, Sanofi, Septerna, Third Harmonic, Trevi Therapeutics, Veradermics. He holds stock in Locus Biosciences, Recens Medical, and ABRAX Japan.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CD25 as a unique marker on human basophils in stable-mildly symptomatic allergic asthma

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Background: Basophils in acute asthma exacerbation are activated as evidenced by their increased expression levels of activation markers such as CD203c and CD63. However, whether basophils of allergic asthmatics who are in stable phase and have no asthma exacerbations display a specific and distinctive phenotype from those of healthy individuals has yet to be well characterized.

Objective: We aimed to identify the phenotype of basophils from allergic asthmatics in the stable phase and investigate whether such a phenotype is affected by *ex vivo* allergen stimulation.

Methods: We determined by flow cytometry, the expression of surface proteins such as CD25, CD32, CD63, CD69, CD203c, and CD300a and intracellular anti-apoptotic proteins BCL-2, BCL-xL, and MCL-1. We investigated these markers in blood basophils obtained from well-characterized patients with stable-mildly symptomatic form of allergic asthma with no asthma exacerbation and from healthy individuals. Moreover, we determined *ex vivo* CD63, CD69, and CD25 on blood basophils from stable-mildly symptomatic allergic asthmatics upon allergen stimulation.

Results: In contrast to all tested markers, CD25 was significantly increased on circulating basophils in the patient cohort with stable-mildly symptomatic allergic asthma than in healthy controls. The expression levels of CD25 on blood basophils showed a tendency to positively correlate with FeNO levels. Notably, CD25 expression was not affected by *ex vivo* allergen stimulation of blood basophils from stable-mildly symptomatic allergic asthma patients.

Conclusion: Our data identifies CD25 as a unique marker on blood basophils of the stable phase of allergic asthma but not of asthma exacerbation as mimicked by *ex vivo* allergen stimulation.

KEYWORDS

CD25, basophils, immunophenotype, stable-mildly symptomatic allergic asthma, *ex vivo* stimulation

1 Introduction

Basophils are essential effector cells in T2-mediated allergic immune responses. Although basophils account for ≤1% of circulating leukocytes, they actively participate in allergic reactions through the release of effector and immunoregulatory mediators, including vasoactive amines (histamine), lipid metabolites (leukotriene C4, LTC_4), and T2 cytokines (IL-4 and IL-13). The role of basophils in allergic responses has become more evident with the recognition and in-depth understanding of late-phase responses in chronic allergic inflammatory disorders such as asthma and rhinitis. Several studies in humans and mice identified that basophils infiltrate into lung tissues (1–4). Studies on induced sputum and lung tissues showed that the numbers of basophils in the airways of asthmatics are elevated (1), and they were further increased during asthma exacerbation (2) or following allergen challenge (3). Moreover, high levels of basophil-derived IL-4 in the lung have been detected after segmental allergen challenge (4). These findings thus confirm that basophils are active players of airway inflammation in allergic asthma.

Several studies from others and us reported that an allergic inflammatory milieu, such as IL-3, IL-5, and GM-CSF, transforms basophils into a primed state (5–7). Exposure to these priming stimuli causes increased sensitivity to activation and also enhances multiple biological functions such as inflammatory cytokine release (IL-4, IL-13, IL-8), LTC_4 formation, chemotaxis, and survival (8). Although other cytokines can induce similar changes, IL-3 is considered the most effective priming factor of human basophils to cause long-term phenotypic and functional changes by itself and in synergy with other stimuli (e.g. FcεRI cross-linking, IL-33) (8–12). For instance, IL-3 boosts the rapid upregulation of CD203c (13) and CD69 (14) on human basophils. Interestingly, prolonged continuous IL-3 receptor-mediated signaling induces the expression of CD25 on human basophils (8).

In addition to its activating effect, IL-3 is also characterized as a pro-survival factor of human basophils. Several studies have indicated that resistance to apoptosis prevails in the allergic airway inflammation of asthma patients (15, 16). Recently, we showed that in the presence of IL-3, basophils are insensitive towards apoptosis induced by IFN-α, extrinsic (TRAIL) (17), and intrinsic (BH3-mimetics) (18) apoptotic stimuli. Furthermore, using BH3-mimetics, we revealed that the observed resistance of basophils to apoptosis in the presence of IL-3 is achieved through the upregulation of anti-apoptotic proteins such as BCL-xL and MCL-1 (19).

Suzuki et al. (20) recently demonstrated that airway basophils show a more activated phenotype than circulating basophils, as evidenced by increased CD203c expression on sputum basophils. Similarly, CD203c was upregulated in blood and sputum basophils after allergen challenge (21) and during asthma exacerbation (13). Thus, basophils undergo several functional and phenotypic alterations during the acute phase of allergic response and asthma exacerbation. Whether such alterations persist during the steady-state of allergic asthma is less well investigated. Hence, in the present study, we investigate whether blood basophils in stable-mildly symptomatic allergic asthma exhibit altered expression levels of surface markers CD25, CD32, CD63, CD69, CD203c, and CD300a, and anti-apoptotic proteins (BCL-2, BCL-xL, and MCL-1) in comparison to blood basophils from healthy controls. Moreover, we study the effect of *ex vivo* allergen challenge on the expression of CD63, CD69, and CD25 on blood basophils from stable-mildly symptomatic allergic asthmatics. Our data confirm that CD25 is a unique marker on human blood basophils in patients with stable or mildly symptomatic of allergic asthma.

2 Materials and methods

2.1 Study design

Eighteen adults who have a history of stable-mildly symptomatic asthma according to Global Initiative for Asthma (GINA) criteria were recruited from the outpatient clinic of the Department of Pulmonary Medicine, Inselspital, Bern University Hospital, Bern, Switzerland. General inclusion criteria of the study were volunteers of age above 18 years, non-smokers, and all genders were eligible. Allergy was confirmed in this study by a positive skin prick test (SPT) and the detection of specific IgE to aeroallergens. A positive SPT was defined as a wheal of ≥3 mm using the extracts of common aeroallergens such as Olea europea, grass pollen (mixture), birch, hazel, beech, dog and cat epithelia, *Dermatophagoid pteronyssinus*, rye (GREER Laboratories), mugwort, short ragweed, adler, common ash, aspergillus, *Dermatophagoid Farinae*, *Cladosporium herbarum* (if not mentioned all from Bencard AG). The specific IgE to aeroallergens (Sx1: timothy-grass, birch, dog and cat epithelia, *Dermatophagoid pteronyssinus*, rye, mugwort, and *Cladosporium herbarum*) was determined by ImmunoCap technology and regarded as positive if it was ≥0.35 kU/L. Exclusion criteria were infection of airways, use of systemic corticosteroids, and immunosuppressants in the four weeks prior to sample collection. In this study, patients with allergic rhinitis are not included and patients with chronic obstructive pulmonary disease or, allergic bronchopulmonary aspergillosis, or eosinophilic granulomatosis with polyangiitis are excluded as well. The healthy controls (n=10) were non-smokers and reported no history of chronic respiratory disease, and their specific IgE to aeroallergen was <0.35 kU/l. All subjects signed informed consent forms approved by the Ethics Committee of the canton of Bern (no. 2016-01571), and experiments were conducted according to the Declaration of Helsinki.

Included patients (n=18) had 1-2 visits during which blood samples were collected. These blood samples were used to determine counts of eosinophils, neutrophils and basophils using an automated hematology analyzer (Sysmex XS-800i), to analyze surface and internal marker expression in basophils by flow cytometry, and to measure specific IgE against common aeroallergens by ImmunoCap technology. In addition, on every visit, asthma patients were evaluated by spirometry including forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC)], asthma control test (ACT) questionnaire score, exhaled nitric oxide fraction (FeNO).

2.2 Flow cytometry analysis of basophils in peripheral blood

100µl of EDTA whole blood samples was prepared for flow cytometric measurements using antibody panels as specified in Table 1. Surface markers were stained for 15 min at room temperature (RT). Subsequently, red blood cells were lysed using BD Lysing Solution (BD Biosciences) for 15min at RT. Afterward, samples were washed and resuspended in 600µl Staining Buffer A, composed of 1xPBS supplemented with 2% heat-activated FCS and 0.05% sodium azide (Merck Millipore).

For intracellular staining, samples were washed with Staining Buffer B, consisting of 1xPBS supplemented with 0.5% BSA and 0.1% sodium azide and permeabilized with Permeabilization solution 2 (BD Biosciences). Next, cells were washed, and intracellular molecules were stained for 30min at RT. Finally, samples were washed and resuspended in 600µl Staining Buffer B. Samples were acquired using BD FACSCanto II. Data were collected using FACSDIVA (all from BD Biosciences) and analyzed by Flowjo software (Treestar Inc). A minimum of 1000 basophils were acquired per sample. The gating strategies used to identify basophils in peripheral blood are described in Figures S1A–C.

2.3 *Ex vivo* stimulation of basophils

Reagents and protocols of Flow2CAST (Blühmann Laboratories) with slightly modified settings (stimulation time, individual antibodies) were used to activate basophils *ex vivo*. The aeroallergens that elicited the strongest positive response in SPT (birch, *Dermatophagoid pteronyssinus*, *Dermatophagoid farinae*, grass, and rye) in the respective allergic asthma patients were chosen for the *ex vivo* stimulation experiment. Briefly, 50µl of EDTA blood from allergic asthma patients who were sensitive was stimulated with either 50µl (100ng/ml) anti-FcεRI cross-linking antibody 29C6 (Roche), 50µl (20ng/ml or 100ng/ml) of the patient's respective aeroallergen in 100µl (or 150µl for unstimulated control) stimulation buffer containing IL-3 for 20min at 37°C, 5% CO_2.

Cells were stained simultaneously with anti-CCR3 Alexa Fluor 647 (5E8; BioLegend), anti-CD63 V450 (H5C6; BD Biosciences), anti-CD69 PerCP (FN50; BioLegend), anti-CD25 APC (BC96;

TABLE 1 Panels for flow cytometric analysis of blood samples.

	Target	Fluorochrome	Clone
Panel #1	LIN (CD3, CD16, CD19, CD20, CD14, CD56)	FITC	SK7, 3G8, SJ25C1, L27, MfP9, NCAM16.2
	CD123	PerCP-Cy5.5	7G3
	CCR3	Alexa Fluor 647	5E8
	CD203c	PE	97A6
	CD32	BV421	FLi8.26
Panel #2	LIN (CD3, CD16, CD19, CD20, CD14, CD56)	FITC	SK7, 3G8, SJ25C1, L27, MfP9, NCAM16.2
	CCR3	APC-Cy7	5E8
	CD300a	PE	MEM-260
	CD63	V450	H5C6
	CD69	PerCP	FN50
	CD25	APC	BC96
Panel of Intracellular Staining	LIN (CD3, CD16, CD19, CD20, CD14, CD56)	FITC	SK7, 3G8, SJ25C1, L27, MfP9, NCAM16.2
	CD123	PerCP-Cy5.5	7G3
	CCR3	Alexa Fluor 647	5E8
	BCL-2 or BCL-xL or MCL-1	PE	124, 54H6, D2W9E

BioLegend). Data were acquired using FACSCanto with FACS Diva software. For gating strategy, debris and doublets were excluded first, followed by gating for $SSC^{low}/CCR3^{pos}$ basophils. Within $SSC^{low}/CCR3^{pos}$ basophils, the cells were further gated for $CD63^{pos}/CCR3^{pos}$ and $CD63^{neg}/CCR3^{pos}$ for degranulated and non-degranulated basophils, respectively as shown in Figure S1D. CD69 and CD25 expression was subsequently analyzed in $CD63^{neg}$ and $CD63^{pos}$ basophils, respectively.

2.4 Statistical analysis

Data were analyzed using GraphPad Prism 8.3 (GraphPad). The alpha level was set to 0.05. If not mentioned, otherwise data are represented as mean ± SEM. Statistical correlations were evaluated by the Spearman rank test. For two-group comparisons, two-tailed Student t-test, and Mann-Whitney test were used. One-way ANOVA with Dunnett`s or Bonferroni`s multiple comparison test was used when more than two groups were analyzed. Results were considered significant if P values were <0.05. P values were defined as **** P< 0.0001, *** P<0.001, ** P<0.01 and * P<0.05. ns, not significant.

3 Results

3.1 Patient characteristics

Demographic and clinical characteristics of the recruited subjects are summarized in Table 2 and Supplementary Table 1. The mean age of the study participants was 37 years (range: 18-59 years), and 72% of them were female. All participants had a history of allergic asthma and were tested positive for at least one among the 16 common aeroallergens (skin prick test, wheal ≥3 mm and ImmunoCap sx1>0.35kU/L). Only one out of eighteen patients showed non-detectable specific IgE (ImmunoCap sx1<0.35kU/L) during one of the visits (Supplementary Table 2). No subject reported or had documented asthma exacerbations during the study period. The asthma control test (ACT) scores (20.97 ± 3.537, mean ± SD) showed that the patients enrolled were suffering from a stable-mildly symptomatic form of allergic asthma. The cut-off for ACT scores used were 20-25: stable, 16-19: mild and < 15: severe. Asthma was not under control for three out of eighteen patients (for three patients in the first visit and for one patient for every visits) (Supplementary Table 1). The fractional exhaled nitric oxide (FeNO) values (28.28 ± 26.89, mean ± SD) indicated that the majority of the sample size (90%) did not associate with severe/chronic lung inflammation (FeNO<50ppb, Supplementary Table 1). Altogether, the ACT scores and FeNO values indicated that the subjects enrolled in this study had stable-mildly symptomatic form of allergic asthma during the majority of visits (Supplementary Table 1).

3.2 Unique upregulation of CD25 expression on blood basophils in patients with stable-mildly symptomatic form of allergic asthma

During asthma exacerbation, blood basophils display an activated phenotype (13). Moreover, anti-apoptotic conditions prevail in basophils under allergic conditions (17, 19). Whether

TABLE 2 Characteristics of the patient cohort.

Characteristics	Values	Sample size
Age, yrs		18
Mean (Range)	37.36 (18-59)	
Gender, n		18
Female	13	
Male	5	
BMI, kg/m^2		18
Mean ± SD	24.83 ± 4.524	
% Atopy (skin prick test)*	100	18
Atopy (specific IgE), kU/L§		18
Mean ± SD	24.96 ± 26.87	
ACT score		18
Mean ± SD	20.97 ± 3.537	
FeNO, ppb		18
Mean ± SD	28.28 ± 26.89	
Blood basophils, G/L		18
Mean ± SD	0.042 ± 0.015	
Blood eosinophils, G/L		18
Mean ± SD	0.246 ± 0.183	
Blood neutrophils, G/L		18
Mean ± SD	3.262 ± 1.22	
% FEV_1 predicted		18
Mean ± SD	91.83 ± 13.92	
% FVC predicted		18
Mean ± SD	100.8 ± 11.81	

BMI, body mass index; FeNO, fractional exhaled nitric oxide; ppb, parts per billion; ACT, asthma control test; G/L, 10^6 cells/L; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; Mean ± SD, mean± Standard deviation (For each clinical parameter, the mean value for each individual during their 1-2 visits was first calculated, and then the mean and standard deviation of these mean values were determined).
*Atopy on skin prick test was defined by at least one positive test (wheal ≥3 mm) against common aeroallergens such as Olea europea, grass pollen (mixture), birch, hazel, beech, dog and cat epithelia, *Dermatophagoid pteronyssinus*, rye, mugwort, short ragweed, adler, common ash, aspergillus, *Dermatophagoid farinae*, *Cladosporium herbarum*.
§At least one of the IgE RAST ≥ 0.35 kU/L (Dermatophagoides pteronyssinis, cat and dog dander, timothy, rye, Cladosporium herbarum, birch, mugwort).

the activated and anti-apoptotic phenotype persist during the steady-state of stable allergic asthma is less well investigated. Hence, we compared the immunophenotype of basophils and their predisposition to apoptosis in the stable-mildly symptomatic allergic asthma patient cohort and healthy controls. As shown in Figures 1A, B, flow cytometry analysis revealed that CD25 expression on blood basophils was significantly higher (P=0.0065 and P=0.0159) for the first and second visit, respectively in stable-mildly symptomatic allergic asthma patients than in healthy control subjects. The proportion of basophils from our patient cohort and healthy controls were comparable (Figure 1C). There was no significant difference in CD25 expression when comparing the first and second visit of the stable-mildly symptomatic allergic asthma patients. For all other analyzed markers, particularly, the activation markers CD63, CD69, CD203c and CD300a no significant difference was observed (Figures 1D–H and Figures S2A-C). This observation indicates that circulating basophils from stable-mildly symptomatic asthmatics were not hyperactivated.

Correlation analysis between CD25 expression on blood basophils and absolute counts (G/L) of eosinophils, basophils and neutrophils, respectively, revealed no significance (Figures S3A-C). FeNO levels of stable-mildly symptomatic allergic asthmatics showed a weak positive correlation (r=0.5186; P=0.0274) when comparing the CD25 expression levels on circulating basophils of the first visit (Figure 1I, left), but not of the second visit (r=0.2091; P=0.5393) (Figure 1I, right). This inconsistent observation in the correlation between CD25 and FeNO in the first and second visits could be due to the lower sample number in the second visit (n=11) than in the first visit (n=18). The levels of CD25 (P=0.03) were significantly higher expressed in patients with intermediate FeNO values (FeNO ≥25 ppb; Figure 1J) according to American Thoracic Society guidelines (22) than in patients with low FeNO (FeNO <25 ppb).

3.3 CD25 expression on basophils is not affected by allergen challenge *ex vivo*

The surface expression of CD63, CD203c and CD69 is commonly used to assess human basophil activation upon acute hypersensitivity allergic reaction. This encouraged us to investigate further whether the expression levels of CD25 on circulating basophils of patients with stable-mildly symptomatic allergic asthma are altered upon *ex vivo* allergen exposure. We confirmed basophil degranulation by a significant increase in the surface expression of CD63 in peripheral basophils, and the increment of expression was found to be dependent on allergen concentration, as shown in Figure 2A. In line with previous studies, CD69 expression was significantly upregulated in CD63pos basophils compared to CD63neg basophils (Figure 2B). Interestingly, the *ex vivo* stimulation using both allergen and anti-FcεRI cross-linking antibody did not affect CD25 expression (Figure 2C). In summary, our data show that the level of CD25 expression is increased in stable-mildly symptomatic allergic asthma but is not affected by *ex vivo* allergen stimulation.

4 Discussion

There is growing evidence on the important functions of basophils in T2-driven inflammation in asthma. Our current study extends this knowledge by investigating the expression levels of multiple surface and intracellular markers of basophils, which indicate their effector and immunoregulatory function in allergic asthma. This is the first report on identifying high levels of spontaneous CD25 expression in circulating basophils in stable-mildly symptomatic allergic asthmatics compared to healthy subjects.

Human basophils express CD25 under physiologic conditions. Its expression at both mRNA and protein levels can be significantly enhanced in the presence of IL-3 stimulation (8, 23). In our previous study, we observed that a continuous IL-3 exposure for up to 5 days was needed to induce CD25 expression. Thus, CD25 can be used as an activation marker to identify late IL-3 priming of basophils. By combining our previous *in vitro* data and current *in vivo* observations, we speculate that circulating basophils from stable asthma patients are in continuous and prolonged exposure to IL-3, secreted by activated T cells, mast cells, or basophils (24), ultimately resulting in the upregulation of CD25. However, as an evidence, the measurement of IL-3 in the sera from the mildly-stable symptomatic allergic asthmatics will be necessary. Nevertheless, we cannot rule out the effects of other cytokines on the expression of CD25 in basophils. Although human basophils express CD25 under physiological conditions, there is only limited knowledge regarding its functional significance in human basophils. A report by Zhao et al. recently showed that IL-2 binds to the CD25 receptor of human basophils, resulting in induced expression of inflammatory cytokines like IL-5 and GM-CSF (23). Interestingly, IL-5 and GM-CSF are also crucial for eosinophil infiltration into the target tissue of allergic inflammation. Thus, such an "*in vivo* IL-3 or IL-2-primed" effect of basophils may further recruit peripheral eosinophils into the airways. Furthermore, in human eosinophils, IL-2 induces enhanced release of eosinophil cationic protein from CD25 expressing but not from CD25 negative eosinophils (25).

In contrast to other reports (13), we did not observe any significant changes in the surface markers such as CD203c and CD63 in our cohort of stable-mildly symptomatic allergic asthma patients. We presume that this difference in the results may be due to the difference in the study cohort, as upregulation of CD203c and CD63 in blood basophils was observed in asthma patients with exacerbation, but not in patients having stable phase of allergic asthma. Furthermore, we stimulated blood basophils with allergen *ex vivo* to mimic asthma exacerbation after allergen challenge. In contrast to the increased CD63 and CD69 levels, we observed no change in the expression levels of CD25 on degranulated, CD63-positive basophils, upon *ex vivo* allergen

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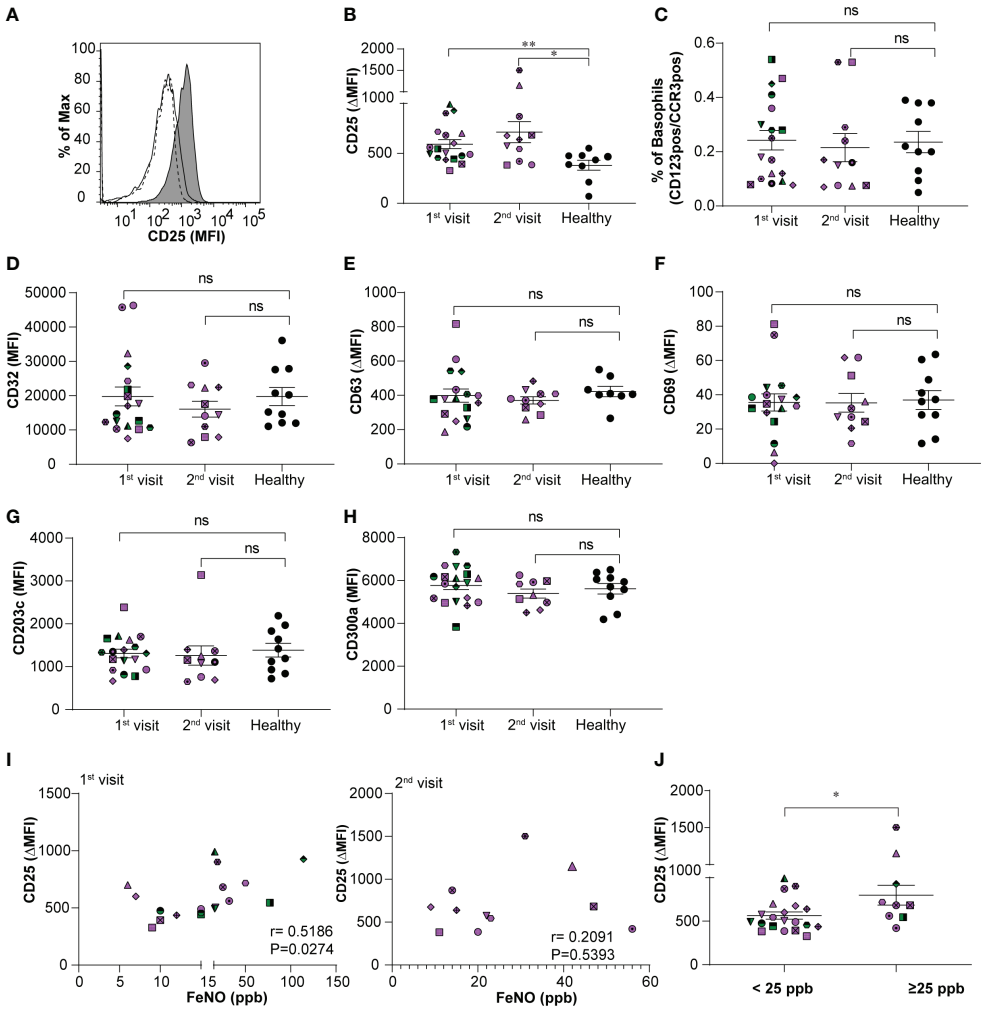

FIGURE 1
Relationship between expression levels of CD25 on basophils and FeNO levels in stable-mildly symptomatic allergic asthma patients. **(A)** Representative histogram showing CD25 expression level (MFI) on blood basophils in stable-mildly symptomatic allergic asthma patient (gray area) compared to healthy control (solid line) and FMO control (dotted line). **(B, D-H)** The expression levels of surface markers on blood basophils of stable-mildly symptomatic allergic asthma patients for the first (n=18) and second visit (n ≤ 11) compared to healthy controls (n≥9) measured by flow cytometry. Data are represented as mean ± SEM and reported as ΔMFI (MFI of test-MFI of FMO control) for CD25, CD63, CD69 and as MFI for CD32, CD203c and CD300a. Results were analyzed by two-tailed unpaired Mann-Whitney test.**P=0.0065, *P=0.0159, ns=non-significant **(C)** The relative percentage of blood basophils (dual positive for CD123 and CCR3) from stable-mildly symptomatic allergic asthma patients during their first (n=18) and second (n=11) visits compared to healthy subjects (n=10) **(I)** Correlation between CD25 expression levels (ΔMFI) and FeNO levels (ppb) in stable-mildly symptomatic allergic asthma patients of the first (left, n=18) and second visit (right, n=11). Spearman`s rank test was used for correlation analysis. Spearman coefficient (r) and level of significance (P) are indicated within the graph. **(J)** Surface expression levels of CD25 (ΔMFI) on blood basophils between <25 ppb (n=20) and ≥25 ppb FeNO level (n= 9) groups within stable-mildly symptomatic allergic asthma patients. Data are represented as mean ± SEM and analyzed using two-tailed unpaired Mann-Whitney test. *P<0.05. **(B-J)** Every patient is represented with specific symbol, while healthy controls are shown in closed black symbols. Patients who had both 1st and 2nd visits are marked in purple and those who had only 1st visit are marked in green color. MFI, median fluorescence intensity; FMO, fluorescence minus one; Healthy, healthy control subjects.

stimulation. Furthermore, although we found a significant higher expression of CD25 in stable-mildly symptomatic asthmatics having FeNO >25ppb compared to patients showing FeNO<25ppb, the correlation of FeNO and CD25 was not consistent between the visits. Altogether, these results suggest that CD25 is not a marker to monitor the asthma exacerbation after allergen challenge, but CD25 can be used as a unique marker for stable-mildly symptomatic allergic asthma patients.

Daclizumab, a humanized monoclonal antibody against CD25, improved pulmonary function and asthma control in moderate to severe asthma patients by IL-2R blockade in activated T cells (26). Its mode of action may also extend to

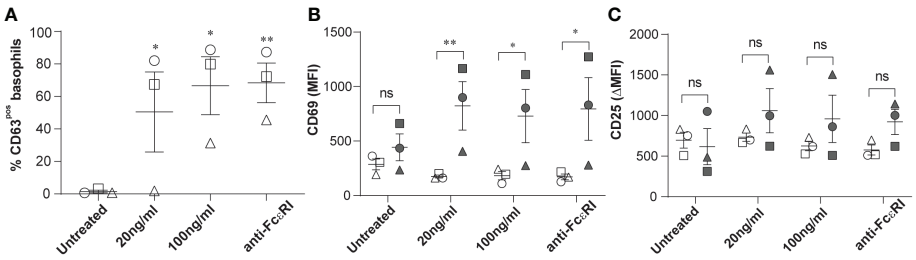

FIGURE 2
Effects of *ex vivo* allergen challenge on CD63, CD69, and CD25 expression in activated and non-activated basophils. Whole blood samples from stable-mildly symptomatic allergic asthma patients (n=3) were stimulated with their respective allergen (20ng/ml or 100 ng/ml) or anti-FcεRI cross-linking antibody 29C6 (100 ng/ml). Unstimulated controls were exposed to stimulation buffer only. **(A)** Percentage of $CD63^{pos}$ basophils in unstimulated controls, 20 ng/ml, and 100 ng/ml allergen and anti-FcεRI cross-linking antibody stimulated samples. **(B, C)** MFI of CD69 **(B)** and ΔMFI (MFI of test-MFI of FMO control) of CD25 **(C)** are shown on $CD63^{neg}$ (open symbols) and $CD63^{pos}$ basophils (closed symbols). **(A-C)** Patients 1, 2, and 3 are represented as circle, square, and triangle symbols, respectively. Data are shown as mean ± SEM and analyzed using one-way ordinary ANOVA tests, with Dunnett`s **(A)** or Bonferroni`s **(B, C)** multiple group comparison test. **P<0.01 and *P<0.05. ns, not significant.

other CD25-expressing effector cells such as basophils and eosinophils. Further studies are warranted to examine the impact of such treatments on basophils *in vivo* functions.

We identified CD25 as a novel biomarker for late *in vivo* priming of human basophils in stable-mildly symptomatic allergic asthma. Our findings highlight the importance of basophils in the pathogenesis of allergic asthma. Further studies are required to investigate the underlying mechanisms and the efficacy of new CD25 and/or basophil targeted therapies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All subjects signed informed consent forms approved by the Ethics Committee of the canton of Bern (no. 2016-01571), and experiments were conducted according to the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JI, LR, SB conducted the experiments, and JI, LR, and MF analyzed data. JI prepared the figures. NP and CG recruited the patients. TH performed FeNO measurements, and the skin prick test. MF designed and supervised the study. JI and MF wrote the manuscript and all the aforementioned authors discussed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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IgE-dependent human basophil responses are inversely associated with the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)

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Basophils crucially contribute to allergies and other Th2-driven diseases by rapidly releasing inflammatory and immunomodulatory mediators following high-affinity IgE-receptor crosslinking. Although these basophil-mediated responses depend on sensitization with antigen-specific IgE, this does not necessarily predict clinical symptom severity. It is thought that the balance of early stimulatory (e.g. SYK) and inhibitory (e.g. SHIP-1) intracellular signals are associated with basophil responsiveness, which is also critically dependent on calcium mobilization. Previous studies suggest that the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), which regulates cytosolic calcium levels, may be inversely associated with airway smooth muscle reactivity in asthma. Since basophils are implicated in asthma severity, our aims were to address whether SERCA2 is implicated in human basophil responses, especially following IgE-mediated activation. Human basophils were obtained from buffy coats, following research ethics approval, and further purified by immunomagnetic cell sorting. Expressions of SERCA2, and other isoforms, were determined by Western blotting in parallel to measuring IgE-dependent histamine releases from the same donors. The effects of a SERCA-activator and inhibitor were also assessed on their abilities to modulate basophil histamine release. We observed an inverse correlation between basophil responsiveness to IgE-dependent stimulation and SERCA2 expression. Thapsigargin, a highly-specific SERCA inhibitor, stimulated basophil histamine release and potentiated IgE-dependent secretion of the amine. Conversely, disulfiram, a SERCA activator, inhibited IgE-dependent basophil activation. The results obtained from this exploratory study indicate that SERCA2 may be an additional regulator of basophil reactivity alongside early excitatory or inhibitory signal transduction pathways.

KEYWORDS

basophils, SERCA, IgE receptor, histamine, inhibitory signaling

1 Introduction

Basophils contribute to the severity of allergic reactions by their capacity to rapidly release histamine and leukotriene C4. They also play a pivotal role in initiating and sustaining Th2-type pro-allergic immune responses by releasing IL-4 and IL-13, alongside various inflammatory cytokines such as TNFα. Despite their relative scarcity, especially to mast cells with which they share the ability to release pro-inflammatory mediators by IgE-dependent mechanisms, animal models of allergy and immunity to parasites have demonstrated that basophils play a non-redundant role in initiating Th2 responses and in chronic allergic inflammation (reviewed in (1). These immunomodulatory attributes are thought to occur following the migration of circulating basophils to tissues affected by allergic inflammation as well as to associated lymphatic tissues. Mouse models have also shown that basophils may also differentially contribute to anaphylaxis (2–4). In humans, basophil activation tests and a rapid decline in circulating basophils have shown a strong correlation to the severity of severe allergic reactions to foods and insect venoms (5–7), further highlighting a role for this cell type in anaphylaxis.

The crosslinking of IgE molecules bound to high-affinity IgE receptors (FcεRI) by allergens crucially determines basophil activation in humans, whereas in mice IgG-mediated anaphylaxis due to basophil activation may also occur (3), underlining the importance for detailed studies of human basophil function. Interestingly, although the degree of human basophil sensitization with antigen-specific IgE is important for enabling allergen-induced cell activation, the severity of clinical symptoms of basophil-driven diseases crucially depends on the basophil phenotype, especially in terms of the concept of "releasability" (8). Previous studies have shown that the releasability of basophils, regarding their strength of activation to IgE-dependent triggers, is fundamentally regulated by various intracellular signals. These include the expression and phosphorylation of early stimulatory kinases, especially the spleen tyrosine kinase SYK (9), and the subsequent activation of downstream kinases such as phosphatidylinositol 3-kinase (PI 3-kinase) (10, 11) and p38 mitogen-activated kinases (p38 MAPK) (12). Basophil stimulation is, however, also downregulated by inhibitory intracellular signals, notably the Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1), which reduces both basophil and mast cell function (13–17). SHIP-1 is also associated with the concept of basophil non-releasers (16), which is observed in up to 20% of donors at any given time where basophils are unable to respond to IgE-dependent stimuli, unrelated to the expression of IgE and FcεRI *per se* (18). This anergic state of human basophils may also be achieved by targeting various inhibitory receptors such as CD300a (19, 20), Allergin-1 (4), FcγRIIb (21) and siglec-7 (22). Basophil responsiveness (releasability) is therefore intricately governed by the balance of stimulatory and inhibitory signaling.

SYK and PI 3-kinase phosphorylation ultimately leads to the activation of phospholipase C and the subsequent synthesis of inositol trisphosphate (IP_3). IP_3 is crucially responsible for the increase in intracellular free calcium that drives the opening of calcium-sensitive calcium channels, allowing for the influx of extracellular calcium ions into basophils, without which no mediator release occurs. Calcium mobilization in basophils is therefore an essential step in determining basophil responses. IP_3-mediated leakage of calcium ions from intracellular stores, such as the sarcoplasmic reticulum, is potentially offset by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2). Interestingly, SERCA2 expressions in airway smooth muscle cells have been reported to be inversely associated with airway inflammation (23) and asthma severity (24). Moreover, it has long been known that Ca^{2+}-ATPase blocker, thapsigargin, activates human basophils (25). These cells have been strongly implicated in asthma severity, particularly during allergic late-phase reactions, where their increased numbers within the lungs and their activation are associated with severe outcomes, including death (26, 27). It is notable that basophils from allergic asthma patients displayed substantially higher magnitudes of histamine release induced by thapsigargin than non-allergic controls (28), indicating that SERCA2 may play a role in the severity of symptoms in basophil-driven diseases. IgE-dependent basophil activation is known to be greatly enhanced by priming cytokines, such as IL-3, IL-5 and GM-CSF, which are elevated in allergic asthma (29–31). Remarkably, these cytokines, which by themselves are poor stimuli of basophil degranulation, can cause substantial release of histamine from basophils in the presence of sub-optimal concentrations of thapsigargin, further indicating that depleting intracellular Ca^{2+} stores critically activates human basophils receptor-mediated histamine release (32).

Given the association of SERCA2 in asthma and that basophils are implicated in asthma severity, our aims were to address the principle of whether SERCA2 governs human basophil responses, especially in relation to IgE-mediated signaling. Our proof-of-concept study suggests that basophil releasability to IgE-dependent activation is, at least in part, determined by SERCA2 expression and possibly by other SERCA isoforms.

2 Materials and methods

2.1 Isolation of human basophils

Basophils were obtained from buffy coats, following ethical approval from the National Health Service (NHS) Research Ethics Committee (reference number 07/Q1206/3), purchased from the NHS Blood and Transfusion service. Basophils were first isolated by Ficoll-density centrifugation (using Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden) and purified further by

immunomagnetic cell selection (negative selection) using commercial isolation kits (EasySep Human Basophil Enrichment Kit, STEMCELL Technologies, Grenoble, France) as previously reported (33, 34). Basophil numbers and purities were verified by light microscopy using alcian blue staining and a Fuchs-Rosenthal haemocytometer. Mean basophil purity obtained following immunomagnetic selection was 91.6 ± 1.2%.

2.2 Western blot analysis

Aliquots of purified basophils (1-2 x 10^5 cells) were pelleted by centrifugation, and then lysed by vigorous mixing with lysis buffer containing 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 10mM EGTA, 5 mM DTT, 1% Nonidet P-40, 1mM PMSF, 100 ug/ml aprotonin, 20 ug/ml leupeptin and 10 mM benzamidine. An equal volume of 2x-concentrated Laemmli sample buffer was then added to the lysed basophils which were then heated to 99° C, with agitation, for 2 min before storage at -80°C. Proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto nitro-cellulose membranes. Prestained molecular weight rainbow markers (Bio-Rad Laboratories Ltd, Watford, UK) were also included for each SDS-PAGE run. Membranes were blocked for 4 h in 5% skimmed milk dissolved in TBST buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20) with gentle agitation. After 3 x 5 min washes in TBST, membranes were incubated overnight (at 4°C) with primary antibodies, directed against human SERCA2 (mouse monoclonal (ab2817) purchased from Abcam, Cambridge, UK). Membranes were then successively washed (4 x 5 min, TBST) followed by incubation with anti-mouse horse radish peroxidase-conjugated secondary antibodies for 2 h with gentle agitation. After washing, unbound secondary antibody proteins were visualized by autoradiography according to the manufacturer's instructions (ECL plus, Amersham, Buckinghamshire, UK). After detection, membranes were stripped for 10-20 min using Re-blot plus reagent (Chemicon, Chandlers Ford, UK), washed in TBST (4 x 5 min) and reprobed. Beta-actin expressions were measured in order to validate equal protein loading using mouse monoclonal HRP-conjugated antibodies (ab20272). Densitometric analysis was performed using ImageJ and the relative band densities of SERCA were normalized to respective band densities of β-actin and adjusted to control samples.

2.3 Cell treatments

The IgE-dependent reactivity of basophils from donors used to detect SERCA expressions by Western blot was determined by assessing histamine release. Briefly, vials containing 50-100 x 10^4 basophils, resuspended in HEPES-buffered Tyrode's solution (buffer), were warmed for 15 min at 37°C before stimulation with 1 μg/ml goat anti-human IgE (ϵ-chain specific, Sigma-Aldrich, St. Louis, USA) for 30 min, alongside unstimulated controls. Reactions were then terminated by adding ice-cold calcium-free buffer and centrifuging vials for 2 min at 1000 x g. Histamine content in the supernatants and cell pellets, which were diluted as required and lysed with perchloric acid (4%), was assessed using a spectrofluorometric autoanalyzer, based on the method reported by Shore et al. (35). Percentage histamine releases were determined from the total histamine content in the sum of pellet and supernatant tubes.

In a separate series of experiments, the effects of disulfiram (a SERCA activator) on basophil histamine release were investigated by preincubating isolated basophils with various concentrations of disulfiram (together with buffer controls) for 15 min at 37°C before stimulation with anti-IgE and assessment of histamine releases as described above. Part of the remaining cell pellets were also subjected to MTS viability assays. Briefly, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and absorbance was measured at 490 nm using a plate reader, according to the manufacturer's instructions (Promega UK Ltd., Southampton, UK).

2.4 Statistical analysis

Experiments were repeated using different basophil donors and results were first tested for normal distribution using the Shapiro-Wilk test. For normally-distributed data, a one-way ANOVA followed by Holm-Bonferroni correction was employed to assess statistically significant differences when multiple comparisons were made and a paired Student's t-test when comparing two events. The potential association between IgE-dependent basophil reactivity and SERCA2 expression was analyzed using linear regression analysis as well as Spearman's rank correlation- and Pearson correlation coefficients. Statistical probabilities (p) were expressed as *, where $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, unless shown otherwise.

3 Results

In agreement with previous reports (25, 28, 32), we first confirmed that thapsigargin activates human basophils and, at sub-optimal concentrations, potentiated IL-3-stimulated basophil histamine release (Supplementary Figure 1). IgE-mediated basophil activation was only weakly potentiated by thapsigargin, whereas mediator secretion induced by the bacterial peptide secretagogue, fMLP, was not at all enhanced. We could further verify that thapsigargin induced substantial calcium mobilization in basophils but, in contrast to the calcium ionophore A23187, the kinetics of calcium mobilization were slower in the presence of extracellular calcium (Supplementary

Figure 2). Interestingly, in the absence of extracellular calcium in the buffer, thapsigargin had very similar effects (and at the same rate) compared to A23187, supporting its mode of action as an intracellular calcium liberator due to SERCA blockade.

Our preliminary investigations indicated that human basophils variably express SERCA2 and, to a lesser extent, also SERCA3, but we failed to observe SERCA1 expressions in these donors (Supplementary Figure 3). Because Mahn et al. (24) previously reported that SERCA2 is inversely associated with asthma severity, and given that basophils show relatively high SERCA2 expressions compared to other isoforms, we wished to more closely examine whether SERCA2 expressions in human basophils is associated with their releasabilty to IgE-dependent stimulation. Indeed, basophils isolated from healthy donor buffy coat blood differentially expressed SERCA2 (Figure 1A) and, overall, this expression appeared to be clearly inversely associated with IgE-dependent histamine release from the same donors (Figure 1B). The negative correlation between SERCA2 expression and corresponding IgE-dependent histamine release was highly statistically significant, despite several outliers (see also Supplementary Table 1 for a summary of all the data shown in Figure 1). In contrast, spontaneous histamine secretion was not significantly associated with SERCA2 expression ($R^2 = 0.1$; $p>0.2$). Because IgE-dependent basophil activation between different donors (even from healthy individuals) is highly variable, we grouped the donors into low, medium and high responders to anti-IgE stimulation. We observed that the expression of SERCA2 was diminished most noticeably in the high responder group (net histamine release >30%; Figures 1C, D), indicating that SERCA2 may, at least in part, be involved in governing low IgE responder phenotypes in addition to other known regulatory signals (especially SYK and SHIP-1).

Since the liberation of intracellular calcium is an essential step for basophils to produce and release pro-allergic and other inflammatory mediators, we hypothesized that agents which activate SERCA could potentially inhibit IgE-mediated basophil activation. We therefore used disulfiram which, in addition to its well-known effects as an inhibitor of aldehyde dehydrogenase, also reversibly stimulates SERCA. We observed that disulfiram strikingly and significantly inhibited IgE-dependent basophil histamine release (Figure 2A, B). Disulfiram did not affect basophil cell viability (Figure 2C) and did not markedly induce histamine release from basophils by itself (Figure 2D).

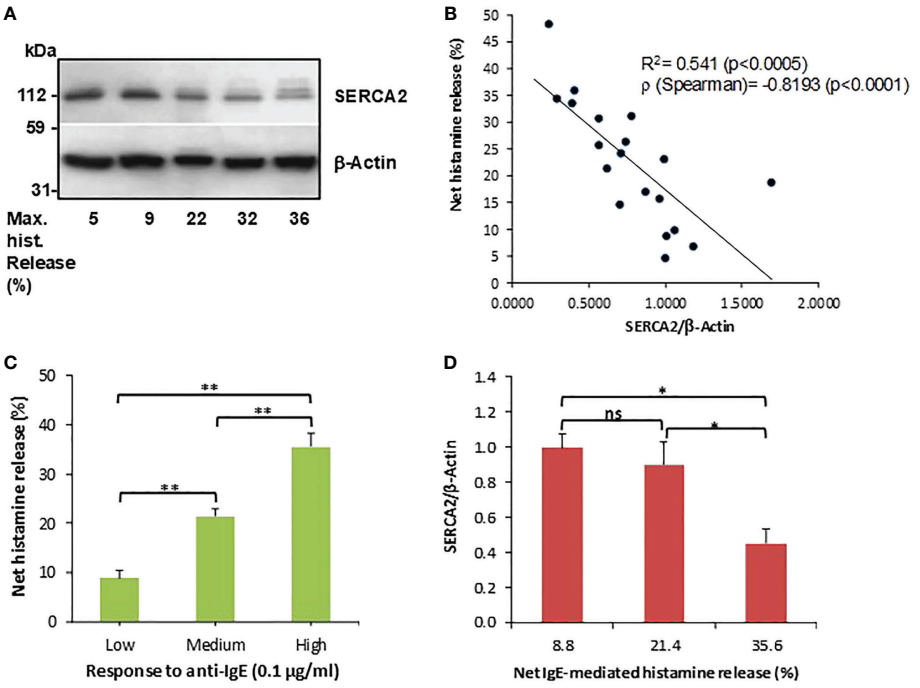

FIGURE 1
SERCA2 expressions and IgE-dependent histamine release from human basophils are negatively correlated. **(A)** SERCA2 expressions in unstimulated purified basophils, as determined by Western blotting, representative for a total of 19 separate basophil donors investigated. **(B)** Densitometric analysis of SERCA2 expressions plotted against the corresponding net anti-IgE-induced histamine release from the same basophil donors (n=19). **(C)** Histamine releasability to IgE-dependent stimulation grouped to low (<15% net release, n=5), medium (15 - 30% net release, n=8) and high (>30% net release, n=6) responders which were employed in **(D)** showing that SERCA2 expressions are clearly and significantly reduced in high responder basophils. Histamine data are shown as means ± SEM. * and ** indicate significant ($p<0.05$ and $p<0.01$, respectively) differences as determined by a one-way ANOVA followed by Holm-Bonferroni correction. ns, not significant.

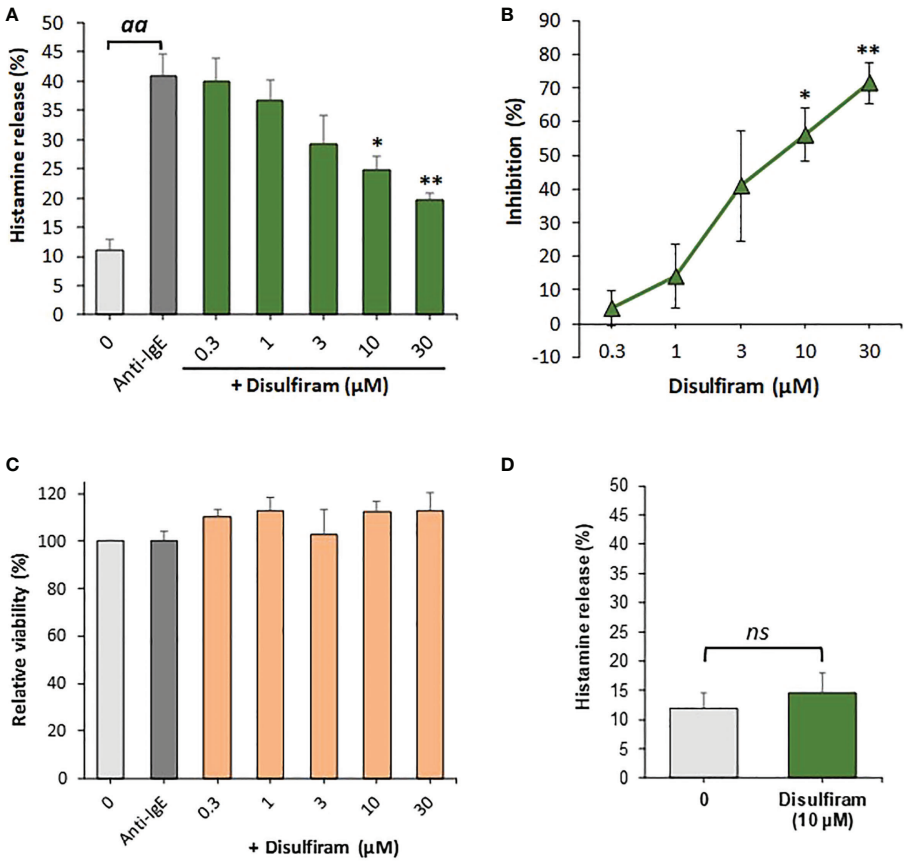

FIGURE 2
Disulfiram inhibits IgE-dependent histamine release from human basophils. **(A)** Basophils were preincubated with or without various concentrations of disulfiram for 15 min at 37°C before stimulation with anti-IgE (1µg/ml) for 30 min (n=5). Results are expressed as relative percentage histamine releases from which **(B)** the percentage inhibition of anti-IgE-induced histamine release was calculated from net histamine releases (corrected for spontaneous release controls). *aa* denotes significant ($p<0.01$) differences between anti-IgE stimulated positive controls from spontaneous release controls, * and ** indicate significant ($p<0.05$ and $p<0.01$, respectively) differences between anti-IgE alone and basophils incubated with disulfiram at the indicated concentrations as determined by a one-way ANOVA followed by Holm-Bonferroni correction. **(C)** Viability, relative to basophils incubated without anti-IgE or disulfiram, as determined by MTS assay, was not reduced by the treatments (n=4). **(D)** Disulfiram did not significantly (ns; determined using a paired Student's t-test) induce histamine release when incubated with basophils for 45 min at 37°C alone (in the absence of anti-IgE; n=4). Results are expressed as means ± SEM for the indicated number of independent experiments using different basophil donors.

4 Discussion

Our findings have identified an inverse association between the ability of human basophils to respond to IgE-dependent stimulation and the expressions of SERCA, particularly the SERCA2 isoform. These observations highlight a potential further tier of control of basophil releasability at the level of intracellular calcium mobilization, alongside other known key upstream signals such as SYK and the inhibitory phosphatase SHIP1. In the histamine-releasing rat mast cell line RBL-2H3, Dráberová et al. (36) reported that the non-T cell activation linker (NTAL) regulates store-operated Ca^{2+} channels in FcεRI signalling. However, it is currently not known whether the regulation mediator release to IgE-dependent triggers by the above signals are linked in human basophils.

It has long been known that the SERCA inhibitor, thapsigargin, activates human basophils (25) and potentiates degranulation upon co-stimulation with basophil priming cytokines, such as IL-3 (32), by depleting Ca^{2+} stores. Our results also broadly confirm these earlier findings (Supplementary Figure 1). The resulting increases in cytosolic free Ca^{2+} ions open calcium release–activated calcium (CRAC) channels, resulting in a substantial influx of extracellular calcium ions into basophils. Without the influx of extracellular calcium, intermediary signalling is considerably abrogated and mediator secretion from basophils does not occur (12). Our investigations confirm that blocking SERCA function by thapsigargin leads to a slow leakage of intracellular calcium in human basophils which, in the presence of extracellular calcium, results in substantial calcium influx which is

comparable to stimulation with the calcium ionophore A23187 (Supplementary Figure 2).

The pharmacological activation of SERCA by disulfiram led to a significant inhibition of anti-IgE-stimulated basophils (Figure 2), further indicating a role for SERCA in IgE-mediated basophil activation. However, these findings need to be interpreted with caution, since disulfiram is not only a SERCA-specific activator but inhibits acetaldehyde dehydrogenase, inositol 1,4,5-trisphosphate 5-phosphatase (37) as well as vacuolar-type ATPase (V-ATPase) (38). In regards to V-ATPase, Pejler et al. (39) showed that the V-ATPase inhibitor bafilomycin A inhibited IgE-dependent beta-hexosaminidase release from bone marrow-derived mouse mast cells. However, these inhibitory effects were moderate (<50% inhibition) and observed only after long (24h) preincubations with bafilomycin A1. It therefore remains questionable whether the potential inhibitory effects of disulfiram on V-ATPase in basophils is relevant considering the short preincubations (15 min) used in the present study where IgE-dependent histamine release was inhibited within previously published concentrations of the drug required for SERCA stimulation (40).

At the level of SERCA protein expression, the inverse association between constitutive SERCA2 and IgE-induced basophil degranulation clearly underlines a potential role for SERCA2 in determining, at least in part, the magnitude of basophil responses. From our exploratory investigation, this appears to be the case for constitutive IgE-mediated releasability in freshly isolated human basophils but it is not presently clear whether SERCA plays a role in governing basophil releasability in basophils which have been primed by IL-3 or other cytokines (e.g. nerve growth factors, IL-33 etc.) which enhance IgE-dependent mediator release. Importantly, basophil releasability to other stimuli, such as the bacterial peptide fMLP, does not correlate with IgE-mediated histamine release, although the initial Ca^{2+} response (caused by a rise in intracellular free calcium from intracellular stores) was reported to probably arise from the same internal source of the ion (41). This suggests that fMLP-induced basophil degranulation should also be dependent on intitial calcium responses and downregulated by SERCA. However, MacGlashan and Botana previously reported that fMLP-induced histamine release does not correlate with calcium responses (41) and, according to previous reports (32) as well as our own data, are not potentiated by thapsigargin. This suggests a differential dependency on intracellular calcium mobilization and subsequent SERCA input regarding basophil mediator secretion caused by different secretagogues, a point which still requires further clarification.

To the best of our knowledge, this is the first study to implicate a role for SERCA in regulating the function of allergic effector cells. A possible role for diminished SERCA expressions in the context of allergic inflammation is currently not well understood, where studies to date have focussed only on its role in airway smooth muscle cells regarding asthma. Here, Mahn et al. (24) reported that SERCA2 deficiency contributes to a hyperproliferative airway smooth muscle phenotype in asthma and is associated with moderate-severe asthma. These findings, however, were disputed by Sweeney et al. (42) who failed to observe differences in SERCA2 mRNA or protein expressions in airway smooth muscle cells between asthmatic patients and controls. Conversely, Qaisar et al. (43) also reported reduced SERCA expressions in asthma and, in a guinea pig model of asthma, reduced SERCA2b expression in airway smooth muscle cells was recently reported to correlate with intrinsic airway baseline tone (44). The proinflammatory cytokines, TNFα and IL-13, which are heavily implicated in asthma severity, were shown to decrease SERCA2 expressions in human airway smooth muscle cells (23).

SERCA proteins are expressed in at least seven different isoforms (SERCA1a/1b, SERCA 2a/2b, and SERCA 3a/3b/3c), of which only SERCA2b and SERCA3 isoforms are expressed in non-muscle cells (reviewed in (45). We primarily focussed on SERCA2 since this isoform was implicated in asthma in previous reports. However, our preliminary data suggest that human basophils may also constitutively express SERCA3 isoforms (Supplementary Figure 3). In contrast, in our preliminary investigations, where we focussed only on constitutive expressions in low or non-responder basophils to IgE-dependent stimulation, we did not observe SERCA1 expression in basophils. Our observations are, in part, supported by previous findings regarding RNA-seq expressions of SERCA2 and 3 (46, 47), which are relatively highly expressed in basophils in comparison to other immune cells (47). However, a gene expression dataset published by Uhlen et al. failed to detect SERCA2 expressions in various granulocytes (including basophils) in contrast to relatively high granulocyte expressions of SERCA3 (48). It is unclear whether the above disparities of SERCA2 gene expressions are related to our observations regarding differential SERCA2 protein expressions with respect to IgE-mediated basophil activation. However, given that human basophils also express SERCA3, further studies are clearly required regarding the possible functional consequences of the differential expressions of various SERCA isoforms in basophils.

Our study was limited to focussing on basophils isolated from buffy coat blood obtained from healthy donors. Further studies are clearly required to determine whether the negative association between SERCA expressions and IgE-mediated basophil releasability has clinical implications, especially regarding allergic diseases. From our exploratory study, there is a clear inverse association between SERCA expression and the ability of human basophils to respond to IgE-dependent stimulation. It remains to be clarified whether SERCA may serve as a new target for therapeutic regulation of basophil responses and is implicated in the functional regulation of other human allergic effector cell types.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by National Health Service (NHS) Research Ethics Committee (reference number 07/Q1206/3). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements

Author contributions

BG conceived the study, conducted most of the experiments, analysed and interpreted the data, and wrote the first draft of the manuscript. AH contributed to the experiments shown in Supplementary Figures 1 and 3, with the assistance of VS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1052290/full#supplementary-material

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Mas-related G protein-coupled receptor MRGPRX2 in human basophils: Expression and functional studies

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Background: Occupancy of MRGPRX2 heralds a new era in our understandings of immediate drug hypersensitivity reactions (IDHRs), but a constitutive expression of this receptor by basophils is debated.

Objective: To explore the expression and functionality of MRGPRX2 in and on basophils.

Methods: Basophils from patients with birch pollen allergy, IDHRs to moxifloxacin, and healthy controls were studied in different conditions, that is, in rest, after stimulation with anti-IgE, recombinant major birch pollen allergen (rBet v 1), moxifloxacin, fMLP, substance P (SP), or other potential basophil secretagogues. In a separate set of experiments, basophils were studied after purification and resuspension in different media.

Results: Resting whole blood basophils barely express MRGPRX2 on their surface and are unresponsive to SP or moxifloxacin. However, surface MRGPRX2 is quickly upregulated upon incubation with anti-IgE or fMLP. Pre-stimulation with anti-IgE can induce a synergic effect on basophil degranulation in IgE-responsive subjects after incubation with SP or moxifloxacin, provided that basophils have been obtained from patients who experienced an IDHR to moxifloxacin. Cell purification can trigger a "spontaneous" and functional upregulation of MRGPRX2 on basophils, not seen in whole blood cells, and its surface density can be influenced by distinct culture media.

Conclusion: Basophils barely express MRGPRX2 in resting conditions. However, the receptor can be quickly upregulated after stimulation with anti-IgE, fMLP, or after purification, making cells responsive to MRGPRX2 occupation. We anticipate

that such "conditioned" basophils constitute a model to explore MRGPRX2 agonism or antagonism, including IDHRs originating from the occupation of this receptor.

KEYWORDS

basophils, allergy, CD63, CD203c, moxifloxacin, MRGPRX2, substance P.

1 Introduction

Human Mas-related G protein-coupled receptor member X2 (MRGPRX2) is expressed by various cell types, including dorsal root ganglion neurons and tryptase- and chymase-containing connective tissue mast cells (MC_{TC}) (1), and can be activated by a variety of basic small molecules, such as the tachykinergic neuropeptide substance P (SP), anaphylatoxins, and compound 48/80, leading to degranulation independent of cross-linking of the high-affinity receptor for IgE (FcεRI) (2, 3). Nonetheless, MRGPRX2 can also be involved in immediate hypersensitivity reactions to drugs (IDHRs), such as icatibant (4, 5), neuromuscular blocking agents (NMBAs) (4, 6–9), fluoroquinolones (4, 6–11), cetrorelix (4, 5), morphine (8, 9, 12), vancomycin (13) and many other antimicrobials/antiseptics (14, 15). However, experimental methodological heterogeneity has significantly hampered the interpretation and generalization of observations on MRGPRX2 involvement in IDHRs. This is in part due to the different experimental mutant animal models and transfected cell lines employed, both human and nonhuman, which exhibit a different level of MRGPRX2 expression, with variable receptor responsiveness and affinity (16).

In this context, a more accessible human model is cultured human mast cells from peripheral blood-progenitor cells (PBCMCs) (9), which has been successfully applied both with and without comparative silencing of MRGPRX2 (8, 17).

Two other putative and attractive candidates for further human MRGPRX2 studies were proposed by Wedi et al. (18), who showed that basophils and eosinophils constitutively express functionally active MRGPRX2 and are responsive to the fluoroquinolone ciprofloxacin. However, with respect to basophils, these data conflict with earlier preliminary findings, that is, whole blood basophils barely express MRGPRX2 (19) and basophils from uneventfully exposed control individuals do not degranulate non-specifically in response to MRGPRX2 agonists such as NMBAs, opiates, fluoroquinolones, and vancomycin (11, 20, 21). Alternatively, we demonstrated that anti-IgE and, to a lesser extent, fMLP, enhance the surface expression of MRGPRX2 by basophils (19).

Here, we investigate the discrepancies in basophilic MRGPRX2 expression and explore the effect of IgE/FcεRI-dependent or -independent stimuli on the surface expression and functionality.

Abbreviations: MRGPR(s), Mas-related G protein-coupled receptor(s); MC_{TC}, connective tissue mast cells; MRGPRX2, Mas-related G protein-coupled receptor member X2; SP, substance P; FcεRI, high-affinity IgE receptor; IDHR(s), immediate hypersensitivity reaction(s) to drugs; NMBA(s), neuromuscular blocking agent(s), MC, mast cells; Mrgprb2, Mas-related G protein-coupled receptor member b2; fMLP, N-formyl-methionyl-leucyl-phenylalanine; IgE, Immunoglobulin E; PBCMC (s), Peripheral blood cultured mast cells; BPA(s), birch pollen allergic patient(s); MOX(s), patient(s) with immediate type hypersensitivity to moxifloxacin; HC(s), healthy control(s); FMO, fluorescence minus one; SSC, side scatter; GPCR(s), G protein-coupled receptor(s).

2 Materials and methods

2.1 Peripheral blood cultured mast cells (PBCMCs)

Human MC_{TC}-like cells were cultured out of peripheral blood progenitor cells according to a protocol earlier described (22), and applied as a positive control for anti-MRGPRX2 staining. Briefly, $CD34^+$ progenitor cells were isolated using magnetic beads (EasySep™ Human CD34 Selection Kit; Stemcell Technologies) and cultured in a serum-free methylcellulose-based medium (MethoCult SF H4236; Stemcell Technologies) supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL) (Gibco, Thermo Fisher Scientific), low-density lipoprotein (10 µg/mL, LDL; Stemcell Technologies), 2-mercaptoethanol (55 µmol/L; Gibco, Thermo Fisher Scientific), stem cell factor (SCF, 100 ng/mL; Miltenyi Biotec) and interleukin-3 (IL-3, 100 ng/mL; PeproTech) during 4-5 weeks.

MCs were stained with anti-human CD117-APC (clone 104D2; BD Biosciences), and anti-human CD203c-PeCy7 (clone NP4D6; BioLegend) and defined as $CD117^+$ and $CD203c^+$.

The PBCMC cultures employed had a purity of 80% for $CD117^+CD203c^+$ cells (23). Before applying the PBCMCs to perform the qPCR experiments, cell debris removal was performed using the EasySep™ Dead Cell Removal (Annexin V) Kit (Stemcell Technologies).

For membrane staining of MRGPRX2, 10 µL anti-human MRGPRX2-PE (clone K125H4; BioLegend) was added before fixing the cells and incubated on ice for 20 minutes in the dark. For intracellular staining of MRGPRX2, PBCMCs were fixed with 4% paraformaldehyde (BioLegend) for 30 minutes at room temperature. Subsequently, cells were washed and permeabilized in PBS (Thermo Fisher Scientific) with 0.05% Triton-X-100 (Avantor (VWR)) (PBS-TX, pH 7.4). Then, 10 µL of anti-human MRGPRX2-PE diluted in PBS-TX was added and incubated for 20 min at 4°C. Cells were washed with 0.3 mL PBS-TX and resuspended in PBS with 0.1% sodium azide (Avantor (VWR)).

2.2 Peripheral whole blood basophils

Heparinized sterile whole blood samples were collected from patients allergic to birch pollen (BPAs) (all presented with rhino-conjunctivitis and/or asthma related to birch pollen exposure and documented sensitization to Bet v 1, the major allergen from *Betula verrucosa*), patients with an IDHR to moxifloxacin (MOXs) [IDHRs to moxifloxacin have been defined in a previous study (11)], and healthy control individuals (HCs) and used for immunophenotyping and activation studies.

For all the activation studies, 200 µL of whole blood were incubated with 200 µL of antigens and prewarmed at 37°C. Reactions were stopped by placing the cells on ice and adding 1 mL ice-cooled PBS-EDTA [10 mmol/L EDTA; Avantor (VWR)]. Supernatants were removed after spinning for 5 min (4°C, 200g).

Basophils were stained with 20 µL monoclonal anti-human IgE (clone GE-1; Sigma Aldrich GmBH) labeled with AlexaFluor 405 (Molecular Probes; Thermo Fisher Scientific), 10 µL monoclonal anti-human CD63-PE (clone H5C6; BD Biosciences), and 10 µL monoclonal anti-human CD203c-APC (clone NP4D6; Biolegend) and incubated on ice for 20 minutes in the dark. For membrane staining of MRGPRX2, 10 µL anti-human MRGPRX2-PE (clone K125H4; BioLegend), was added before fixing the cells and incubated on ice for 20 minutes in the dark. Cells were lysed/fixed with 2 mL BD FACS Lysing solution for 20 min at room temperature. Cells were washed twice with PBS with 0.1% sodium azide and measured. Basophils were gated as $SSc^{low}aIgE^{+}$ cells. Resting basophils were defined as $CD203c^{+}CD63^{-}$, whereas degranulating basophils were identified as $CD203c^{++}CD63^{+}$. Analyses were performed at predetermined time points.

For intracellular staining of MRGPRX2, basophils were fixed with 2 mL Phosflow Lyse/Fix Buffer (BD Biosciences) for 20 min at 37°C. Subsequently, cells were washed and permeabilized in PBS with 0.1% Triton-X-100 (PBS-TX, pH 7.4). Then, 10 µL of anti-human MRGPRX2-PE diluted in PBS-TX was added and incubated for 20 min at 4°C. Cells were washed with 0.3 mL PBS-TX and resuspended in PBS with 0.1% sodium azide.

2.3 Purified basophils

Basophils were isolated from EDTA-anticoagulated whole blood samples obtained from HCs using magnetic beads (EasySep™ Human Basophil Enrichment Kit; Stemcell Technologies) according to the manufacturer's instructions and used for a different set of immunophenotyping and activation studies.

After purification, cells were resuspended in RPMI 1640 (Thermo Fisher Scientific) 10% fetal calf serum (Thermo Fisher Scientific) + gentamycin 0.5% (Thermo Fisher Scientific) + glutamine 1% (Thermo Fisher Scientific) (RPMI medium) or in Tyrode buffer 10% autologous serum (Thermo Fisher Scientific) (Tyrode medium) for 30 minutes before starting the experiments. For all the activation studies, 100 µL of purified basophils were incubated with 100 µL of antigens and prewarmed at 37°C before use. Reactions were stopped by placing the cells on ice and adding 1 mL ice-cooled PBS-EDTA (10 mmol/L EDTA). Supernatants were removed after spinning for 5 min (4°C, 200g). Basophils were stained before fixation with 20 µL monoclonal anti-human IgE (clone GE-1; Sigma Aldrich GmBH) labeled with AlexaFluor 405 (Molecular Probes; Thermo Fisher Scientific), 10 µL monoclonal anti-human CD63-PE (clone H5C6; BD Biosciences), 10 µL monoclonal anti-human CD203c-APC (clone NP4D6; Biolegend), and 10 µL anti-human MRGPRX2-PE (clone K125H4; BioLegend). Cells were lysed/fixed with 2 mL BD FACS Lysing solution for 20 min at room temperature. Cells were washed twice with PBS with 0.1% sodium azide and measured. Basophils were gated as $SSc^{low}aIgE^{+}$ cells. Resting basophils are defined as $CD203c^{+}CD63^{-}$, whereas degranulating basophils are identified as $CD203c^{++}CD63^{+}$. Analyses were performed at different predetermined time points.

2.4 Experiments with peripheral whole blood basophils

Basophils from BPAs and HCs were incubated with mouse anti-human monoclonal anti-IgE antibodies (10 µg/mL, clone G7-18; BD Bioscience) as a positive control or rBet v 1 (0.01 µg/mL, rBet v 1; Biomay) to assess activation/degranulation through IgE/FcεRI cross-linking as previously described (24).

To study their IgE/FcεRI-independent activation, basophils from BPAs and HCs were stimulated with N-formyl-methionyl-leucyl-phenylalanine (0.5 µg/mL, fMLP; Sigma-Aldrich), LPS (10 µg/mL; Sigma-Aldrich, Merck) and staphylococcus enterotoxin B (SAB 1-100 µg/mL; Sigma-Aldrich, Merck) or incubated with IL-3 (10 ng/mL; PeproTech).

To study the functionality of basophilic MRGPRX2 expression, basophils from individuals who were responsive to positive control stimulation with anti-IgE in the CD63 basophil activation test (BAT) were separately or simultaneously stimulated with anti-IgE (10 µg/mL) and the natural MRGPRX2 ligand SP (15 µmol/L, Sigma-Aldrich, Merck). MRGPRX2-mediated activation/degranulation induced by SP (1.5 µmol/L, 15 µmol/L, 150 µmol/L, and 300 µmol/L) alone or after 20 minutes of priming with IL-3 (2 ng/ml and 10 ng/ml) was also assessed.

To study moxifloxacin-induced activation/degranulation, basophils from MOXs and HCs were separately or simultaneously challenged with anti-IgE (10 µg/mL) and moxifloxacin (0.025 mmol/L or 2.5 mmol/L, Sigma-Aldrich, Merck) as previously described (11).

Whole blood basophils were also separately or jointly incubated with IL-3 (10 ng/mL), IL-33 (30 ng/mL, PeproTech), and moxifloxacin (0.025 mmol/L and 2.5 mmol/L, Sigma-Aldrich, Merck).

2.5 Experiments with purified basophils and preincubation with cytokines

Purified basophils were resuspended in RPMI medium or Tyrode medium and analyzed *via* a BAT. Purified basophils resuspended in the two different media were also stimulated with anti-IgE (10 µg/mL).

MRGPRX2-mediated activation/degranulation induced by SP (1.5 µmol/L, 15 µmol/L, 150 µmol/L, and 300 µmol/L) alone or after 20 minutes of priming with IL-3 (2 ng/ml and 10 ng/ml) was assessed in purified basophils resuspended in RPMI medium.

These were also separately or jointly incubated with IL-3 (10 ng/mL), IL-33 (30 ng/mL), and moxifloxacin (2.5 mmol/L).

2.6 Flow cytometric analysis

Flow cytometric analysis was performed on a FACSCanto II™ flow cytometer (BD Immunocytometry Systems) equipped with three lasers (405, 488, and 633 nm). Correct compensation settings for the antibodies conjugated with fluorochromes were performed using BD CompBeads (BD Biosciences). Fluorescence minus one (FMO) samples were used to set a marker for positivity according to the 99th percentile. Flow cytometric data were analyzed using Kaluza Analysis 2.1 software (Beckman Coulter). Flow cytometric characterization of basophils relied upon a combination of side scatter (SSC), anti-IgE and CD203c. At least 1,000 basophils were counted and analyzed. Activation is expressed in net percentages of upregulation of CD63, CD203c, and MRGPRX2, that is the percentage of CD63, CD203c, and MRGPRX2 positive stimulated cells minus the percentage of positive CD63, CD203c, and MRGPRX2 resting cells.

2.7 RT-qPCR and gel electrophoresis

RNA isolation from PBCMCs and purified basophils at rest or after stimulation with anti-IgE (10 µg/mL) from 3 HCs was performed using the Nucleospin RNA XS kit, according to the manufacturer's protocol (Macherey-Nagel). Sample RNA concentration and quality were determined using the Agilent Bioanalyzer 2100 platform (Agilent Tech.). An RNA integrity number (RIN) cut-off of 6 was applied to exclude inadequate samples. A total of 200 ng RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules), and the resulting cDNA was diluted 1:5. Bio-Rad PrimePCR assays containing validated primer pairs were used to perform a qPCR analysis on the expression of the target gene MRGPRX2 (PrimePCR Assay ID qHsaCID0023564), and two housekeeping genes, namely HPRT1 (PrimePCR Assay ID qHsaCID0016375) and RPS29 (PrimePCR Assay ID qHsaCED0038808) (25), applied as positive controls. Each sample was run in triplicate.

RT-qPCR was performed on 2 µL of cDNA using the SSO Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories), with a total of 40 amplification cycles and the PCR protocol according to the manufacturer's instructions. Next, qPCR products underwent 2% agarose gel electrophoresis and UV visualization (GelRed Nucleic acid stain, Biotium) to evaluate the presence of amplicons of the expected band size.

2.8 Statistical analysis

Two-way analysis of variance (ANOVA), paired Student's t-tests, Tukey's multiple comparisons test, Mann Whitney test and Pearson's correlation coefficient were applied, where appropriate, using JMP Pro 13 (SAS, Cary, NC, USA). P-values < 0.05 were considered as significant. Figures were developed in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3 Results

3.1 MRGPRX2 expression in resting basophils and PBCMCs

Resting PBCMCs express MRGPRX2, both intracellularly and on their surface membrane (Figure 1). In contrast, resting whole blood basophils from BPAs and HCs invariantly show intracellular staining for MRGPRX2, but barely express the receptor on their surface membrane.

Whereas MRGPRX2 mRNA is detected in PBCMCs by qPCR, it was not found in resting purified basophils at rest or after stimulation with anti-IgE, as shown after gel electrophoresis of the qPCR products (Supplementary Figures 1, 2).

For both housekeeping genes (HPRT1 and RPS29) qPCR was successful and showed clear expression in all samples indicating that positive control genes are clearly expressed in both PBCMCs and resting or anti-IgE-stimulated purified basophils.

3.2 MRGPRX2, CD63, and CD203c expression by activated whole blood basophils

Stimulation with anti-IgE and fMLP induces an upregulation of MRGPRX2. This significant upregulation peaks after 3 minutes and reaches a plateau at 60 minutes. The appearance of the degranulation marker CD63 and upregulation of CD203c display slightly dissimilar time kinetics, since both events peak later, i.e., after 5 minutes (Figure 2). MRGPRX2 upregulation after stimulation with fMLP is significantly less pronounced compared to anti-IgE stimulation.

Representative plots of MRGPRX2 upregulation in HC and BPA after 20 minutes stimulation with anti-IgE, fMLP, and Bet v 1 are shown in Figure 3.

No difference in MRGPRX2 expression was observed between resting basophils from BPAs (n=16; median 4%; range 0-14%) and HCs (n=10; median 6%; range 4-20%). On the other hand, a significantly higher MRGPRX2 upregulation was observed in anti-IgE stimulated basophils from BPAs (n=16; median 50%; range 13-99%) when compared to HCs (n=10; median 19%; range 12-31%) (p = 0.02, Mann Whitney test).

IL-3 triggers upregulation of CD203c without any upregulation of MRGPRX2 or CD63 (Supplementary Figure 3). We could not detect any upregulation of CD203c, CD63 or MRGPRX2 after stimulation with LPS and Staphylococcus enterotoxin (data not shown).

3.3 Co-incubation experiments with anti-IgE and substance P

In whole blood basophils from individuals who are responsive to anti-IgE, SP alone does not induce upregulation of CD63, CD203c, or MRGPRX2 surface expression. In contrast, co-incubation with anti-IgE and SP exerts a numeric synergistic effect on CD63 upregulation reaching significance after 3 minutes (p = 0.0104 at 3 minutes; p = 0.0017 at 5 minutes; p = 0.0026 at 20 minutes) (Figure 4).

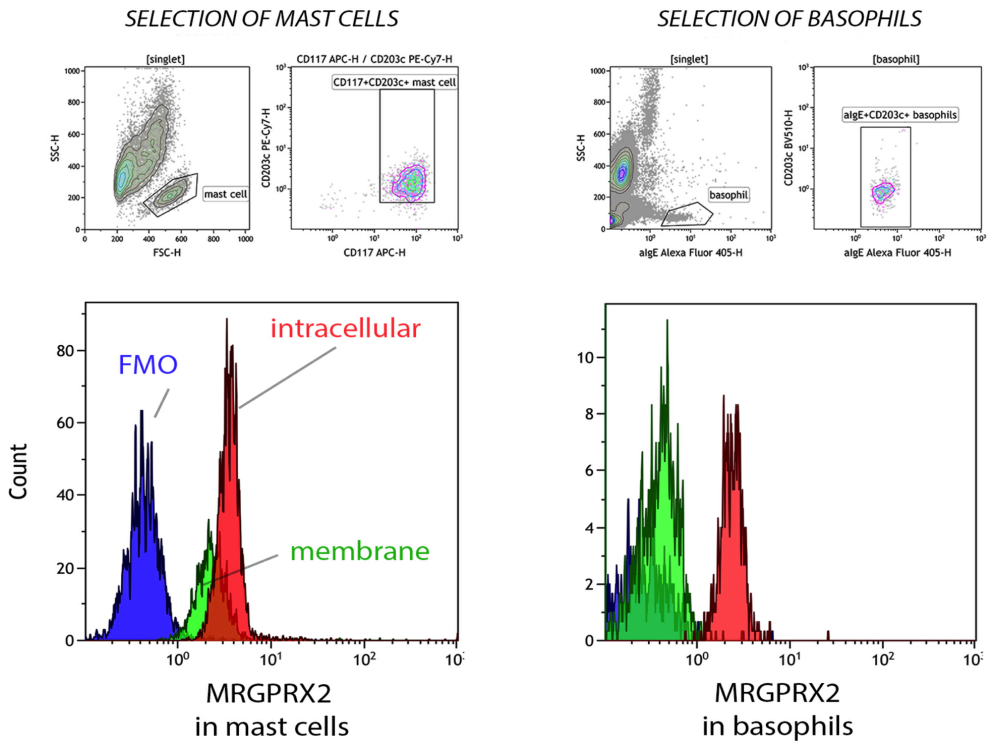

FIGURE 1
Membrane and intracellular expression of MRGPRX2 in peripheral blood cultured mast cells (PBCMCs) and whole blood basophils. Representative plots of membrane (green histogram) and intracellular (red histogram) MRGPRX2 expression in PBCMCs and resting whole blood basophils. FMO = fluorescence minus one sample (blue histogram). PBCMCs = peripheral blood cultured mast cells.

3.4 Co-incubation experiments with anti-IgE and moxifloxacin

Based on the appearance of CD63, stimulation with anti-IgE reveals two distinct basophil reactivity patterns, i.e., "CD63 responders" and "CD63 non-responders". HCs and CD63-responding MOXs show an anti-IgE-induced appearance of CD63 (Figures 5A, G) and upregulation of surface MRGPRX2 (Figures 5B, H). CD63-non-responding MOXs fail to demonstrate an increase of CD63 expression (Figure 5D) but also show surface upregulation of MRGPRX2 (Figure 5E). In all three groups, upregulation of CD203c is observed (Figures 5C, F, I).

Moxifloxacin alone does not induce degranulation with the appearance of CD63 or surface upregulation of MRGPRX2 in either MOXs, regardless of CD63-responding status, (Figures 5D, E, G, H) or HCs (Figures 5A, B).

Like for SP, co-incubation of the cells with anti-IgE and moxifloxacin 0.025 mmol/L exerts a synergistic effect with enhanced upregulation of CD63, which is strictly restricted to the CD63-responding MOXs and reaches statistical significance from 5 minutes onwards ($p = 0.0007$ at 5 minutes; $p = 0.004$ at 20 minutes) (Figure 5G). In contrast, in CD63-non-responding MOXs (Figure 5D) and in responsive HCs (Figure 5A), co-incubation with anti-IgE and moxifloxacin 0.025 mmol/L does not result in a CD63 expression higher than with anti-IgE alone. No significant difference is observed in all three groups on the expression of MRGPRX2 (Figures 5B, E, H) and CD203c (Figures 5C, F, I) in comparison to experiments with anti-IgE alone. A representative plot is shown in Supplementary Figure 4.

In contrast, coincubation with anti-IgE and moxifloxacin 2.5 mmol/L seems to have an antagonistic effect on anti-IgE-mediated CD63 upregulation at 3 minutes in CD63-responding MOXs ($p = 0.005$), but not in CD63-non-responding MOXS, and from 3 minutes onwards ($p = 0.0241$ at 3 minutes; $p = 0.0026$ at 5 minutes; $p = 0.006$ at 20 minutes) in responsive HCs (Figures 5A, D, G). Interestingly, the magnitude of inhibition seems to be higher in HCs than in CD63-responding MOXs. Furthermore, coincubation with anti-IgE and moxifloxacin 2.5 mmol/L has a synergistic effect on MRGPRX2 upregulation at 3 minutes only in responsive HCs ($p = 0.0256$) (Figure 5B). No correlation between the magnitude of the inhibitory effect of coincubation with moxifloxacin 2.5 mmol/L on anti-IgE-mediated CD63-upregulation and MRGPRX2 upregulation was observed for any subpopulation (Supplementary Figure 5). With respect to CD203c, coincubation with anti-IgE and moxifloxacin 2.5 mmol/L triggers significant synergistic upregulation only in CD63-responding MOXs ($p = 0.0019$ at 1 minute; $p = 0.0039$ at 5 minutes) (Figure 5I).

3.5 MRGPRX2 expression in purified basophils

As shown in Figure 6A and Supplementary Figure 5, purification of basophils induces a significantly higher MRGPRX2 expression as compared to whole blood basophils ($p \leq 0.0001$).

Purified basophils, resuspended in both Tyrode medium and RPMI medium, show upregulation of CD203c but not CD63.

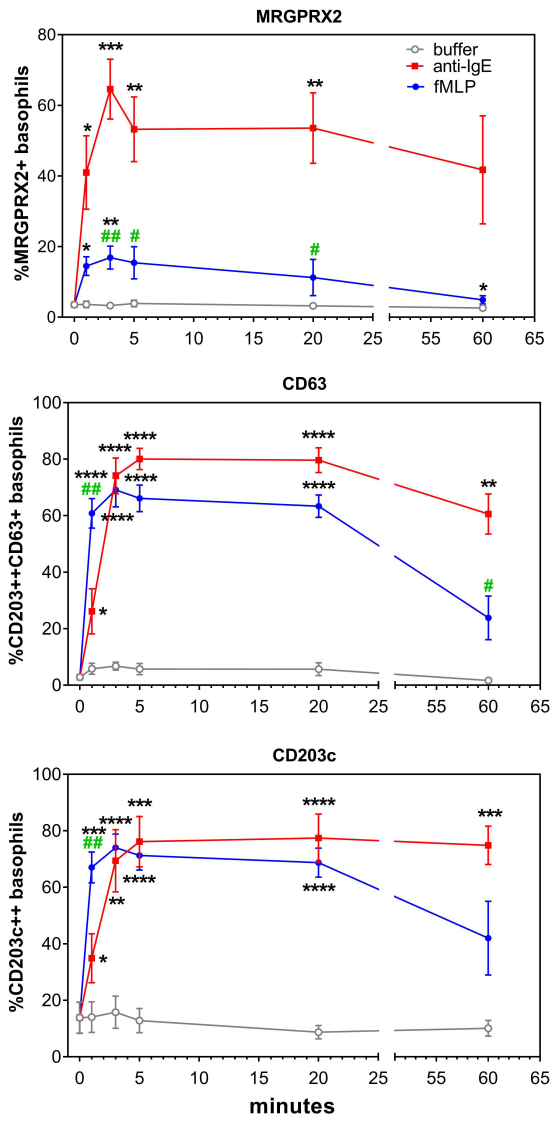

FIGURE 2
Effect of anti-IgE and fMLP stimulation on membrane MRGPRX2 expression in whole blood basophils. **(A)** Representative plots on the effect of anti-IgE and fMLP stimulation of 20 min on membrane MRGPRX2 expression of whole blood basophils from one healthy control. Resting cells are identified as $IgE^{+}CD203c^{+}CD63^{-}$ (green). $MRGPRX2^{+}$ expressing cells are indicated in purple. **(B)** Time kinetics of MRGPRX2, CD63 and CD203c membrane expression of whole blood basophils after stimulation with anti-IgE (red), fMLP (blue) or buffer (grey) (n=8). * p<0.05; **p<0.01 ***p<0.001; ****p<0.0001 compared to the buffer. $^{\#}$p<0.05; $^{\#\#}$p<0.01 compared to the anti-IgE stimulation; Tukey's multiple comparisons tests.

Purified basophils incubated in RPMI medium are no longer stimulable with anti-IgE and their MRGPRX2 density is higher than that observed for separated basophils suspended in Tyrode medium (Supplementary Figure 7).

3.6 MRGPRX2 functionality in purified basophils

Stimulation of purified basophils resuspended in RPMI medium with SP, primed with two different concentrations of IL-3 for 20 minutes, induces upregulation of CD63 after 3 minutes with a direct SP-dependent dose effect that reaches significance for the highest tested concentration of SP (p = 0.0275 for SP 300 µmol/L after priming with IL-3 2 ng/mL compared to buffer after priming with IL-3 2 ng/ml; p = 0.0042 for SP 300 µmol/L after priming with IL-3 10 ng/mL compared to buffer after priming with IL-3 10 ng/mL). At 20 minutes the effect is significant only for experiments conducted jointly with the highest IL-3 and SP concentrations (p = 0.0394 for SP 300 µmol/L after priming with IL-3 10 ng/ml compared to buffer after priming with IL-3 10 ng/mL) (Figure 6B).

No significant SP-induced CD63 upregulation is observed in the same set of experiments performed without co-incubation with IL-3 or on whole-blood basophils (Figure 6C and Supplementary Figure 8).

Priming with IL-3 has no effect on MRGPRX2 expression, neither on purified nor on whole blood basophils (Supplementary Figure 9).

3.7 Moxifloxacin-induced degranulation of purified basophils

As shown in Figure 7, stimulation of whole blood basophils or purified basophils resuspended in RPMI medium with moxifloxacin alone results in a CD63 expression comparable to spontaneous expression of CD63.

IL-3 and IL-33 separately can significantly (but slightly) increase CD63 expression in purified basophils at 20 minutes with a (not significant) synergistic effect when combined. In purified basophils, co-incubation of IL-3 and/or IL-33 with moxifloxacin seems to cause an additive effect on CD63 upregulation which is not statistically significant.

Almost no upregulation of CD63 is observed in whole blood basophils obtained from the same patients, regardless of incubation with IL-3, IL-33, and/or moxifloxacin.

Co-incubation of both purified and whole blood basophils with IL-3, IL-33, or both, fails to induce upregulation of MRGPRX2 after 3 and 20 minutes (Supplementary Figure 10).

4 Discussion

Here we confirm that human resting peripheral blood basophils only rarely express functionally active MRGPRX2 on their surface membrane and, consequently, are unresponsive to endogenous and exogenous MRGPRX2 agonists. Although unclear, this probably correlates with the lack of exposure to specific tissue factors and allows the prevention of potent and potentially harmful nonspecific activation of these cells, as MRGPRX2 can also be activated by various exogenous and endogenous substances including serum albumin fragments (26).

Our data are not in line with the observations by Wedi et al. (18), who demonstrated that MRGPRX2 is constitutively expressed on resting isolated basophils, ciprofloxacin induces basophil degranulation, and basophils contain MRGPRX2 mRNA. Net of methodological differences, our study confirms that basophils express MRGPRX2. However, according to our results, extracellular expression of the receptor and its engagement by endogenous and exogenous ligands seems to require a previous conditioning (that can

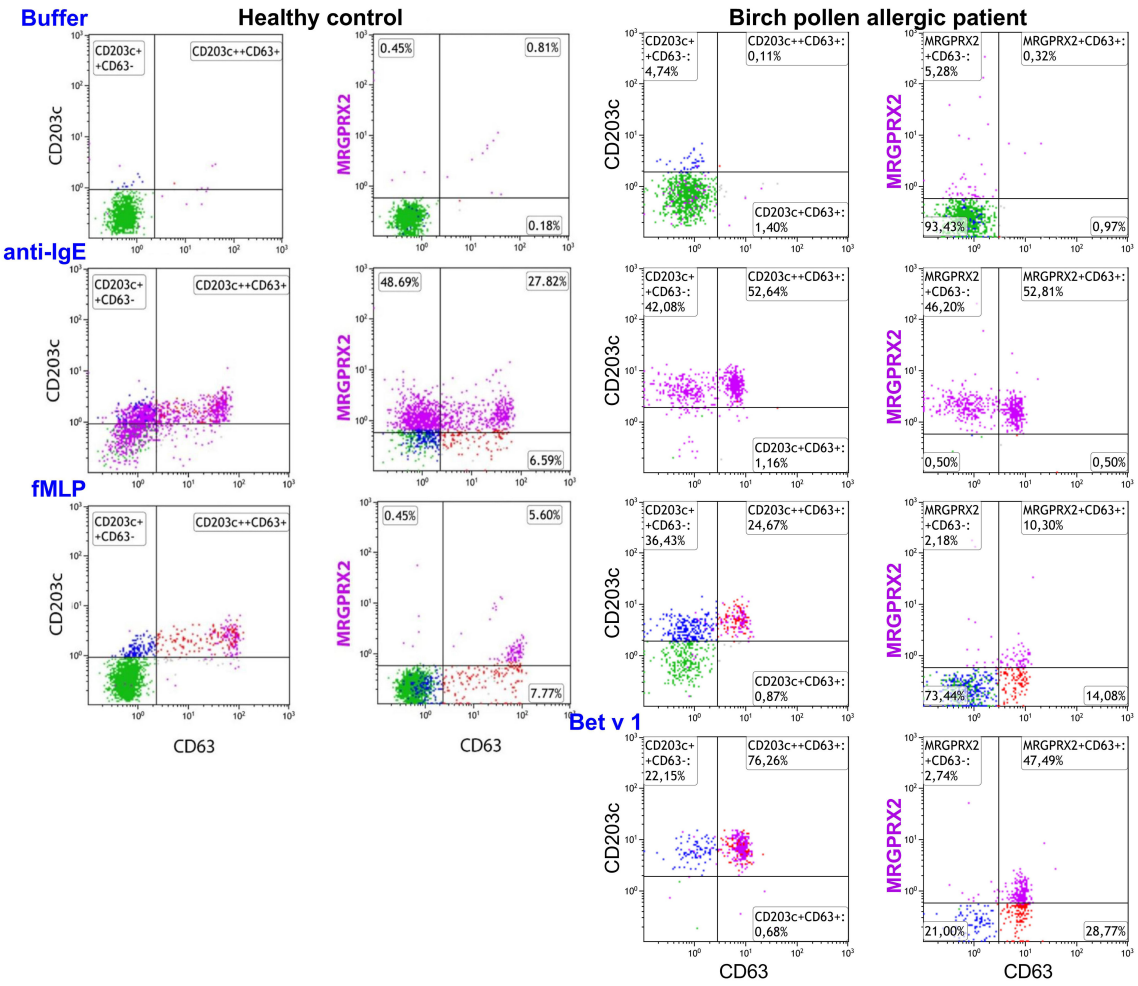

FIGURE 3
Effect of anti-IgE and fMLP stimulation on membrane MRGPRX2 expression in birch allergic patients. Representative plots on the effect of anti-IgE, fMLP or Bet v 1 stimulation of 20 min on membrane MRGPRX2 expression of whole blood basophils from one birch pollen allergic individual. Resting cells are identified as $IgE^{+}CD203c^{+}CD63^{-}$ (green). $MRGPRX2^{+}$ expressing cells are indicated in purple.

also be elicited by the experimental conditions themselves). In fact, constitutive surface expression of MRGPRX2 by basophils would be difficult to align with previous results on moxifloxacin, other fluoroquinolones, opioids, and neuromuscular blocking agents, all known MRGPRX2 agonists (11, 20, 21, 27–32).

In support of our hypothesis, we show that cell purification with magnetic beads actively induces surface expression of MRGPRX2 in basophils. Furthermore, purified basophils resuspended in the same culture medium as used by Wedi et al. (18) showed a higher density of MRGPRX2 than those resuspended in a different solution and became unresponsive to an IgE-mediated stimulus. This might imply that this specific culture medium may further enhance the expression of MRGPRX2. However, even after purification and resuspension in the same culture medium as that used by Wedi et al. (18), the basophils in our experiments did not exhibit spontaneous surface upregulation of CD63 and were still unable to degranulate after stimulation with an MRGPRX2 agonist. Indeed, further conditioning with IL-3 (and/or IL-33) was necessary to achieve significant degranulation with substance P or moxifloxacin, but neither IL-3 nor IL-33 was responsible for an increase in MRGPRX2 expression that might have underpinned such basophil activation.

Overall, some subtle differences in the two experimental settings may have played a role in these discrepancies. However, as demonstrated by the series of experiments performed with substance P and moxifloxacin with whole blood basophils versus purified basophils from healthy donors, MRGPRX2 ligands appear to be able to selectively induce degranulation in purified basophils. This suggests that cell purification is responsible for the expression of functional MRGPRX2.

Unlike Wedi et al, but in agreement with the data from the FANTOM 5 project (33), we could not demonstrate the presence of MRGPRX2 mRNA in resting basophils. The reason for these conflicting findings is unclear. The observation that the levels of mRNA and protein are poorly correlated is not an infrequent phenomenon. For example, it has been shown that the expression of FcεRI does not necessitate large numbers of mRNA molecules (34). The same could also be true for MRPGRX2 in basophils.

Alternatively, MRGPRX2 is confirmed to be ubiquitously and abundantly expressed intracellularly in resting whole blood basophils

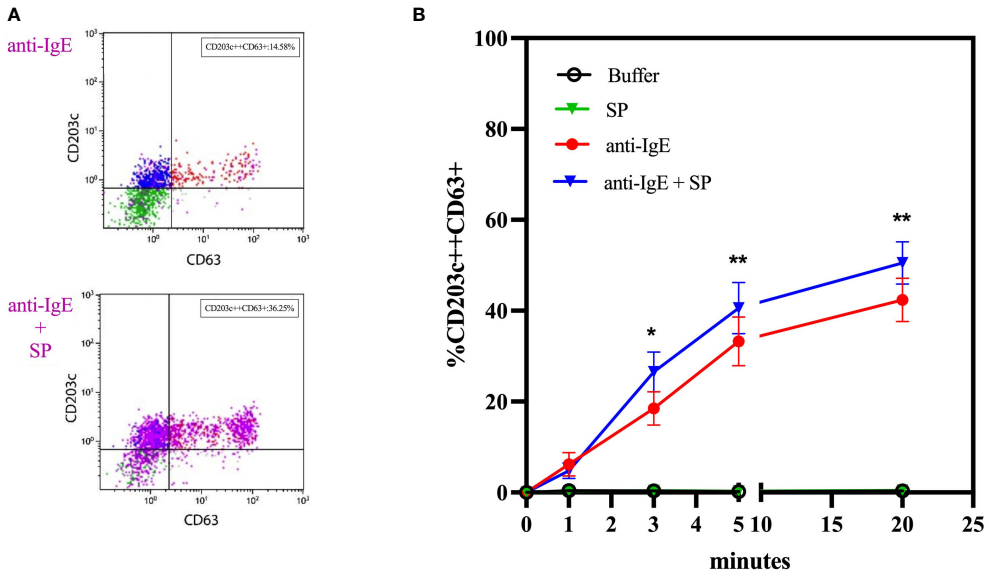

FIGURE 4
Co-incubation experiments with anti-IgE and substance P. **(A)** Representative plots of the effect of co-incubation of anti-IgE and SP on the membrane expression of CD63 of whole blood basophils in comparison to anti-IgE alone. **(B)** Time kinetics of the effect of SP, anti-IgE and SP in co-incubation with anti-IgE on the membrane expression of CD63 of whole blood basophils (n=10). SP = substance P. * $p \leq 0.05$; ** $p \leq 0.01$ anti-IgE compared to anti-IgE + SP at each time point; paired Student's t-tests.

and surface expression is rapidly upregulated in response to IgE/FcεRI-dependent and IgE/FcεRI-independent activation of the cells. At present, the exact intracellular localization of MRGPRX2 in basophils remains unknown. Its upregulation displays different time kinetics and magnitudes depending on the activation mode. All three readings, i.e., the ectoenzyme CD203c, the lysosomal degranulation marker CD63, and MRGPRX2, reveal fMLP, which acts *via* FPR-1, another G-protein-coupled receptor (GPCR) (35), to trigger a faster but transient and significantly less pronounced activation of the cells as compared to cross-linking of IgE/FcεRI by anti-IgE or a relevant allergen (e.g., rBet v 1 in BPAs). These data parallel the findings by Knol et al. (36), who showed basophilic histamine release by IgE/FcεRI cross-linking to be slower than the almost instantaneous release in response to fMLP and the observations in MCs by Gaudenzio et al. (5), who demonstrated that IgE-independent activation triggers a more rapid but transient degranulation as compared to IgE/FcεRI cross-linking. Furthermore, priming with IL-3 selectively induces CD203c upregulation without upregulation of CD63 or MRGPRX2 and, in CD63-non-responding moxifloxacin hypersensitive patients, MRGPRX2 upregulation occurs independently of CD63 appearance. Collectively these data suggest that MRGPRX2 is localized in a third intracellular compartment.

Furthermore, our experiments show that basophilic expression of MRGPRX2 could also contribute to pathological conditions such as IDHRs resulting from the off-target occupation of non-immune receptors. At present, most studies on the ability of drugs to activate MRGPRX2 have been conducted with murine MCs (4), transfected HEK cells (4), and human MC lines (e.g., LAD2) (4, 6, 12), or *in vitro* $CD34^+$-derived human MCs (5, 7–9, 22). Based on these studies it has been proposed that the occupation of MRGPRX2 could be responsible for IDHRs to several drugs and that the murine orthologue MrgprB2 might serve as a model for the development of therapeutic strategies aimed at preventing or treating a subset of IDHR. However, a comparison of the data of McNeil et al. (4) with the findings by Azimi et al. (6) and our own observations (8), reveals significant species-specific differences, which might hinder the translation of findings in mice to humans making this model not suitable for the development of therapeutic strategies. Alternatively, the LAD2 cells have been shown to be intermediately differentiated as compared to human mature skin MCs and to variably express MRGPRX2 (37).

Based on our data, we anticipate that "conditioned" basophils could serve as a human model to explore IDHRs resulting from the MRGPRX2 occupation. Moreover, as such MRGPRX2-dependent IDHRs only occur in a minority of exposed individuals, and do not necessarily involve all drugs with MRGPRX2-agonistic properties, our approach using "conditioned" patients' basophils could allow capturing data that are inaccessible when using animal models or techniques based upon cell lines or healthy donor cells. The reason why not all individuals exposed to a substance capable of activating MRGPRX2 react with an IDHR has not yet been elucidated with certainty but is probably attributable to polymorphisms in the receptor. For instance, mutations in the carboxyl terminus of MRGPRX2, the portion responsible for phosphorylation and desensitization of the receptor, can make mast cells more responsive to ligands such as SP (38). Admittedly, using humanized cell cultures expressing a specific variant of MRGPRX2, while not equally easily accessible, could be another possibility for an individualized study of the receptor.

In this context, we show that in patients with immediate hypersensitivity to moxifloxacin, co-incubation of the basophils with anti-IgE and moxifloxacin induces a more pronounced degranulation as compared to IgE/FcεRI cross-linking by anti-IgE alone. This is in accordance with the recent findings that IgE-

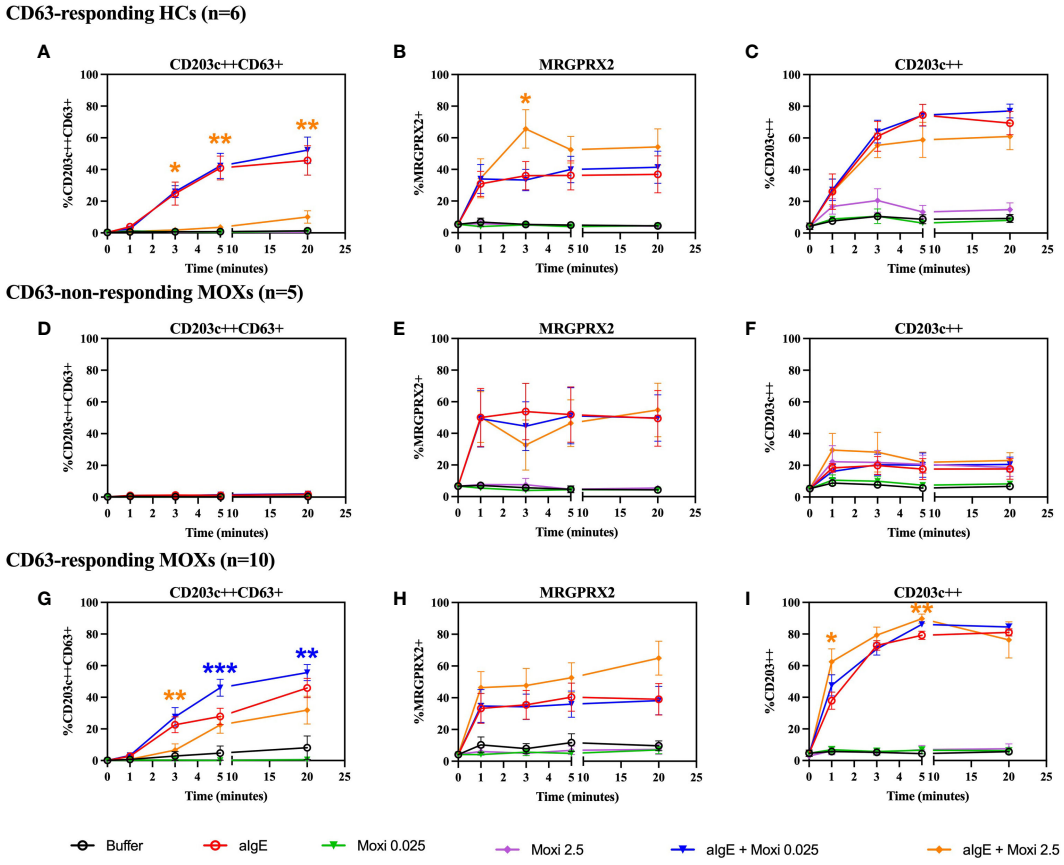

FIGURE 5
Time kinetics of co-incubation experiments with anti-IgE and moxifloxacin. Whole blood basophils are stimulated with anti-IgE and moxifloxacin (0.025 mmol/L and 2.5 mmol/L) alone or in co-incubation with anti-IgE. The figure shows the effect on CD63, MRGPRX2 and CD203c membrane expression from CD63-responding HCs (n=6) **(A-C)**, CD63-non-responding MOXs (n=5) **(D-F)** and CD63-responding MOXs (n=10) **(G-I)**. For experiments with moxifloxacin 2.5 mmol/L, alone or in coincubation with anti-IgE, in CD63-responding MOXs, n=7. aIgE=anti-IgE; Moxi: moxifloxacin; MOXs = patients with immediate type hypersensitivity to moxifloxacin; HCs = healthy controls. Blue asterisks: statistical significance of aIgE + Moxi 0.025 mmol/L compared to aIgE; orange asterisks: statistical significance of aIgE + Moxi 2.5 mmol/L compared to aIgE; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; paired Student's t-tests.

mediated and MRGPRX2 activation can synergistically combine to boost the exocytosis of cutaneous MCs (39).

Remarkably, even though IgE-mediated upregulation of MRGPRX2 is observed in all the subjects, synergistic degranulation is restricted to patients with immediate-type hypersensitivity to moxifloxacin with a "CD63-responder" status of the basophils. The reasons why patients with a "CD63-non-responder status" and controls with a "CD63-responder status" do not show this synergistic effect remain elusive. To some extent, the different behavior between CD63-responding patients and CD63-responding controls could relate to polymorphisms, mutations, and epigenetic modifications affecting MRGPRX2-driven signaling (40–42).

Similarly unclear, there seems to be a conundrum with co-stimulation with anti-IgE and moxifloxacin, with opposite findings for low and high stimulation concentrations of this drug. The reasons for the antagonistic effect of the highest tested concentration of moxifloxacin on anti-IgE induced degranulation observed in CD63-responders, regardless of their clinical status, remain elusive. Babina et al. recently described a synergistic effect on the degranulation of mast cells for low concentrations of two different MRGPRX2 ligands, namely SP and codeine. Of note, for higher concentrations of the same ligands, no agonistic effect on the degranulation of one stimulus on the other was observed (39). Whether extremely high concentrations of MRGPRX2 ligands could lead to the initiation of a counterregulatory mechanism, which in our case may have influenced IgE-mediated degranulation, remains speculative. The existence of extensive crosstalk between IgE-mediated and non-IgE-mediated pathways and their intracellular signaling has recently been described in detail (43). For instance, despite the existence of a redundant and overlapping signaling network between the two pathways, calcium channels differentially affect PI3K activation in FcεRI- compared to MRGPRX2-mediated signaling, which is a crucial intracellular signal transducer for both (44). This could result in a counterregulatory mechanism that avoids noxious degranulation (43). Particularly interesting is that while in HCs there is no synergistic effect of moxifloxacin 0.025 mmol/L, moxifloxacin 2.5 mmol/L still manages to have an inhibitory effect on anti-IgE-mediated degranulation; moreover, this effect is visibly higher than that seen in CD63-responding patients. One could therefore speculate that high concentrations of moxifloxacin succeed in stimulating

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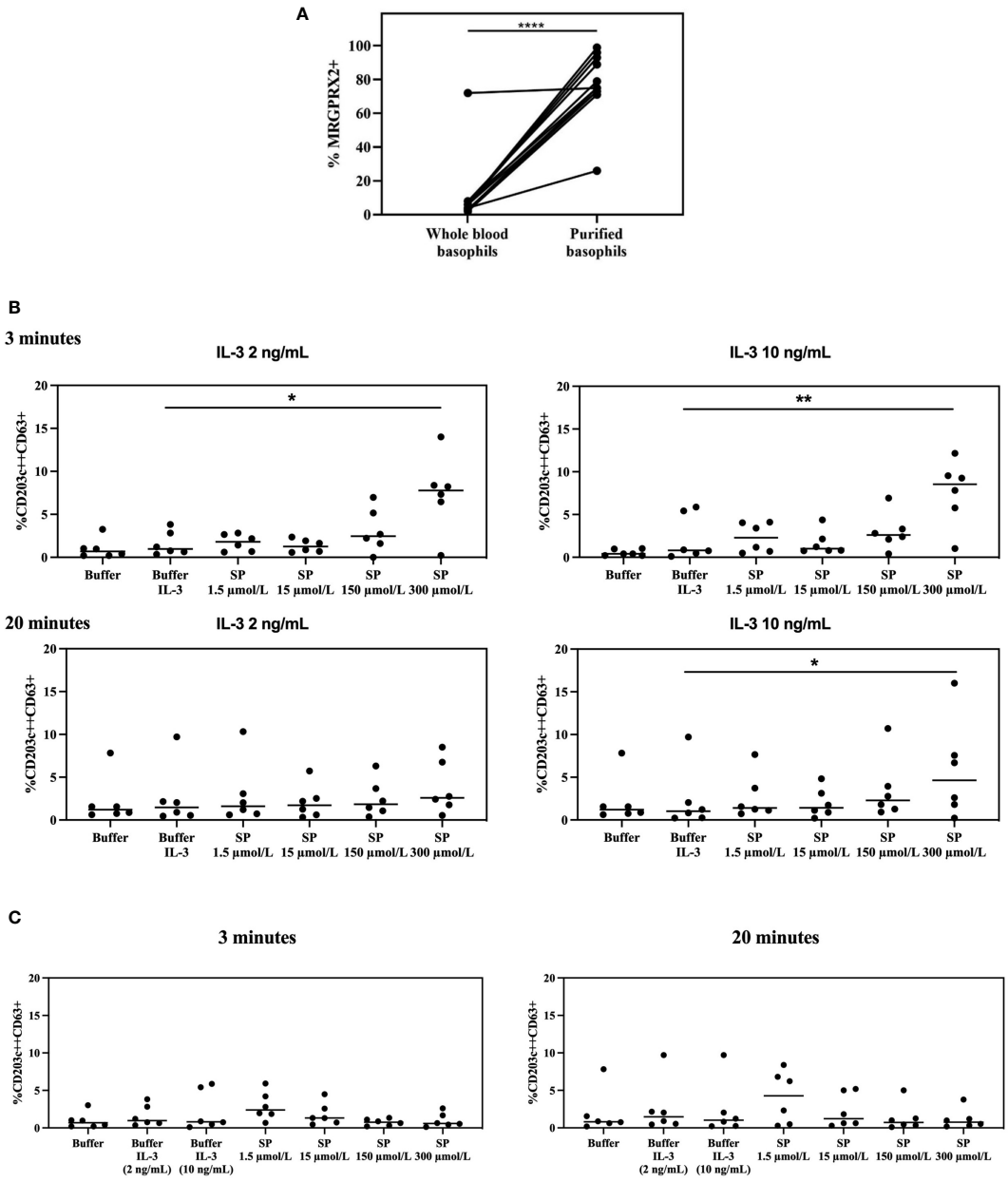

FIGURE 6
MRGPRX2 expression in whole blood vs purified basophils and MRGPRX2 functionality in purified basophils. **(A)** Effect on MRGPRX2 expression on the surface membrane of basophils from HCs after purification using magnetic beads (n=12). **** $p \leq 0.0001$; paired Student's t-tests. **(B)** Membrane expression of CD63 on purified basophils from HCs resuspended in RPMI medium, primed with IL-3 (2 ng/mL and 10 ng/mL) and subsequently stimulated with SP for 3 and 20 minutes (n=6). * $p \leq 0.05$; ** $p \leq 0.01$ SP 300 µmol/L after priming with IL-3 compared to buffer after priming with IL-3; paired Student's t-tests. **(C)** Membrane expression of CD63 on purified basophils from the same HCs as in Figure 6B resuspended in RPMI medium, without priming with IL-3, and stimulated with SP for 3 and 20 minutes (n=6). HCs = healthy controls. SP: substance P.

MRGPRX2 in these healthy subjects without causing degranulation, but with an exclusive counterregulatory effect, whereas a full degranulation effect is already observed at lower concentrations in patients. It might be precisely the result of residual MRGPRX2-mediated degranulation that accounts for the lower inhibition observed in CD63-responding patients. Not to be overlooked, a direct pharmacological effect of high concentrations of moxifloxacin on calcium channels may have led to this effect, because of their key role and differential effects on IgE- and non-IgE-mediated pathways. Fluoroquinolones can interact directly with calcium channels. In fact, they induce a multi-ion channel–blocking action in the heart within the supra-therapeutic dose range and can exert insulin secretion *via* the Ryanodine receptor activation and the active influx of calcium from the extracellular space in pancreatic β-cells (45, 46). Clearly, MRGPRX2 signaling remains unclear and definitely is more than currently meets the eye.

Admittedly, our *ex vivo* model of co-incubation with anti-IgE and moxifloxacin does not exactly mirror *in vivo* conditioning of the cells during infection/inflammation. However, for the time being, except for fMLP and rBet v 1 in patients with birch pollen allergy, we failed to

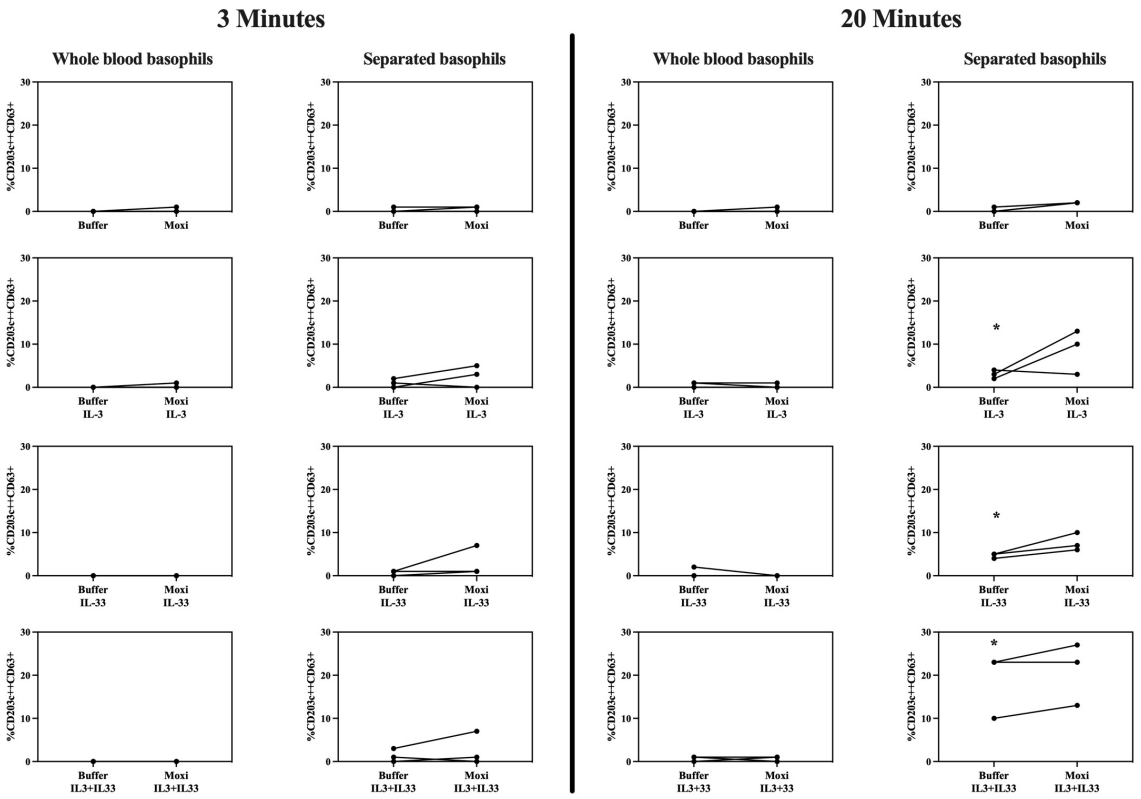

FIGURE 7
Stimulation with moxifloxacin of whole blood basophils vs purified basophils. Membrane expression of CD63 of whole blood basophils and purified basophils resuspended in RPMI medium from 3 HCs after incubation with IL-3 (10 ng/mL), IL-33 (30 ng/mL), and moxifloxacin (2.5 mmol/L) alone or in co-incubation with IL-3 (10 ng/mL) and/or IL-33 (30 ng/mL) (analyses performed at 3 and 20 minutes) (n=3). HCs = healthy controls. * $p \leq 0.05$ compared to buffer; paired Student's t-tests.

identify other substances that promote surface upregulation of MRGPRX2 by basophils. Neither LPS nor Staphylococcus enterotoxin seems to have any effect. Similarly, no significant upregulation of MRGPRX2 could be induced in MCs either, despite numerous efforts (47). Wedi et al. described a dose-dependent increase of MRGPRX2 surface expression in purified basophils after 30 minutes of incubation with IL-3, a well-established primer of basophils (48). Upregulation of the receptor was observed also after 24 hours of incubation with IL-3, anti-IgE, C5a, or fMLP (18). However, we failed to observe an IL-3 induced upregulation of MRGPRX2, neither in whole blood nor in purified basophils.

In conclusion, we show that circulating basophils can be rapidly "conditioned" to respond to the occupation of *de novo* MRGPRX2 surface expression. Moreover, since resting basophils of uneventfully exposed control individuals do not respond non-specifically to drugs requiring MRGPRX2 involvement (11, 20, 21, 28–32), it is tempting to hypothesize that comparative studies with and without "conditioned" cells might enable discrimination between IDHRs from genuine cross-linking of IgE/FcεRI and MRGPRX2 occupation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics committee of the Antwerp University Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

VS designed the study. AV, MB, and M-LvP enrolled the patients. CM, who also contributed to the experimental design, performed the experiments with CB and MVH. The experiments with the polymerases chain reaction were performed by SVR and J-PT. AT and JE performed the data analysis and wrote the first draft of the paper. DE, MH, and VS coordinated and supervised the project and contributed to writing the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1026304/full#supplementary-material

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Basophils control T cell priming through soluble mediators rather than antigen presentation

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Basophils play an important role in the development of type 2 immunity and have been linked to protective immunity against parasites but also inflammatory responses in allergic diseases. While typically classified as degranulating effector cells, different modes of cellular activation have been identified, which together with the observation that different populations of basophils exist in the context of disease suggest a multifunctional role. In this review we aim to highlight the role of basophils play in antigen presentation of type 2 immunity and focus on the contribution basophils play in the context of antigen presentation and T cell priming. We will discuss evidence suggesting that basophils perform a direct role in antigen presentation and relate it to findings that indicate cellular cooperation with professional antigen-presenting cells, such as dendritic cells. We will also highlight tissue-specific differences in basophil phenotypes that might lead to distinct roles in cellular cooperation and how these distinct interactions might influence immunological and clinical outcomes of disease. This review thus aims to consolidate the seemingly conflicting literature on the involvement of basophils in antigen presentation and tries to find a resolution to the discussion whether basophils influence antigen presentation through direct or indirect mechanisms.

KEYWORDS

basophil, dendritic cell, allergy, Type 2 immunity, antigen presentation

Introduction

Basophils were discovered by Paul Ehrlich in 1879 during staining experiments with peripheral blood and represent the least common granulocyte population in mammals, accounting for 0.5-1% of circulating leukocytes. They differentiate from hematopoietic progenitor cells ($Lin^-CD34^+Fc\varepsilon RI^{high}c\text{-}kit^-$) in the bone marrow under the control of the transcription factors C/EBPα and GATA-2 and leave the bone marrow as mature circulating basophils (1). Basophils were traditionally considered to be circulating counterparts of tissue-resident mast cells based on their expression of the high-affinity IgE receptor (FcεRI),

mechanisms of degranulation and histamine release upon activation. Facilitated by the discovery of distinct developmental pathways that are controlled by the key transcription factor C/EBPα (2), the distinct expression of c-kit/CD117 on human and murine mast cells but not basophils (3), and the development of basophil-specific transgenic knockout strains (4), a specialized role for basophils in various diseases and protective immunity have become better understood. Basophils have been shown to play an important role in allergic diseases, autoimmunity, parasitic infections and tissue homeostasis through the production of key cytokines and their interaction with immune and non-immune cells both in pro-inflammatory and anti-inflammatory contexts (5). Basophils are best studied in the context of allergy, where they have been implicated in several disease mechanisms, such as delayed IgE-mediated chronic allergic inflammation (6, 7), eosinophil entry (8), itch (9), and alternative macrophage activation (10), but also wound healing (11) and microbial dysregulation (12). Basophil activation is also used in the clinical diagnosis of allergic diseases and in monitoring the therapeutic response to immunomodulatory treatments (13). Basophils can be activated via various IgE-dependent and -independent pathways leading to the release of effector molecules like histamine, amphiregulin, eicosanoids (e.g. LTC4), granzyme B and a variety of different cytokines (e.g. IL-3, IL-4, IL-5, IL-6, IL-13, IL-25, IL-31) (14) (Figure 1).

The 'classical' activation of human and murine basophils in the context of allergy is caused by the crosslinking of FcεRI via IgE and leads to rapid degranulation and the release of preformed histamines and proteases, followed by a secondary *de novo* synthesis of lipid mediators and cytokines and their secretion (15). Alternative activation is readily achieved *in vitro* and independent of IgE crosslinking and mediated by innate stimuli including epithelial derived inflammatory cytokines, growth factors, eicosanoids, metabolites and TLR ligands (16).

Basophils can promote allergic immune responses by producing substantial amounts of pro-allergic IL-4 and IL-13 upon allergen stimulation (17, 18), thus representing an important accessory cell type to promote Th2-like responses (19, 20). Basophils can also contribute to a Th2 bias in pro-inflammatory environments, as basophil recruitment into tumor-draining lymph nodes was found to correlate with Th2 inflammation and reduced survival in pancreatic cancer patients (21). Basophils can however also contribute to pro-inflammatory immune responses through the production of IL-6, influencing Th17 immunity. In murine models of pro-inflammatory lung inflammation basophils and their production of IL-6

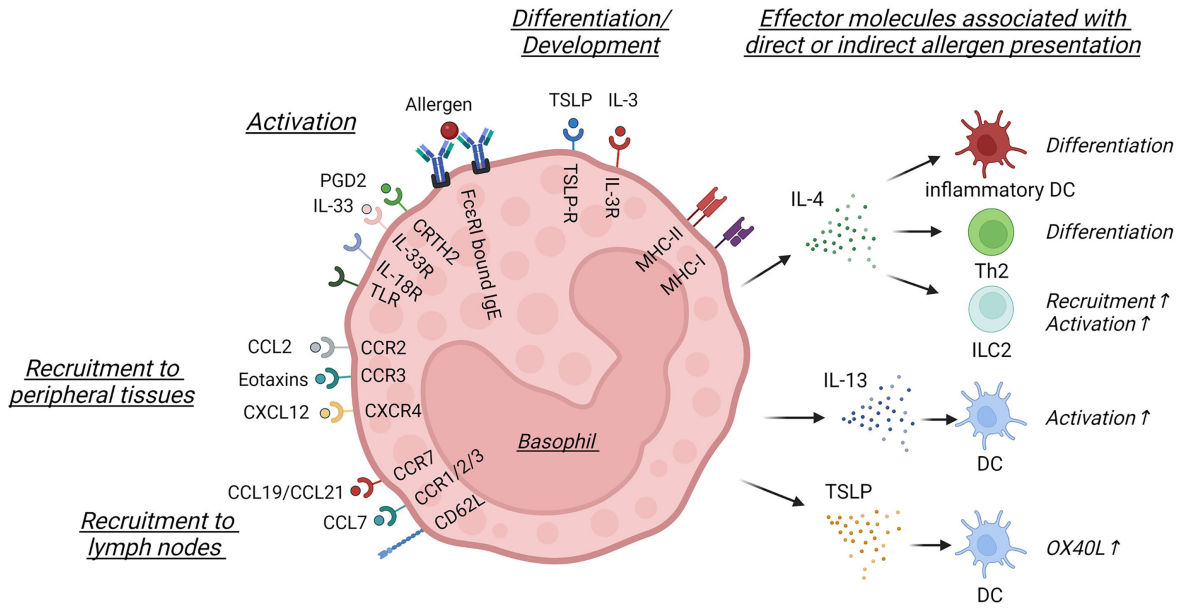

FIGURE 1
Basophil activation and effector signals involved in direct or indirect allergen presentation. In this schematic only surface and secreted molecules discussed within this review are shown. Basophil differentiation and development is controlled by TSLP and IL-3, which leads to the differential expression of cytokine and chemokine receptors, such as CRTH2, IL-33R, IL-18R, different TLRs, CCR2, CCR3 or CXCR4. Basophils can be activated by crosslinking of FcεRI-bound IgE or by different soluble mediators, such as PGD2 or IL-33. Basophils are recruited into peripheral tissues *via* CCL2, eotaxins or CXCL12, while CCL7 signaling or CCR7 and CD62L expression facilitate lymph node entry. While in specific contexts basophils can express MHC-I and MHC-II, they are best known for the secretion of soluble mediators. IL-4 can influence the differentiation of inflammatory dendritic cells (DC) and Th2 cells or activate innate lymphoid cell type 2 (ILC2), while IL-13 and TSLP secretion activates DC and induces OX40L upregulation, indirectly influencing the priming of Th2 cells. This figure was created using biorender.

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contributed to the differentiation of Th17 cells (22), while in models of kidney fibrosis CXCR2$^+$ basophils, recruited into the inflamed kidney, were an important source of IL-6 and controlled the number of Th17 cells (23). In human patients, basophils have also been identified in Th17-associated disorders, such as kidney fibrosis (23), IBD (24) and cystic fibrosis (25), indicating that basophils influence both Th2 and Th17 immunity through the release of key cytokines.

Beyond their role as cytokine-producing cells, basophils have also been suggested to influence the priming of adaptive immune responses by acting as unconventional antigen-presenting cells. In this review we will therefore discuss if basophils can influence antigen-presentation through direct and indirect mechanisms and correlate experimental evidence obtained in murine studies with clinical observations.

Subsets of basophils

Four populations of circulating basophils can be identified in the blood of healthy individuals based on their surface marker expression of CD16, CD244 and FceRI (26). FceRI-expressing basophils are highly responsive to IgE and IL-3 stimulation, while FceRI^{low} basophils respond poorly to those stimuli *in vitro* (26). Resting and activated human basophils also express distinct chemokine receptors, potentially supporting their migration towards sites of inflammation or the draining lymph nodes (dLN) (27). In the context of local inflammation, murine models have shown that eotaxin-CCR3, CCR2-CCL2 and CXCR4-CXCL12 interactions are the most common (28) (Figure 1). Chemokine receptor upregulation can be induced by different molecular mechanisms. CXCR4 upregulation is regulated by thymic stromal lymphopoietin (TSLP) and IL-3, cytokines essential for the development and activation of basophils (29, 30), and leads to basophil migration towards a CXCL12 gradient in inflamed skin (31). In *Lyn*$^{-/-}$ lupus prone mice CXCR4 surface expression is however controlled by PGD2 signaling and leads to the accumulation of basophils in secondary lymphoid organs impacting the severity of disease (32) (Figure 1).

Importantly, murine basophils can be differentiated into distinct basophils subsets by *in vitro* stimulation with certain cytokines, indicating that the cytokine milieu can influence basophil maturation and effector function of basophils differently. TSLP-cultured basophils showed higher expression of IL-3R, IL-33R and IL-18Rα and less degranulation, while producing higher levels of IL-4, IL-6, CCL3 and CCL12 in the context of IL-3, IL-18 and IL-33 activation (33) (Figure 1). IL-3-cultured basophils showed higher expression of CD11b and CD62L, higher production of chemokines and produced more TNFα, suggesting a pro-inflammatory differentiation (33). A similar heterogeneity was observed in human basophils, which developed in a TSLP-elevated environment during food allergy-associated eosinophilic esophagitis (EoE) (30). While expression levels of HLA-DR, CD28, CD40, CD86, CD69 and CD203c were similar to those observed in healthy donors, basophils from EoE patients expressed significantly higher levels of the IL-33R, indicating that different basophil populations are associated with an altered susceptibility to allergic inflammation (33). In patients with mild to moderate asthma, basophils were strongly activated by TSLP leading to secondary production of IL-3, suggesting that in certain contexts TSLP and IL-3 can also act in concert (34).

Phenotypically different subgroups of basophils have also been observed in patients with chronic urticaria when analyzing both the frequency of peripheral basophils and their reactivity to certain stimuli. Here, stimulation of peripheral blood basophils with anti-FcεRI revealed distinct reactivity patterns. While one group of patients exhibited a concentration-dependent activation of basophils (responders), FcεRI stimulation failed to activate basophils in the non-responder group (35, 36). This incapability to induce IgE-mediated reactions despite sufficient FcεRI might be due to a lack of expressing the tyrosine kinase Syk and/or an overexpression of the Src-homology 2-containing-5'-inositol phosphatases (SHIP)-1 and SHIP-2, pathways which control FceRI signaling (35, 37). Among the nonreactive patients, a subgroup with pronounced basopenia (basophils accounting for less than 0.1% of peripheral blood cells) could been identified (38). The basophils of this clinically most severely affected cohort were characterized by a significantly augmented background activation, reduced receptor-bound IgE and a decrease in surface expression of FcεRI (39). Basopenia was associated with more severe disease, whereas the basophil responder phenotype was associated with longer disease duration.

Decreased frequencies of circulating basophils are furthermore observed in other disorders, such as allergic contact dermatitis, bullous pemphigoid, systemic lupus erythematosus or atopic dermatitis (AD) (40–42), and are likely caused by their migration into the affected tissues or secondary lymphoid organs (32). This is supported by evidence that transient basopenia reflects basophil migration to the skin during skin irritation (43) or the bronchoalveolar lavage fluid upon aeroallergen challenge (44) and might be controlled by similar or distinct chemotactic pathways compared to anaphylaxis (45).

Within tissues, basophils not only drive classical symptoms of allergic inflammation *via* histamine and leukotriene release, but also impact a number of immunological mechanisms *via* cytokine production, making them a highly immunologically relevant cell type (46) (Figure 1).

Direct mechanisms of basophil-enhanced antigen presentation

Whether basophils have antigen-presenting capacity is still debated and has been reviewed before (47, 48). Mice deficient in

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interferon-regulatory factor 2, a transcription factor believed to suppress basophil differentiation, show a marked increase in basophil numbers and develop spontaneous Th2 responses (49). Another molecule, Lyn kinase controls basophil GATA3 expression and *Lin*$^{-/-}$ mice exhibit basophilia and a basophil-dependent Th2 bias (50), indicating an important role for basophils in driving type 2 immunity. In *Lin*$^{-/-}$ mice but also in the context of parasite infection and certain allergy models, murine basophils have been reported to express MHC-II (20, 51–53), suggesting their involvement in antigen-presentation. While MHC-II expression of murine basophils could also be observed in certain hapten-induced models of type 2 immunity (53, 54), basophils examined in models of airway and skin allergy did not express MHC molecules (55, 56). Similar observations were made in allergic patients, where no expression of HLA-DR was observed in patients allergic to house dust mite (HDM), birch pollen as well as in healthy individuals before or after *in vitro* stimulation (57–60). Yet, patients from an allergen-rich environment displaying aFUT6 deficiency (effectively reducing the ability of basophils to egress from the blood stream and infiltrate tissues) developed reduced itch sensitivity and lower amounts of HDM-specific IgE, indicating that basophils influence Th2 immunity (61). While the mechanisms of antigen-presentation were not investigated further in this study, MHC-II expression by basophils might be regulated by the cytokine milieu or affect the development of distinct basophil subsets with distinct expression patterns. However, the reported MHC-II surface expression in murine basophils was several orders of magnitude lower than those observed in B cells and dendritic cells (DC) (51), highlighting that carefully controlled isolation and analysis protocols are necessary to avoid contaminated readouts (62).

While the tools to assess antigen uptake *in vivo* are limited, uptake of natural and model antigens has not been observed in murine and human basophils (29, 55, 58), while antigen-processing could be observed in certain *in vitro* settings (54, 63). Bone marrow-cultured murine basophils generated *in vitro* using IL-3 and GM-CSF showed a substantial increase of MHC-II molecules on their surface. While no corresponding increase in MHC-II transcript levels could be measured in basophils, it was observed that DC, which expressed high levels of MHC-II and were also developing under the same culture conditions, provided a possible source for MHC-II protein (47). Further experiments between purified bone marrow-derived basophils and DC confirmed that MHC-II molecules were derived from DC and acquired by basophils through cell contact-dependent trogocytosis (63) (Figure 2A). While the molecular requirements facilitating basophil-specific trogocytosis are not well understood, trogocytosis has been observed in other immune cells, either involving uptake of cellular membrane from dead cells, resulting in killing or active cellular membrane transfer (64). The process most similar to trogocytosis observed between basophils and DC is the interaction between T cells and DC. Here, trogocytosis requires ligand-receptor interaction between the T cell receptor (TCR) and a matching peptide-MHC complex (65). This interaction leads to the formation of an immunological synapse resulting in the internalization of the TCR and the transfer of peptide-MHC complexes, together with membrane fragments of DC onto the surface of the T cell (66, 67). This mechanism has been observed for both CD4$^+$ and CD8$^+$ T cells (68) and TCR-mediated trogocytosis is dependent on both actin polymerization and the TCR signaling pathway (69) and can be impaired by blocking costimulatory molecules or integrin interactions (70). While TCR-mediated trogocytosis can be excluded as a mechanism for basophils, it remains to be determined if integrin binding facilitates trogocytosis between basophils and DC.

Trogocytosis might also enhance the expression of costimulatory molecules by basophils. Naïve as well as stimulated murine basophils can express several costimulatory markers like CD40, CD80 and CD86 (20, 53, 71), but in contrast to DC do not upregulate these markers upon stimulation (54). While murine basophils constitutively express CD80 and CD86, co-culturing with DC further increases surface CD86, which might be linked to cell membrane trogocytosis (63). While trogocytosis has not been studied in the context of human basophils, basophils extracted from healthy individuals or allergic patients did not express costimulatory molecules, neither after being freshly isolated nor when stimulated with cytokines, IL-3, antigens or TLR agonists (58–60).

Several studies have shown that basophils can drive Th2 polarization *in vitro*, when purified from immunized mice and pulsed with OVA peptide (18, 20). While not being able to process full length proteins, murine basophils can present and cross-present OVA peptides efficiently and induce CD4 as well as CD8 T cell proliferation *in vitro* (53, 71), indicating that basophils have a certain capacity for antigen presentation. After depletion of basophils using an anti-FcεRI-directed MAR-1 antibody, Th2 responses were also decreased *in vivo* in an MHC-II-dependent manner (19, 20), suggesting a direct role of basophil-mediated antigen presentation. However, Hammad et al. demonstrated that *in vivo* basophil depletion with an anti-FcεRI MAR-1 antibody had strikingly different effects on subsequent Th2 challenge with HDM allergen compared to anti-CD200R3 (Ba103) antibody treatment, because of the depletion of FcεRI$^+$ inflammatory DC (55). While originally classified as monocyte-derived DC, these inflammatory DC have recently been identified as FcεRI$^-$ FcγRIV-expressing cDC2, which are depleted by the MAR-1 antibody due to its cross-reactivity with FcγRIV (72, 73). More specific depletion models of basophils using the anti-CD200R3 antibody or transgenic mouse models under the control of *Mcpt8* could show that basophils were not required for the development of Th2 cells in models of parasite infection (29, 56, 74) and models of airway or skin allergy (55, 75, 76), despite cellular interactions between basophils and T cells being observed (77). These studies made clear that DC were essential for T cell proliferation and Th2

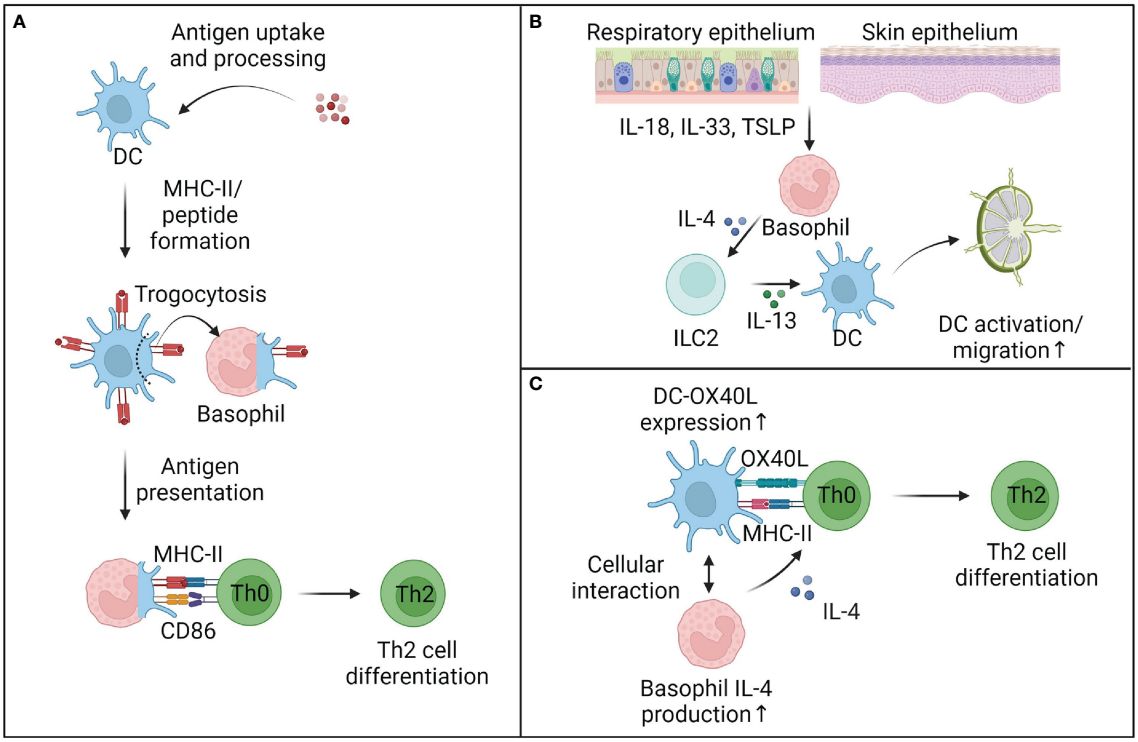

FIGURE 2
Mechanisms of basophil-enhanced antigen presentation. **(A)** Basophils can cooperate with dendritic cells (DC) to prime T cells. While basophils cannot take up and process complex antigens, they can trogocytose parts of cell membranes and antigen-loaded MHC-II complexes from DC and thus directly influence Th2 cell differentiation. It remains unclear to what extent trogocytosis plays a role *in vivo*, but other mechanisms of basophil-enhanced antigen presentation are well described. **(B)** In tissues, basophil-derived IL-4 activates murine innate lymphocytes type 2 (ILC2), which produce IL-13 and other mediators and activate DC to migrate to the draining lymph nodes. **(C)** Within lymph nodes, basophils can enhance DC activation and OX40L expression, while also providing early IL-4 to support the differentiation of Th2 cells. Although the requirement of early IL-4 for the differentiation of Th2 cells is debated, multiple studies provide evidence that basophils directly support the priming of Th2 cells, at least in the murine system. This figure was created using biorender.

priming, disproving earlier claims (54). In addition, these results also aligned with findings from patients samples, which showed that antigen-pulsed basophils purified from PBMC of healthy donors or allergic patients could not drive T cell proliferation in CFSE assays in contrast to other antigen-presenting cells (57–60). While these studies cannot exclude a cooperation between basophils and other cell types, basophils seem to have a limited capacity to drive T cell responses independently.

Indirect mechanisms of basophil-enhanced antigen presentation

Several mechanisms have been reported, which describe how basophils cooperate with other immune cells to enhance antigen presentation. In particular, the cooperation between basophils innate lymphoid cell type 2 (ILC2) and DC has been defined as an important immune axis in type 2 immunity (Figure 2B). Tissue ILC2 have been shown to play a complex role in allergic inflammation of both the lung and the skin (78, 79) and are found in close proximity with basophils in skin biopsies of AD patients and in pre-clinical models of AD. It could be observed that basophils and ILC2 form clusters in inflamed skin, with basophil accumulation preceding ILC2 activation and proliferation (80). Similar to IL-4-dependent accumulation of lung ILC2 during parasite infection (81), skin ILC2 accumulation was dependent on basophil-derived IL-4 in the murine MC903-induced model of AD (80). Basophil-derived IL-4 also controls the function of ILC2 in allergic lung inflammation through the production of IL-13 and the recruitment of eosinophils (82). IL-13 has in turn been shown to be major activator of DC both in the skin and lung (78, 83, 84), suggesting an indirect cooperation between basophils and DC *via* ILC activation in the skin and lung.

Basophils have also been reported in dLN, where they are localized within the T cell zone (19, 85). Basophils recruitment to the dLN is driven by TSLP signaling, although it remains unclear if TSLP acts on DC or T cells to recruit basophils or drives the

development of a dLN-migratory basophil subset (33, 76, 86, 87). Basophil entry into the dLN is facilitated by CD62L and CCL7, which support basophil binding to high endothelial venules and migration into the T cell zone (19, 75). Similarly, CD62L and CCR7 were upregulated in basophils from newly diagnosed systemic lupus erythematosus patients and associated with their accumulation in secondary lymphoid organs (42). Basophils have also been shown to enhance humoral immunity and together with $CD4^+$ T cells, profoundly enhanced B cell proliferation and immunoglobulin production (88).

It has been suggested that basophils can present antigen under certain contexts, but this mechanism might be less relevant for initial Th2 cell priming, as much fewer basophils are found in the dLN compared to DC and are recruited to the dLN at later timepoints (55). These findings are supported by observations that basophils isolated from healthy human spleens showed no expression of HLA-DR or costimulatory molecules at steady state or after *in vitro* stimulation and could not drive T cell proliferation, indicating that human basophil function is restricted to the secretion of soluble mediators (89). However, other studies have suggested that basophils provide help to DC for optimal Th2 induction (75, 90, 91). As basophils are major producers of IL-4, while DC are not (92), basophils could provide an early source of IL-4 (93), especially in dLN (Figure 2C). IL-4 has also been suggested to activate DC and induce the differentiation of inflammatory DC (94) observed in allergic and viral inflammation (55, 73). *In vitro* co-cultures between IL-4-deficient basophils, DC and OT-II T cells showed that Th2 cell differentiation was reduced and OX40L expression by DC was decreased in the absence of basophils or basophil-derived IL-4 (95). Furthermore, Di et al. underline the importance of OX40L signaling by DC and basophils. Blocking OX40-OX40L interactions with an anti-OX40L antibody strongly reduced allergic airway inflammation following OVA sensitization and adoptive transfers of OVA-challenged basophils into $OX40^{-/-}$ mice or blockade of OX40L led to reduced lung inflammation (96). As the requirement for an initial source of IL-4 in Th2 priming continues to be critically debated (97–100), regulation of OX40L expression through basophils might represent an additional mechanism of how basophils can influence antigen presentation (Figures 1, 2C).

Discussion

In the early 2000s an interesting hypothesis developed, which suggested that basophils could drive Th2 immunity independently of DC, and supply signals for antigen presentation, costimulation and Th2 polarizing cytokine secretion (20, 54, 101). This led to multiple studies investigating this hypothesis in different models of parasite infection, skin and lung allergy, which found that basophils could not process and present complex protein antigens, where present in dLN in much lower numbers than DC and arrived at later timepoints (55, 56, 74). Similarly, basophils collected from allergic patients, were not able to internalize, process or present allergen and thus failed to induce proliferation and cytokine secretion in T cells (57, 58). In line with this, basophils are unlikely directly involved in the priming of *de novo* Th2 cells, but could enhance DC activation and Th2 priming through the production of IL-4, the activation of ILC or other mechanisms of cellular cooperation [as reviewed in (47, 89, 90, 102)].

In patients, different populations of basophils have been observed in a range of human diseases including tumors, fibrosis, infection and chronic inflammation (5), and it is unknown if under certain conditions human basophils obtain antigen-presenting capacities, especially in the context of antigen challenge or chronic disease. Multiple murine studies have shown that basophils enhance T cell responses after antigen challenge (22, 103), yet little is known regarding human diseases, due to limited studies in affected tissues. While many studies agree that basophils do not express MHC-II or HLA-DR transcript, cell contact-dependent acqusition of MHC-II through trogocytosis could represent an additional molecular mechanism that allows basophils to be involved in antigen presentation. While trogocytosis has been studied in murine bone marrow-derived basophils (63), it is unknown if it also occurs *in vivo*, affects human basophils and also other surface molecules reported on basophils, including costimulatory molecules or MHC-I (20, 53, 71).

Additional studies to understand the molecular mechanisms that lead to the differentiation of basophil populations in the context of disease are therefore urgently necessary. While it is difficult to follow basophil differentiation during the progression of disease, seeding of basophils into tissue organoids from control- or patient-derived samples might offer new opportunities to study cellular differentiation and mechanisms of cellular cooperation and trogocytosis.

As basophils represent very rare immune cells, improved protocols to isolate basophils from affected tissues are also necessary to characterize basophils with novel technologies like single-cell sequencing. These analyses should however not only focus on transcriptomic signatures (e.g. by using single-cell RNA sequencing), but be combined with surface protein detection, such as site-seq or high-dimensional flow cytometry, to capture functional molecules that might have been acquired from other cells types. These studies might highlight tissue- and disease-dependent differences between basophil populations that contribute to disease and indicate their relationship to basophils within tissues in comparison to circulating basophil populations (104). As basophils have a multifaceted immunological role, these studies might ultimately define subpopulations that drive specific disease phenotypes through direct or indirect antigen presentation, cytokine secretion or histamine/leukotriene release, and allow for their selective targeting in the context of disease.

Author contributions

CM, MS, FB, MW and JM were involved in the writing of the original manuscript. MS, FB, and JM were involved in creating the figures. CM and JM were responsible for revisions and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IL-3 produced by T cells is crucial for basophil extravasation in hapten-induced allergic contact dermatitis

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Basophils have been recognized as a characterized cellular player for Th2 immune responses implicated in allergic diseases, but the mechanisms responsible for basophil recruitment to allergic skin remain not well understood. Using a hapten fluorescein isothiocyanate (FITC)-induced allergic contact dermatitis (ACD) mouse model, we show that basophils in FITC-treated IL-3-knockout mice are defective in crossing the vascular endothelium to enter the inflamed skin. By generating mice in which IL-3 is selectively ablated in T cells, we further demonstrate that IL-3 produced by T cells mediates basophil extravasation. Moreover, basophils sorted from FITC-treated IL-3-knockout mice exhibit a decreased expression of integrins Itgam, Itgb2, Itga2b and Itgb7, which are potentially implicated in extravasation process. Interestingly, we observed that these basophils had a reduced expression of retinaldehyde dehydrogenase 1 family member A2 (Aldh1a2), an enzyme responsible for the production of retinoic acid (RA), and administration of all-trans RA restored partially the extravasation of basophils in IL-3-knockout mice. Finally, we validate that IL-3 induces the expression of ALDH1A2 in primary human basophils, and provide further evidence that IL-3 stimulation induces the expression of integrins particularly ITGB7 in an RA-dependent manner. Together, our data propose a model that IL-3 produced by T cells activates ALDH1A2 expression by basophils, leading to the production of RA, which subsequently induces the expression of integrins crucially implicated in basophil extravasation to inflamed ACD skin.

KEYWORDS

basophil, IL-3, allergy, skin, extravasation, integrin, retinoic acid

Introduction

Basophils, one type of circulating granulocytes that account less than 1% of peripheral blood leukocytes, represent a characteristic cellular component in parasite infection and allergic skin inflammation. Basophils complete their maturation in the bone marrow, circulate in the blood and migrate to tissue under inflammatory conditions. They have been shown to infiltrate skin lesions in certain skin disorders such as allergic contact dermatitis (ACD), acute atopic dermatitis (AD), prurigo, urticaria and bullous pemphigoid, but are absent in other skin disorders like psoriasis vulgaris (1).

Despite of being the least abundant circulating leukocytes, basophils have been recognized to play important roles in physiological and pathological contexts. Basophils are recruited to inflamed tissues and activated in an IgE-dependent or -independent manner to release a variety of effector molecules, such as histamine and leukotriene C4, chemotactic factors, and cytokines including IL-4, IL-13 that are involved in immediate and late-phase reactions of the immune system (2). In addition, basophils were reported to crosstalk with other inflammatory cells, for example to mediate eosinophil recruitment to allergic skin (3, 4) or to confer an M2-like phenotype on macrophages (5).

Although our knowledge on basophil function has been rapidly expanded, how these cells infiltrate to inflammatory sites remains not well understood. IL-3 has been implicated in basophil survival *in vitro* (6), and activation (7, 8), in regulating basophil expansion in blood, or basophil production from the bone marrow in *Nippostrongylus brasilensis (N.b.)* parasite infection mouse models (9, 10). IL-3 was also reported to play a role for basophil recruitment to the mesenteric lymph nodes in *N.b.* infection (Kim et al., 2010), or to skin-draining lymph nodes in an AD mouse model (11). Yet, it remained not defined how important IL-3 is for basophil recruitment to allergic skin site and what are underlying mechanisms.

Tissue inflammatory immune response develops upon the extravasation of leukocytes into the tissue by crossing blood vessels. For circulating leukocytes to enter a tissue under inflammatory conditions, a cascade of events is required that involves an interaction between the leukocyte and endothelial cells (ECs), comprising essential sequential steps including chemo-attraction, rolling, adhesion to the blood vessel wall and trans-endothelial migration (TEM): first, triggering of the activation of leukocyte rolling and adhesion by chemokines (12); second, the binding of selectins (P-and E-selectins on the endothelium) to their ligands such as P-selectin glycoprotein ligand 1 (PSGL-1) expressed by leukocytes, and regulation of leukocyte rolling on the endothelium; third, adhesion of leukocytes to blood vessels by intergrins expressed on leukocyte surface to bind to their ligands expressed on ECs (e.g. ICAM-1, VCAM-1…); finally, TEM where leukocytes cross ECs lining the blood vessels (13, 14).

Integrins have been identified as important molecules implicated in leukocyte extravasation. Integrins are composed of a complex family of αβ heterodimers that can assemble into different receptors in vertebrates (15). For example, ITGAL/ITGB2 and ITGAM/ITGB2 were shown to be involved in neutrophil extravasation (16, 17) and ITGA4/ITGB7 for T cell migration (18). As to basophil extravasation, *in vitro* studies have shown that IL-3 receptor complex is expressed in ECs or basophils (19, 20), and treatment of ECs (21) or basophils (22, 23) with IL-3 enhanced basophil rolling, adhesion and TEM. Antibodies against PSGL-1, P-selectin, ITGAM, ITGB2 or ITGB1 were shown to inhibit basophil adhesion and migration to ECs (21–23). However, all these studies were performed *in vitro* and there was little *in vivo* study to explore basophil extravasation to inflamed tissues.

In this study, we investigated basophil recruitment in allergic skin by using hapten FITC-induced ACD mouse model (24), where basophil infiltration is a characterized feature. We demonstrate a crucial role of IL-3 produced by T cells in mediating basophil extravasation to the inflamed skin, and show that in the absence of IL-3 signaling, basophils exhibit reduced expression of a number of integrins that was accompanied by a reduced expression of retinoic acid (RA)-producing enzyme ALDH1A2. We tested whether the supplement of RA restores basophil skin extravasation in IL-3-knockout mice, and further examined the potential role of RA signaling in the regulation of integrins in IL-3-stimulated human primary basophils. Our data thus provide insights on a central role of IL-3 in the interaction between T cells, basophils and ECs in mediating basophil extravasation to the inflamed skin.

Materials and methods

Mice

Wild-type BALB/c mice were purchased from Charles River Laboratories. CD4-Cre$^{Tg/0}$ mice (25) were purchased from the Jackson laboratory and were backcrossed into Balb/c background (>99%).

IL-3-ablated (*Il3*$^{-/-}$) mice and -floxed (*Il3*$^{L2/L2}$) mice (all in pure Balb/c background) were generated by us at the Institut Clinique de la Souris (ICS) (Figure S1). In order to obtain an Il3 "2 in 1" allele (tm1a, Figure S1), we acquired and modified an IMPC plasmid ETPG00275_W_2_F02 (https://www.mousephenotype.org/data/genes/MGI:96552). This plasmid was digested with a RsrII restriction enzyme to remove the LacZ and the 5' region of the NeoR cassette, and a DNA fragment containing the eGFP cDNA and the deleted part (5' region) of the NeoR cassette (ordered from GeneArt) was amplified with primers containing 25 bps homology for the IMPC vector and cloned to the plasmid using the SLIC method (26). The resulting plasmid was fully sequenced to confirm the presence of all the desired components including *in frame* eGFP, Lox and FRT sites and NeoR cassette. After cutting with PvuI, the linearized construct was electroporated in in-house derived BALB/CN mouse embryonic stem cells (ESCs). After selection, targeted clones were identified by PCR using external primers and were further confirmed by Southern blot using both a Neo probe (5' and 3' digests) as well as a 3' external probe. Two positive ES clones were microinjected into C57BL/6N blastocysts. Resulting male chimeras were bred with wildtype C57BL/6N females. Germline transmission of the tm1a allele was obtained. The tm1c allele (or "L2" allele) was obtained after breeding of the heterozygous animal with a PHENOMIN-ICS BALC/CN Flp delete mouse line (Figure

S1). The tm1b allele (GFP-KI/Il3-KO, or mutant "-" allele) was obtained after breeding the heterozygous animals with a PHENOMIN-ICS Cre deleter mouse line (Figure S1).

Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the animal care and ethics committee of animal experimentation of the IGBMC n°017 and by the Ministère de l'enseignement supérieur, de la recherche et de l'innovation.

FITC treatment

Fluorescein isothiocyanate (FITC, ≥97.5% (HPLC) (Sigma) was first dissolved in acetone (to a concentration of 2%), then mixed with equal volume of dibutyl phthalate (DBP, Sigma) to get a final concentration of 1% FITC (in 1:1 DBP/acetone). Mice were sensitized with 25 µl of FITC (in 1:1 DBP/acetone) on the left ear (LE) followed by the challenge on the right ear (RE) with 25 µl of FITC (in 1:1 DBP/acetone), as indicated in experimental schemes in figures. RE thickness was measured using Digimatic Caliper (Mitutoyo).

All-trans RA treatment

All trans-RA (at-RA; MP Biomedicals) was dissolved in ethanol for a stock solution (5 mg/ml; 16 mM). For topical treatment, at-RA was diluted in ethanol to a final concentration of 40 µM and topically applied on mouse ears (25 µl per ear); for intraperitoneal (i.p.) injection, 0.1 ml of RA (5 mg/ml in ETOH) was mixed with 4.9 ml of sunflower oil; vortexed and sonicated to make a solution with final concentration of 0.1 mg/ml for injection (10 µl/g mouse) (27).

Cell preparation for FACS analyses

For preparation of dermal cells, ears were split into two halves, floated on a solution of Dispase (4mg/ml in PBS, Gibco) with epidermis side up, and incubated at 37°C for 1 h. Dermis was then separated from epidermis and was further incubated on an agitator at 37°C for 1 h in a solution containing 1 mg/ml collagenase D (Roche), 0.25 mg/mL DNaseI (Sigma) and 2.5% of foetal calf serum (FCS) (ThermoFisher) in PBS, then passed through a cell strainer (EASYstrainer 70 µm, Greiner bio-one). Cells were then centrifuged at 1200 rpm, 4°C for 5 min, resuspended in FACS buffer (1% of FCS + 2 mM EDTA in PBS), counted and used for FACS staining ($2x10^6$ cells) or for sorting.

For preparation of blood cells, 400 µl of blood was collected from mice by retro-orbital bleeding in EDTA-coated tubes, mixed with the same volume of Dextran (2% in PBS, Sigma-Aldrich) and incubated for 30 min at 37°C. The upper phase was transferred into new tubes, 600 µl of FACS buffer was added, then centrifuged at 4000 rpm for 4 min at 4°C. The pellet was resuspended in 0.3 ml of ACK lysis buffer (Ammonium-Chloride-Potassium: NH4Cl 0.15 M; KHC03 1 mM; Na_2EDTA 0.1 mM), incubated for 2 min at room temperature (RT), and then added 1ml of FACS buffer and centrifuged 4000 rpm for 4 min at 4°C. The pellet was resuspended in FACS buffer and used for FACS staining.

Antibody staining and FACS analyses

Cells were first incubated with anti-mouse CD16/CD23 (Fc block) for 10 min on ice, then washed and stained with the surface antibodies (Abs, listed below), starting with biotinylated Abs in 25 µl of FACS buffer for 10 min on ice, then washed and stained with streptavidin mixed with other surface Abs in 25 µl of FACS buffer for 10 min on ice (except for CD34 Ab which was incubated for 90 min on ice). Cells were then washed with FACS buffer, incubated for 3 min with DAPI (final concentration: 1 µg/ml) for exclusion of dead cells before passing on LSRII (BD).

For intracellular staining, dermal cells were cultured in RMPI medium w/o HEPES, + 10% FCS +1% P/S and 2 mM Glutamin, in presence or absence of GolgiSTOP (BD) and Cell Stimulation Cocktail (eBioscience) at 37°C for 2 h. Cells were then washed with FACS buffer then incubated with anti-mouse CD16/CD23 (Fc block) for 10 min on ice, then washed with FACS buffer and stained with the surface Abs (listed below) as described above. Cells were then washed and resuspended with 100 µl of Fixation/Permeabilization solution (BD Cytofix/Cytoperm kit) for 20 min on ice, then washed twice with the wash buffer (BD Cytofix/Cytoperm kit). IL-3 Ab (listed below) was added and incubated on ice for 30 min. After washing, cells were finally resuspended with FACS buffer and passed on LSRII analyser.

Antibodies used for Flow cytometry are described in Table 1.

RNA extraction of cells sorted from ears and quantitative RT-PCR

Ear dermal cells were prepared as described above. After antibody staining, cells were FACS-sorted: Endothelial cells ($CD45^-CD34^+ESAM\text{-}1^+$), Hematopoietic cells ($CD45^+$), TCRβ cells ($CD45^+TCR\beta^+$), Neutrophils ($CD45^+TCR\beta^-Gr1^{hi}$), Eosinophils ($CD45^+TCR\beta^-Siglec\text{-}F^+SSC^{hi}$), Basophils ($TCR\beta^-Siglec\text{-}F^-Gr1^-CD45^{lo}CD49b^+$). RNA was extracted with NucleoSpin RNA XS kit following the manufacturer's instruction.

RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by means of quantitative PCR with LightCycler 480 (Roche Diagnostics) and QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl-transferase (HPRT) as an internal control. Sequences of PCR primers for mouse genes are described in Table 2.

Histology

Mouse ears were fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) were stained with haematoxylin and eosin.

TABLE 1 Antibodies used for Flow cytometry.

Name	Fluorophore	Clone	Company	Dilution
CD16/CD32 (Fc block)		93	eBioscience	0.5:25
CD49b-biotin		DX5	eBioscience	0.5:25
IgE-biotin		R35-72	BD Biosciences	0.5:25
Streptavidin	BV605		Invitrogen	0.5:25
CD45	APC-eFluo780	30-F11	eBioscience	0.05:25
TCR-beta	PerCP-Cy5.5	H57-597	eBioscience	1:25
Siglec-F	Alexa Fluor647	E50-2440	BD Biosciences	1:25
Gr1	PE	RB6-8C5	eBioscience	0.02:25
CD34	eFluor 700	RAM34	eBioscience	4:25
ESAM-1	APC	1G8/ESAM	Biolegend	1.25:25
CD19	PerCP-Cy5.5	eBio1D3	eBioscience	1:25
CD3	FITC	145-2C11	eBioscience	1:25
CD45R/B220	PE-Cy7	RA3-6B2	Biolegend	1:25
IL-3	PE	MP2-8F8	Biolegend	1.25:50
FcεRIα	Alexa Fluor 647	Fc23cpg	eBioscience	1:25

Immunohistochemistry staining

For immunohistochemistry (IHC) staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), 5 µm paraffin sections were treated with 0.6% H_2O_2 to block endogenous peroxidase activity before antigen retrieval with either Pepsin (Life technologies; for IHC of MBP) or citric buffer (10 mmol/L citric acid, pH 6; for IHC of MCPT8). Slides were then blocked with normal rabbit serum (Vector Laboratories) and incubated overnight with rat anti-mouse MBP (1:2000, provided by Dr James J Lee, Mayo Clinic, Rochester) and rat anti-mouse MCPT8 (1:500, clone TUG8, Biolegend). Slides were then incubated with biotinylated rabbit anti-rat IgG (1:300) and treated with AB complex (Vector Laboratories, Cat No. PK-6104). Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako) and counter-stained with hematoxylin.

In vitro culture of human basophils and quantitative RT-PCR analyses

Human basophils were isolated from the buffy bags of healthy donors (Centre Trinité, L'Établissement Français du Sang, Paris; EFS-INSERM, 18/EFS/041) as previously described (28) by using Basophil Isolation Kit (Miltenyi Biotec, Paris, France). Basophils were then cultured in X-Vivo medium, with 100 ng/0.5 M cells/ml of IL-3, or with 10 nM all-trans RA for 6 hr with or without prior treatment with 1 µM each of retinoic acid receptors (RAR) antagonists CD2665 (RARβ/γ antagonist; Tocris, Cat. 3800) and BMS614 (RARα antagonist; Sigma, Cat. SML-1084) for 1 hr or with RAR antagonists for 1h followed by IL-3 for 6h or with RAR antagonists alone for 1h. Untreated basophils (Baso alone) were used as control.

Total RNA from the different experimental conditions was isolated using the RNeasy minikit (Qiagen, Hilden, Germany). cDNAs were synthesized using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Courtaboeuf, France), and quantitative PCR was performed with LightCycler 480 (Roche Diagnostics) and QuantiTect SYBR Green Kit (Qiagen) using the primers as described in Table 3. Relative RNA levels were calculated with human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) as an internal control.

Statistical analysis

Data were analysed using GraphPad Prism 9. Comparison of two groups was performed either by Student's two-tailed unpaired t-test with Welch's correction or the two-tailed Mann–Whitney rank sum nonparametric test depending on results from the Kolmogorov–Smirnov test for normality.

Results

Basophil accumulation in FITC-induced ACD skin is dependent on adaptive immunity

To induce allergic contact dermatitis (ACD) in mice, we employed an experimental protocol (24) in which Balb/c wildtype (WT) mice were first sensitized on one ear (left ear, LE) at Day (D)

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TABLE 2 Sequences of PCR primers for mouse genes.

Gene name	Sequence 5′ to 3′
Hprt	TGGATACAGGCCAGACTTTG GATTCAACTTGCGCTCATCTTA
Il3	TGAAGGACCCTCTCTGAGGA CGCAGATCATTCGCAGAT
Il4	GGCATTTTGAACGAGGTCAC AAATATGCGAAGCACCTTGG
Il13	GGAGCTGAGCAACATCACACA GGTCCTGTAGATGGCATTGCA
Il17a	CCAGGGAGAGCTTCATCTGT ACGTGGAACGGTTGAGGRTAG
Ifng	AACGCTACACACTGCATCTTGG GACTTCAAAGAGTCTGAGG
Ccr3	TAAAGGACTTAGCAAAATTCACCA TGACCCCAGCTCTTTGATTC
Mcpt8	GTGGGAAATCCCAGTGAGAA TCCGAATCCAAGGCATAAAG
Selp *(P-selectin)*	AAAAGGTTCCTGGACGCCAA GACGTCATTGAGGTGAGCGA
Sele *(E-selectin)*	ACGGATAGAGAGAAGCAGGAGC TCATGAGCTCACTGGAGGCA
Icam1	GCTCAGTATCTCCTCCCCA GCTGTGCTTTGAGAACTGTG
Vcam1	CCCAAACAGAGGCAGAGTGT CAGGACTGCCCTCCTCTAGT
Itgb1	GCTGGGTTTCACTTTGCTGG TGTGCCCACTGCTGACTTAG
Itgb2	CAACAACGTCAAGAAGCTGGG GCCTTCTCCTTGTTGGGACA
Itgb3	GTGTGGGCCTCAAGATTGGA AGGCACAGTCACAGTCGAAG
Itgb7	GACGACTTGGAACGTGTGCG TGGGTGGTGAAGCTTGGAGG
Itgam	AAACAAGGATGCTGGGGAGG GTCTCATCAAAGAAGGCACGG
Itgal	CTGGACCTGCGTGAAGACC GGTACCGTGGGGCTCCTG
Itga2b	AGACACCAGTCAGCTGCTTC CCTGACGGGGCTTCTGTAAG
Itga4	TAGCGAATCTTGGCGACATT ACCAACGGCTACATCAACAT
Itga5	ATGCCCTGAAGCCAAGTGTT TATTCCCGCTGCAAGAAGGT
Itgae	AGCCGGGACATTAACGCCTC ACCACCATGACCTTCAATGCTT

TABLE 3 Sequences of PCR primers for human genes.

Human Genes	Sequence 5′ to 3′
GAPDH	GTCAAGGCTGAGAACGGGAA AAATGAGCCCCAGCCTTCTC
ALDH1A2	TATGTGGATTTGCAGGGCGT ACATCAGCAGGGGGAAGTTC
ITGB2	CGACATCATGGACCCCACAA GCATGGAGTAGGAGAGGTCC
ITGAM	AGTGCTGGGGGACGTAAATG CCCACTCAGTGACTGACCAA
ITGA2B	CTCCTGCTGACTGGCACAC TCAGCCCCTCACTCTGACC
ITGB7	ACAGGGGATGCCACAGAATG GCCAGCAGCTCCTCTCGT

0, 1 and 2, with fluorescein isothiocyanate (FITC, a hapten with potential to induce ACD when combined to dibutyl phthalate DBP), and challenged with the same solution on the other ear (right ear, RE) at D6 (Figure 1A). This treatment led to an increase in the thickness of RE from FITC-sensitized and challenged mice (Figure 1B, compare untreated WT and WT+FITC), but not from mice with only sensitization or only challenge (Figure S2). Hematoxylin and eosin (H&E) staining of RE at D7 showed that the FITC treatment induced an inflammatory response with an epidermal hyperplasia and an immune infiltrate in dermis (Figure 1C). Immunohistochemistry (IHC) analyses using an antibody against MCPT8 (mast cell protease 8) (29) and an antibody against MBP (major basic protein) (30) revealed the dermal accumulation of basophils and eosinophils, respectively (Figure 1C, compare untreated WT and WT+FITC). RT-qPCR analyses showed an increase in RNA levels of cytokines IL-3, IL-4, IL-13, IL-17A and IFN-γ, as well as of MCPT8 (expressed by basophils) and CCR3 (expressed mainly by eosinophils and basophils) in RE from FITC-treated WT compared to untreated WT mice (Figure 1D, compare untreated WT and WT+FITC). FACS analyses of dermal cells showed an increased $CD45^+$ hematopoietic cells in FITC-treated WT ears compared to untreated ears. These include $TCR\beta^+$ T cells (identified as $CD45^{hi}TCR\beta^+$), eosinophils (identified as $CD45^{hi}TCR\beta^-SiglecF^+$), neutrophils ($CD45^{hi}TCR\beta^-Gr1^{hi}$), basophils (identified as $TCR\beta^-Gr1^-SiglecF^-CD45^{lo}CD49b^+$), as well as $TCR\beta^-Gr1^-SiglecF^-CD45^{hi}CD49b^+$ cells (which represent a heterogeneous resident cell population containing skin mast cells, called hereafter $CD45^{hi}CD49b^+$ cells) (Figures 1E, F, compare untreated WT and WT+FITC).

It has been reported that in different inflammatory contexts, basophil expansion and accumulation in tissues are adaptive immunity dependent (11, 31–33) or independent (34, 35). To examine whether basophil recruitment in FITC-induced ACD skin is dependent on adaptive immunity, $Rag1^{-/-}$ mice which lack mature T- and B-lymphocytes were subjected to FITC treatment. Results showed that FITC-induced ACD inflammation was abolished in $Rag1^{-/-}$ mice, with no increase in RE thickness (Figure 1B), largely diminished accumulation of eosinophils, basophils, neutrophils and $CD45^{hi}CD49b^+$ cells (which contain mast cells) (Figures 1C, E, F), and no increase in cytokine expression (Figure 1D). These results thus indicate that skin recruitment of basophils, as well as other immune cells in FITC-induced ACD are dependent on adaptive immunity.

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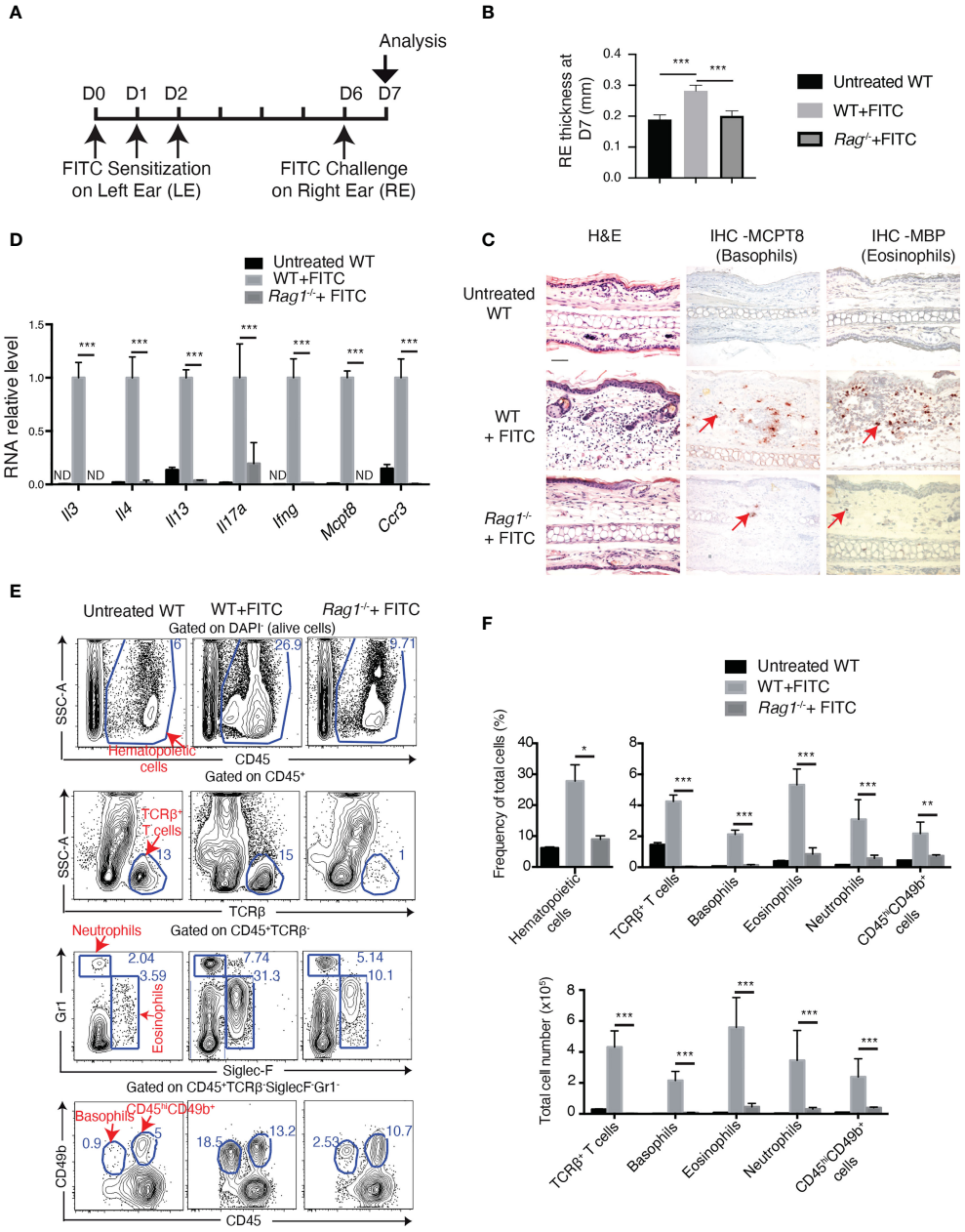

FIGURE 1
FITC treatment induces basophil accumulation in ACD skin in Rag1-dependent manner. **(A)** Experimental protocol. Eight to twelve-week-old female mice were sensitized with FITC on left ear (LE) at Day **(D)** 0, D1 and D2. Right ears (RE) were then challenged at D6 with FITC and sampled for analyses at D7. **(B)** RE thickness at D7. **(C)** Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining of RE sections. Arrow points to one of the positive cells of IHC staining. Scale bar: 50 µm. **(D)** RT-qPCR analyses of cytokines in RE. ND, non-detected. **(E)** FACS analyses of dermal cells of RE for $CD45^+$ (hematopoietic cells), $CD45^+TCR\beta^+$ T cells, $CD45^+TCR\beta^-Siglec\text{-}F^+Gr1^{low\text{-}neg}$ (eosinophils), and $CD45^+TCR\beta^-Gr1^{hi}$ (neutrophils), $CD45^{low}CD49b^+Siglec\text{-}F^-Gr1^-$(basophils) and $CD45^{hi}CD49b^+Siglec\text{-}F^-Gr1^-$ cells (which contain mast cells). **(F)** Comparison of frequency of total cells and total cel numbers. *P≤0.05 **P≤0.01, ***P≤0.001 (Student's t-test). Values are mean ± SEM [**(B)**, n=7; **(D, F)**, n=4 mice per group].

IL-3 is crucial for basophil accumulation in FITC-induced ACD skin

Based on the above observation that IL-3 expression in ACD skin was totally abolished in *Rag1*$^{-/-}$ mice, we next examined the role of IL-3 in ACD skin inflammation. *Il3*$^{-/-}$ and their wildtype control (CT) littermate mice were subjected to the FITC treatment. Measurement of RE thickness showed a modest but significant decrease in FITC-treated *Il3*$^{-/-}$ mice compared to FITC-treated WT mice (Figure 2A). FACS analyses showed that the number of basophils was highly reduced in RE from FITC-treated *Il3*$^{-/-}$ mice compared to that from FITC-treated CT mice (Figures 2B, C), which was also confirmed by IHC staining for basophils and eosinophils (see Figure 3A). In contrast, no decrease was observed in the number of $TCR\beta^+$ T cells, eosinophils, neutrophils or $CD45^{hi}CD49b^+$ cells (which contain mast cells) (Figures 2B, C), indicating a specialized requirement of IL-3 for basophil recruitment and accumulation in ACD skin.

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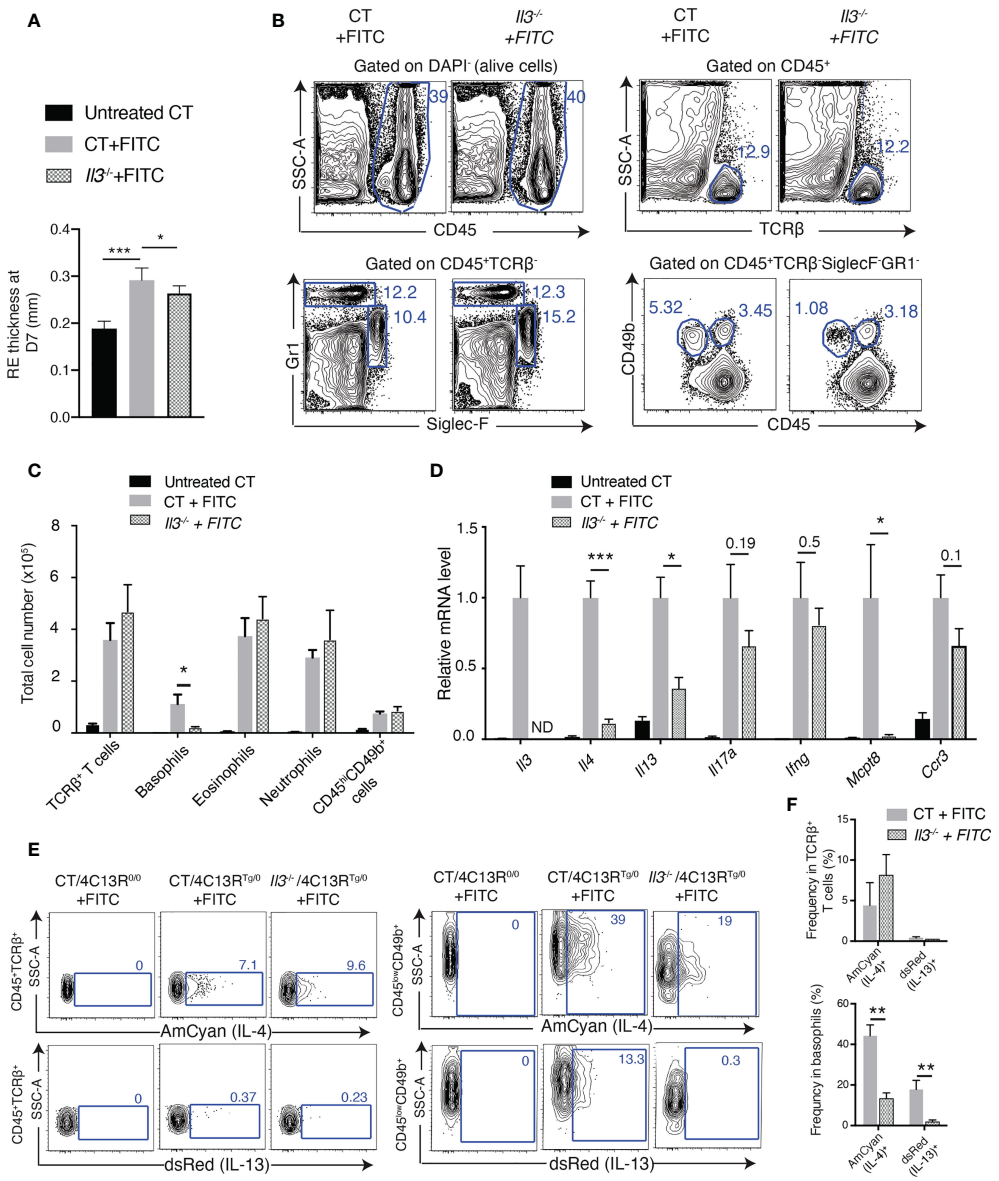

FIGURE 2
IL-3 plays a crucial role for basophil recruitment to FITC-induced ACD skin. **(A)** Thick- ness of FITC-challenged right ear (RE) at D7. **(B)** FACS analyses of dermal cells of RES from FITC-treated 113 and wildtype control (CT) littermate mice, for $CD45^+$ hematopoietic cells, $CD45^+TCR\beta^+$ T cells, $CD45^+TCR\beta^-Siglec\text{-}F^+Gr1^{low\text{-}neg}$ (eosinophils), and $CD45^+TCR\beta^-Gr1^{hi}$ (neutrophils), $CD45^{low}CD49b^+TCR\beta^-Siglec\text{-}F^-Gr1^-$ (baso- phils) and $CD45^{hi}CD49b^+TCR\beta^-Siglec\text{-}F^-Gr1^-$ cells (which contain mast cells),. **(C)** Comparison of total cell numbers in RE. **(D)** RT-qPCR analyses of REs. ND, non detectable. **(E)** FACS analyses of Amcyan (IL-4) and dsRed (IL-13) expression by $CD45^+TCR\beta^+$ T cells (left panel) and by $CD45^{low}CD49b^+$ basophils (right panel), in the dermis of FITC-treated CT/4C13R$^{Tg/0}$ and *Il3*$^{-/-}$/4C13R$^{Tg/0}$ mice. FITC-treated CT/4C13R$^{0/0}$ was used to set the gating for AmCyan and dsRed. **(F)** Comparison of frequencies of AmCyan $(IL\text{-}4)^+$ cells and dsRed$(IL\text{-}13)^+$ cells in $TCR\beta^+$ T cells or in basophils. *P≤0.05 **P≤0.01, ***P<0.001 (Student's t-test). Values are mean ± SEM [**(A)**, n=7; **(C, D, F)**, n=4 mice per group].

In addition, RT-qPCR analyses of RE showed that RNA levels of MCPT8, IL-4 and IL-13 were significantly decreased in FITC-treated *Il3*$^{-/-}$ mice compared to FITC-treated CT mice (Figure 2D). As IL-4 and IL-13 have been reported to be produced by various cell types including Th2 cells and basophils (36), we further investigated the cells in which IL-4 and IL-13 expression was reduced in FITC-treated *Il3*$^{-/-}$ skin. For this purpose, we bred *Il3*$^{-/-}$ mice with *4C13R* dual reporter mice (which have transgenic expression of the cyan fluorescent protein AmCyan under the control of Il4 regulatory elements and the red fluorescent protein dsRed under the control of Il13 regulatory elements) (37). FACS analyses of RE showed that first, IL-4 (AmCyan) and IL-13 (dsRed) were detected in both $TCR\beta^+$ T cells and basophils in FITC-treated CT/4C13R$^{Tg/0}$ mice (Figures 2E, F); second, AmCyan (IL-4) and dsRed (IL-13) expression in $TCR\beta^+$ T cells was comparable between FITC-treated CT/4C13R$^{Tg/0}$ and *Il3*$^{-/-}$/4C13R$^{Tg/0}$ skin (Figures 2E, F). In contrast, their expression in basophils was diminished in FITC-treated *Il3*$^{-/-}$/4C13R$^{Tg/0}$ skin (Figures 2E, F), indicating that basophils but not Th2 cells were responsible for the reduction of IL-4 and IL-13 expression detected in RE from FITC-treated *Il3*$^{-/-}$ mice.

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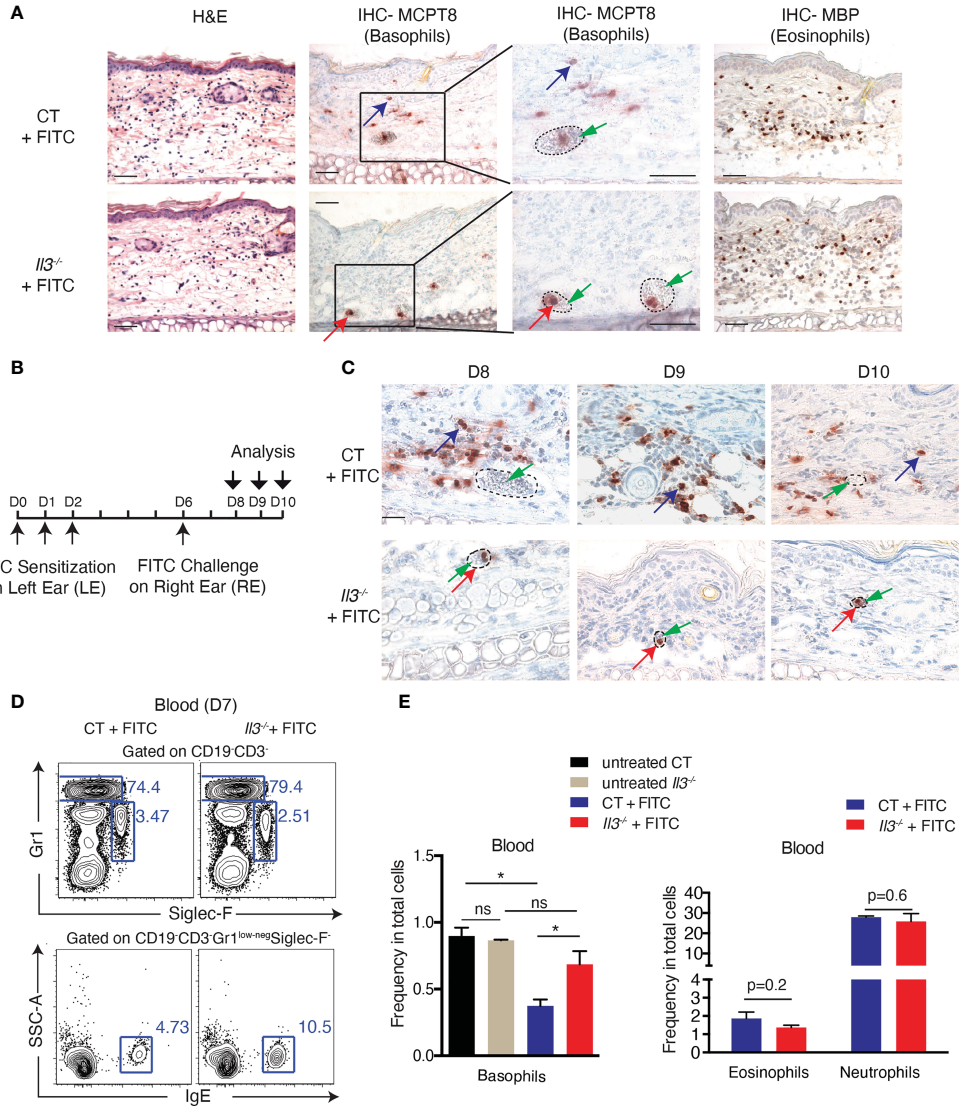

FIGURE 3
IL-3 is crucial for basophil extravasation to FITC-induced ACD skin. **(A)** HE and IHC staining of RE sections at D7. Red arrow points to one of basophils inside blood vessels, whereas blue arrow points to one of basophils out of blood vessels. The green arrow points to red blood cells inside the vessel. Dashed circles outline blood vessels. **(B-C)** *Il3*$^{-/-}$ and CT mice were sensitized at D0, D1 and D2 on LEs and challenged at D6 on REs, which were analysed at D8, D9 and D10 **(B)** for Mcpt8 IHC staining **(C)**. Scale bar, 50 µm. **(D)** Representative FACS plots of blood cells for CD19^{-} CD3^{-}Gr1^{-}SiglecF^{-}IgE^{+} (basophils), CD19^{-}CD3^{-}Siglec-F^{+}Gr1$^{low-neg}$ (eosinophils), and CD19^{-}CD3^{-}Gr1^{hi} (neutrophils) from FITC-treated CT and *Il3*$^{-/-}$ mice. **(E)** Comparison of frequency of basophils (left panel), eosinophils and neutrophils (right panel) in total cells. *P≤0.05 (Student's t-test). ns, non significant. Values are mean ± SEM (n≥3 mice per group).

Together, these results suggested that in ACD skin, IL-3 was specifically and crucially required for the accumulation of basophils, which were the major cell type contributing to the induced expression of Th2 cytokines IL-4 and IL-13 in FITC-induced ACD.

IL-3 is crucial for basophil extravasation to ACD skin

To examine basophils in FITC-treated WT and *Il3*$^{-/-}$ skin in histological level, we performed MCPT8 IHC staining. Of interest, we observed that in addition to the decrease in basophil number, all the detected basophils were strikingly restricted inside blood vessels in RE of FITC-treated *Il3*$^{-/-}$ mice (Figure 3A; MCPT8-labled basophils were immersed in red blood cells), indicating a defect in basophils for crossing the vascular endothelium. In contrast to basophils, no difference was observed in eosinophil extravasation to skin between FITC-treated *Il3*$^{-/-}$ and CT mice (Figure 3A).

To examine whether this observation could reflect a delayed basophil recruitment in *Il3*$^{-/-}$ mice, we performed FITC treatment and analysed RE at later time points (D8, D9 and D10) (Figure 3B). Similar phenotype (restriction of basophils inside blood vessels in the skin) was observed as at D7, indicating that basophils in FITC-treated *Il3*$^{-/-}$ mice were not able to cross vascular endothelium at any of these time points (Figure 3C). Thus, basophil extravasation was not delayed but defective in FITC-treated *Il3*$^{-/-}$ mice.

Next, we performed FACS analyses of blood basophils, which were identified as $CD19^-CD3^-Gr1^-Siglec\text{-}F^-IgE^+$ cells (Figure 3D). Blood eosinophils and neutrophils were identified as $CD19^-CD3^-Siglec\text{-}F^+Gr1^{low\text{-}neg}$, $CD19^-CD3^-Gr1^{hi}$, respectively (Figure 3D). Results showed that first, the frequency of basophils in the blood was comparable between untreated CT and *Il3*$^{-/-}$ mice (Figure 3E, compare untreated CT with untreated *Il3*$^{-/-}$), indicating that IL-3 was not necessary for the development of baseline levels of basophils in mice, in agreement with previous reports (9, 38, 39); second, the frequency of basophils in the blood of FITC-treated CT mice was lower compared to untreated CT mice (Figure 3E, compare CT+FITC with untreated CT), likely due to the skin recruitment of basophils; and third, such decrease was not observed in FITC-treated *Il3*$^{-/-}$ mice (Figure 3E, compare *Il3*$^{-/-}$ + FITC with untreated *Il3*$^{-/-}$), which was fitting with the observation that basophils were not able to cross vascular endothelium to enter the skin in these mice. In contrast to basophils, no difference was observed for frequency of eosinophils and neutrophils in the blood between FITC-treated *Il3*$^{-/-}$ and CT mice (Figure 3E). Altogether, these data suggested that basophil extravasation to inflamed ACD skin was defective in mice lacking IL-3.

IL-3 produced by T cells mediates basophil extravasation to ACD skin

By performing intracellular staining, we showed that IL-3 was detected in both $TCR\beta^+$ T cells and basophils of FITC-treated WT skin (Figure 4A). To examine whether IL-3 produced by T cells mediates basophil recruitment to ACD skin, we generated mice in which IL-3 is ablated selectively in both $CD4^+TCR\beta^+$ and $CD8^+TCR\beta^+$ T cells, by breeding *Il3*$^{L2/L2}$ with *CD4-Cre*$^{Tg/0}$ mice (25). Results showed that similar to what was observed in FITC-treated *Il3*$^{-/-}$ skin, all basophils detected by IHC-MCPT8 were confined inside blood vessels (Figure 4B). FACS analysis of FITC-challenged *CD4-Cre*$^{Tg/0}$/*Il3*$^{L2/L2}$ skin showed a diminished frequency of basophils, while no decrease was observed in $TCR\beta^+$ T cells, eosinophils, neutrophils or $CD45^{hi}CD49b^+$ cells (which contain mast cells) (Figure 4C). In addition, a higher frequency of basophils in blood was seen in FITC-treated *CD4-Cre*$^{Tg/0}$/*Il3*$^{L2/L2}$ mice compared to FITC-treated wildtype CT mice (Figure 4D), again suggesting a defective extravasation of basophils to ACD skin in these mice. Together, these results indicated that IL-3 produced by T cells was crucial for basophil extravasation in ACD skin.

Decreased expression of integrins in basophils from FITC-treated *Il3*$^{-/-}$ skin

It has been recognized that leukocyte extravasation is regulated by a concerted multistep actions between leukocytes and endothelial cells (ECs) including rolling, adhesion and TEM (13). IL-3 receptor was previously shown to be expressed by both human ECs (19, 20) and human/mouse basophils (40, 41). *In vitro* studies have suggested that basophils or ECs could respond to IL-3 signalling: IL-3 stimulation of human basophils enhances their adhesiveness to ECs (23) and their TEM (22); on the other hand, stimulation of human ECs by IL-3 induced the expression of P-selectin and selective basophil accumulation (21, 42).

We thus sorted ECs and basophils by FACS from FITC-challenged mouse RE and analysed by RT-qPCR the expression of molecules potentially implicated in basophil-EC interaction. First, the expression of *Selp* (P-selectin), *Sele* (E-selectin), *Icam1* and *Vcam1* was much higher in ECs compared to $CD45^+$ hematopoietic cells, however, no decrease in these genes was observed in ECs from *Il3*$^{-/-}$ compared to CT mice (Figure S3). On the other hand, analyses of the sorted basophils (note that basophils sorted from the FITC-treated *Il3*$^{-/-}$ RE corresponded to those stuck inside blood vessels) revealed a significant decrease in *Itgam*, *Itgb2*, *Itga2b* and *Itgb7* from FITC-treated *Il3*$^{-/-}$ compared to CT mice (Figure 5), whereas no significant difference was observed for *Itga4*, *Itga5*, *Itgae*, *Itgb1*, *Itgal* and *Itgb3* (Figure 5). Importantly, the decrease in *Itgam*, *Itgb2*, *Itga2b* and *Itgb7* was specific for basophils, as no change was observed for neutrophils, eosinophils or $TCR\beta^+$ T cells from FITC-treated *Il3*$^{-/-}$ compared to CT mice (Figure 5). It is also notable that these genes were all highly expressed in basophils compared to neutrophils, eosinophils and $TCR\beta^+$ T cells from FITC-treated CT mice (Figure 5). Together, these data revealed an IL-3-dependent expression of integrins ITGAM, ITGB2, ITGA2B and ITGB7 in basophils, which are potentially implicated in basophil-EC interaction during the extravasation process in FITC-induced ACD.

Retinoic acid signaling promotes basophil extravasation to ACD skin

We next sought to explore how IL-3 signalling regulates the expression of integrins by basophils. Of interest, it was previously reported that in human basophils co-cultured with mast cells, mast cell-derived IL-3 induces the expression of the retinaldehyde dehydrogenease ALDH1A2 (also called RALDH2), an enzyme that catalyses the last oxidative step of the cascade leading retinol to produce retinoic acid (RA) (43). It was shown that RA produced by basophils promotes the expression of ITGA4/ITGB7 heterodimer on T cells in a paracrine manner, thus influencing T cell polarisation (43). Other studies reported the induction of ALDH1A2 in human basophils (44) or ALDH1A3 (also called RALDH3) in mouse basophils (45) upon the stimulation of IL-3 and IL-33/IgE stimulation, respectively. We then examined the expression of *Aldh1a1*, *Aldh1a2* and *Aldh1a3* in basophils, eosinophils, neutrophils and $TCR\beta^+$ T cells sorted by FACS from FITC-treated WT and *Il3*$^{-/-}$ RE. Results show that RNA levels for *Aldh1a2*, but not *Aldh1a1* or *Aldh1a3*, were significantly decreased in basophils from FITC-treated *Il3*$^{-/-}$ compared to CT mice (Figure 6A), whereas its levels in eosinophils, neutrophils or $TCR\beta^+$ T cells were all low and remained unchanged between FITC-treated *Il3*$^{-/-}$ and CT mice (Figure 6A). These results thus suggested that *Aldh1a2* expression by basophils from FITC-treated skin is dependent on IL-3. Notably, RT-qPCR analyses of naïve basophils sorted from spleen in steady state showed that *Aldh1a2* was undetectable (qPCR cross point >50) in basophils from both wildtype control (CT) and *Il3*$^{-/-}$ mice (Figure S4).

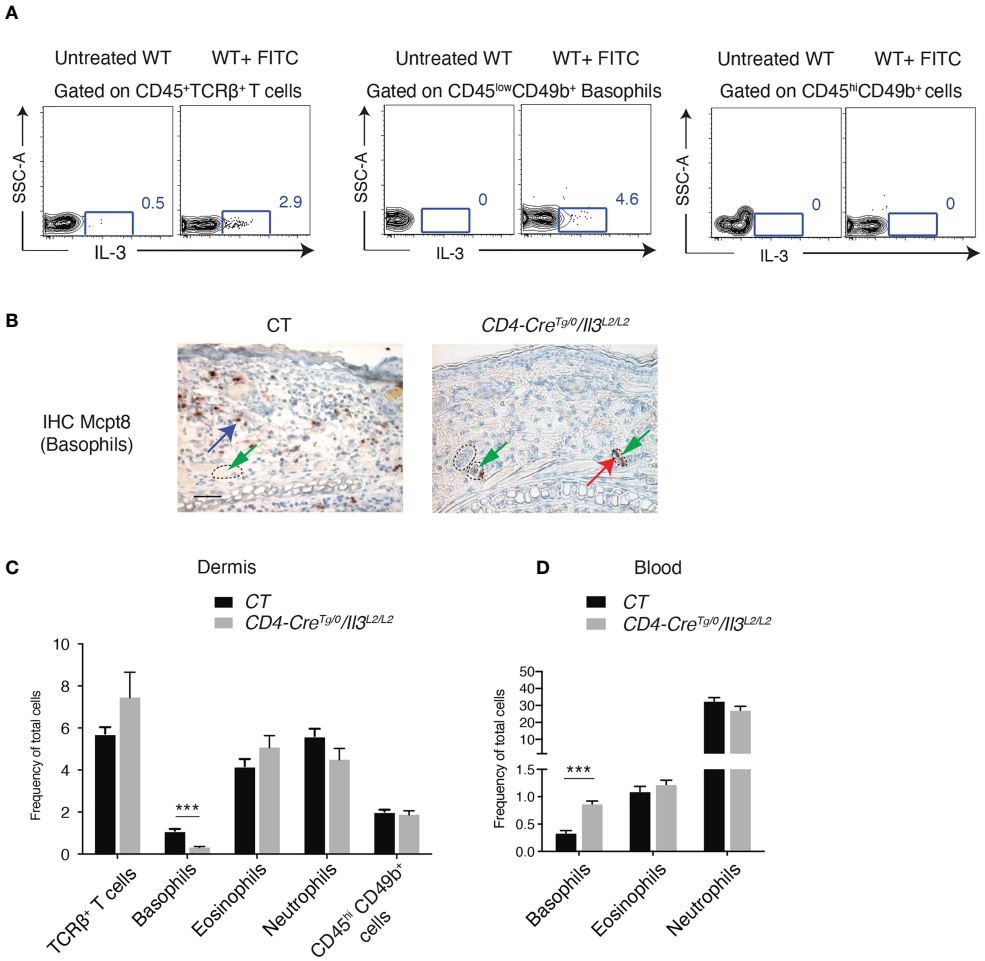

FIGURE 4
IL-3 produced by T cells mediates basophil extravasation to FITC-induced ACD skin. **(A)** Intracellular staining of IL-3 in dermal cells of RES from untreated or FITC-treated Balb/c WT mice. **(B)** IHC staining with Mcpt8 antibody in RE of FITC-treated CD4-Cre$^{0/0}$/*Il3*$^{L2/L2}$ (CT) and CD4-Cre$^{Tg/0}$/*Il3*$^{L2/L2}$ mice. Red arrow points to one of basophils inside blood vessels and blue arrow points to one of basohils of blood vessel. Dashed circles outline blood vessels. Scale bar, 50μm. **(C)** FACS analyses of dermal cells of REs from FITC-treated CT and CD4-Cre$^{Tg/0}$/*Il3*$^{L2/L2}$ mice, for CD45$^+$ hematopoietic cells, CD45$^+$TCRβ$^+$ T cells, CD45$^+$TCRβ- Siglec-F$^+$Gr1$^{low\text{-}neg}$ (eosinophils), and CD45$^+$TCRβ$^-$Gr1^{hi} (neutrophils), CD45^{low}CD49b$^+$Siglec-F$^-$Gr1$^-$(basophils) and CD45^{hi}CD49b$^+$Siglec-F$^-$Gr1$^-$ cells (which contain mast cells). **(D)** FACS analyses of blood cells from FITC-treated CT and CD4-Cre$^{Tg/0}$/*Il3*$^{L2/L2}$ mice for CD19$^-$CD3$^-$Gr1$^{low\text{-}neg}$SiglecF$^-$IgE$^+$ (basophils), CD19$^-$CD3$^-$Siglec-F$^+$Gr1$^{low\text{-}neg}$ (eosinophils), and CD19$^-$CD3$^-$Gr1^{hi} (neutrophils). ***P≤0.001 (Student's t-test). Values are mean ± SEM (n=4 mice per group).

In addition, RT-qPCR analyses showed that except for *Itgam*, which was significantly lower in basophils from *Il3*$^{-/-}$ mice compared to CT mice, the other ITGs analyzed including *Itgb2*, *Itga2b*, *Itgb3*, *Itgae* and *Itgb7* (a slight tendency; p=0.07) did not exhibit significant difference between basophils from CT and *Il3*$^{-/-}$ mice (Figure S4). These results thus suggested that in contrast to the inflamed context where IL-3 played a significant role in regulating the RNA expression of *Aldh1a2* and ITGs, in steady state, IL-3-ALDH1A2 axis was minimally implicated in regulating the expression of ITGs in basophils.

We further tested whether RA administration restores basophil extravasation in *Il3*$^{-/-}$ mice. Wildtype CT and *Il3*$^{-/-}$ mice were treated with FITC as described in Figure 1A, and all-trans RA (at-RA) was either topically applied to RE 2 h before the FITC-challenge, or injected i.p. 24 h before the FITC-challenge. Results show that upon at-RA topical treatment, more basophils were accumulated in FITC-treated CT skin (Figure 6B, compare CT +FITC w/o at-RA and CT +FITC + topical at-RA). Moreover, while basophils were stuck inside blood vessels in FITC-treated *Il3*$^{-/-}$ (w/o at-RA) mice, at-RA topical treatment resulted in more basophils detected outside the blood vessels (Figures 6B, C). Similarly, i.p. injection of at-RA led to an increased number of basophils outside the blood vessels of FITC-treated *Il3*$^{-/-}$ skin, although such effect appeared to be relatively weaker compared to topical RA treatment (Figures 6B, C). Taken together, these data suggested that the administration of at-RA has an effect to restore basophil extravasation in *Il3*$^{-/-}$ mice.

IL-3 stimulation of human basophils upregulates integrin particularly ITGB7 in RA signaling-dependent manner

To examine the human relevance of the above findings in mouse, we performed *in vitro* culture of human primary basophils isolated from healthy donors. We first confirmed that

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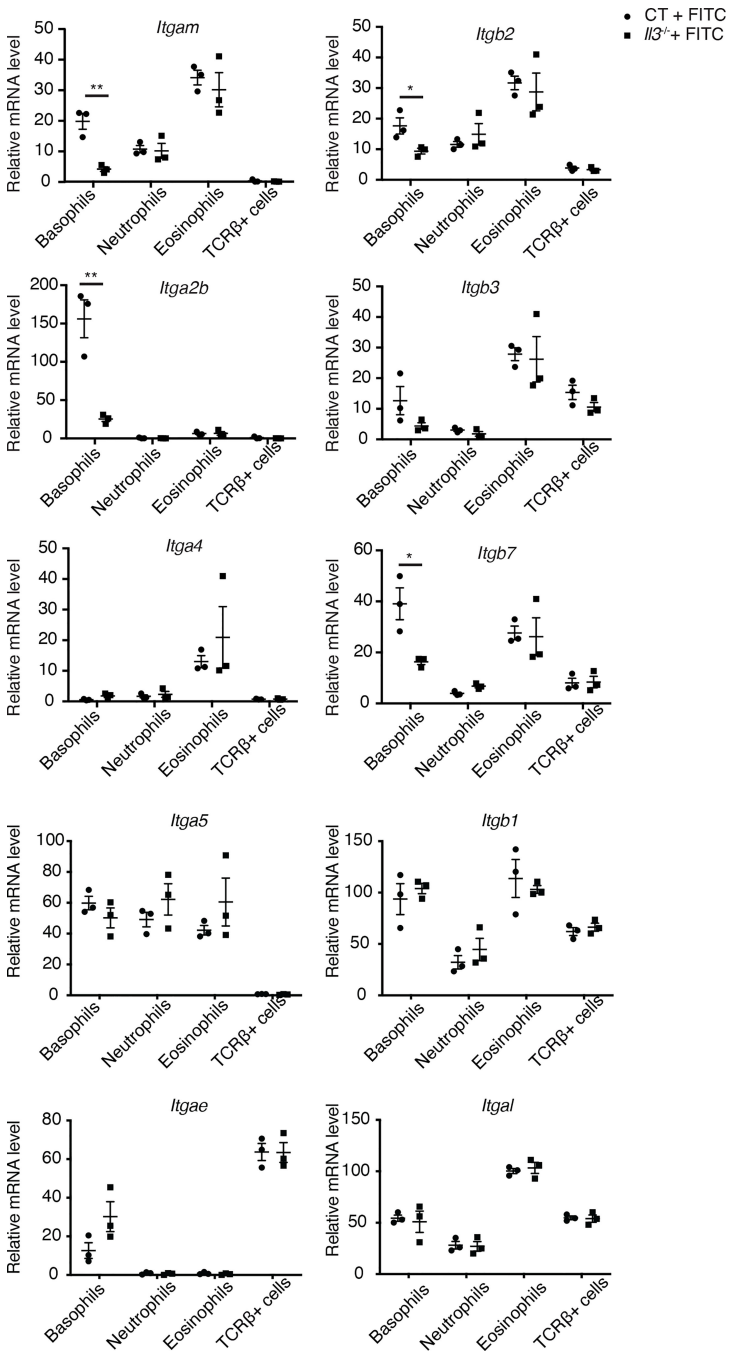

FIGURE 5
Decreased expression of integrins by basophils sorted from FITC-treated *Il3*$^{-/-}$ skin. RT-qPCR analyses of the sorted basophils, neutrophils, eosinophils and TCRβ^{+} T cells. CT, wildtype controls. *P≤0.05 **P≤0.01 (Student's t-test). Values are mean ± SEM (n=3 mice per group).

ALDH1A2 expression was highly induced by IL-3 in basophils particularly from Donor 1, 2 and 3, while the Donor 4 exhibited relatively less induction of *ALDH1A2* (Figure 7A). Further examination of integrin expression showed that basophils from Donor 1 and Donor 2 showed an increased expression of *ITGAM*, *ITGB2*, *ITGA2B* and *ITGB7* upon IL-3 stimulation (Figure 7A), while the induction of aforementioned integrins was less clear in the basophils from Donor 3 and Donor 4 (Figure 7A). Moreover, when stimulated with RA, basophils from Donor 1 and Donor 2 also showed an increased expression of these integrins, which was reduced upon the treatment with RAR antagonists (Figure 7A). Particularly, *ITGB7* expression was increased in basophils from all the 3 donors (Donor 1-3) upon RA stimulation, which was antagonized by RAR antagonists (Figure 7A). Though Donor 4

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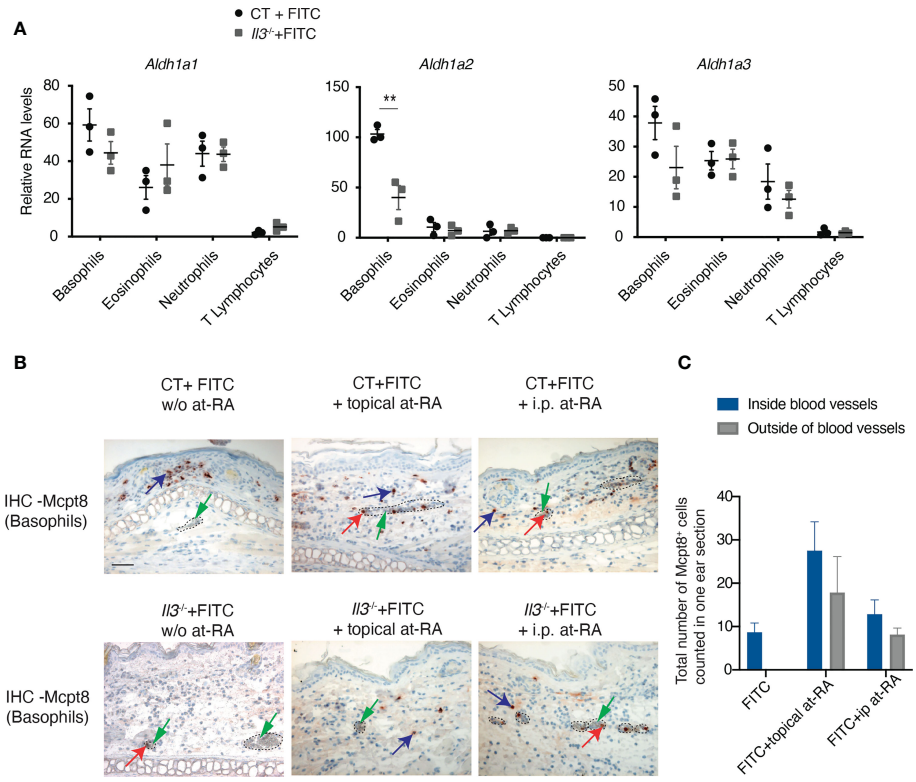

FIGURE 6
All-trans retinoic acid treatment prior to FITC challenge promotes basophil recruitment to ACD skin. **(A)** RT-qPCR analyses of retinaldehyde dehydrogenase genes Aldh1a1, Aldh1a2, and Aldh1a3 in basophils, neutrophils, eosinophils and T cells sorted from FITC-treated *Il3*$^{-/-}$ and CT ears. **P≤0.01 (Student's t-test). Values are mean ± SEM (n=3). **(B)** *Il3*$^{-/-}$ and CT mice were FITC-sensitized and -challenged as described in Figure 1A. Mice were either topically treated with all-trans retinoic acid (at-RA) on RE at 2 hr before FITC-challenge, or intraperitoneally (i.p.) administrated with at-RA at 24 hr before FITC-challenge. RE sections from *Il3*$^{-/-}$ and CT mice were used for MCPT8 IHC. Blue arrows point to one of the positive cells outside of blood vessels; red arrows point to one of the positive cells inside blood vessels; green arrows point to red blood cells inside the vessel. Dashed circles outline blood vessels. Scale bar: 50 µm. **(C)** Comparison of total number of Mcpt8 basophils inside and ourside of blood vessels from ear sections of *Il3*$^{-/-}$ mice treated with FITC, FITC+topical at-RA, or FITC+i.p. at-RA. Values are mean ± SEM (n=6).

did not show a dramatic increase in ITGB7 expression, its expression was completely antagonized by RAR antagonists.

In another set of experiment, basophils were treated with IL-3 plus RAR antagonists. As shown in Figure 7B, *ALDH1A2* expression was induced by IL-3 and was not affected by the addition of RAR antagonists. *ITGB7* expression was induced by IL-3, and such induction was suppressed by the addition of RAR antagonists (Figure 7B). These data thus suggested that IL-3 stimulation upregulates ITGB7 expression in human basophils in an RA signaling-dependent manner.

Discussion

In this study, we investigated basophil recruitment to allergic skin with a hapten-induced ACD mouse model. Making use of our newly generated IL-3-knockout and conditional knockout mouse lines, our data demonstrated a crucial role for IL-3 produced by T cells in mediating basophil extravasation to the inflamed skin. Moreover, we found that basophils from FITC-treated IL-3-knockout mice had a decreased expression of several integrins including *Itgam*, *Itgb2*, *Itga2b* and *Itgb7*, which was associated with the failure of basophils in crossing ECs to enter inflamed skin site of these mice. Interestingly, basophils from FITC-treated IL-3-knockout mice exhibited a reduced expression of *Aldh1a2*, and administration of at-RA restored basophils extravasation in these mice. Finally, we show that as observed in mice, human primary basophils express *ALDH1A2* upon IL-3 stimulation, and that IL-3-induced expression of integrins particularly *ITGB7* was dependent on RA signaling.

Our data point to a central role of IL-3 in basophil extravasation into the inflamed ACD skin, which involves a cooperation between T cells, basophils and ECs. Yet, it remains to be determined when and how IL-3 is induced in CD4$^+$ T cells upon the sensitization and challenge. Though we show that IL-3-expressing TCRβ$^+$ T cells are accumulated in RE from FITC-treated mice but not from untreated mice, thus suggesting that IL-3 at the challenge phase is likely responsible for its effect on basophil extravasation, it does not exclude a possible role of IL-3 during the sensitization phase of ACD. To explore this, temporal knockout of IL-3 (e.g. using tamoxifen-inducible Cre-ER^{T2} system), or blockade of IL-3 signaling using neuralizing antibody or antagonists to IL-3 or IL-3Rα (IL-3 specific receptor subunit), during sensitization or challenge phase would be useful. This will also provide information on the appropriate time window to target IL-3 axis

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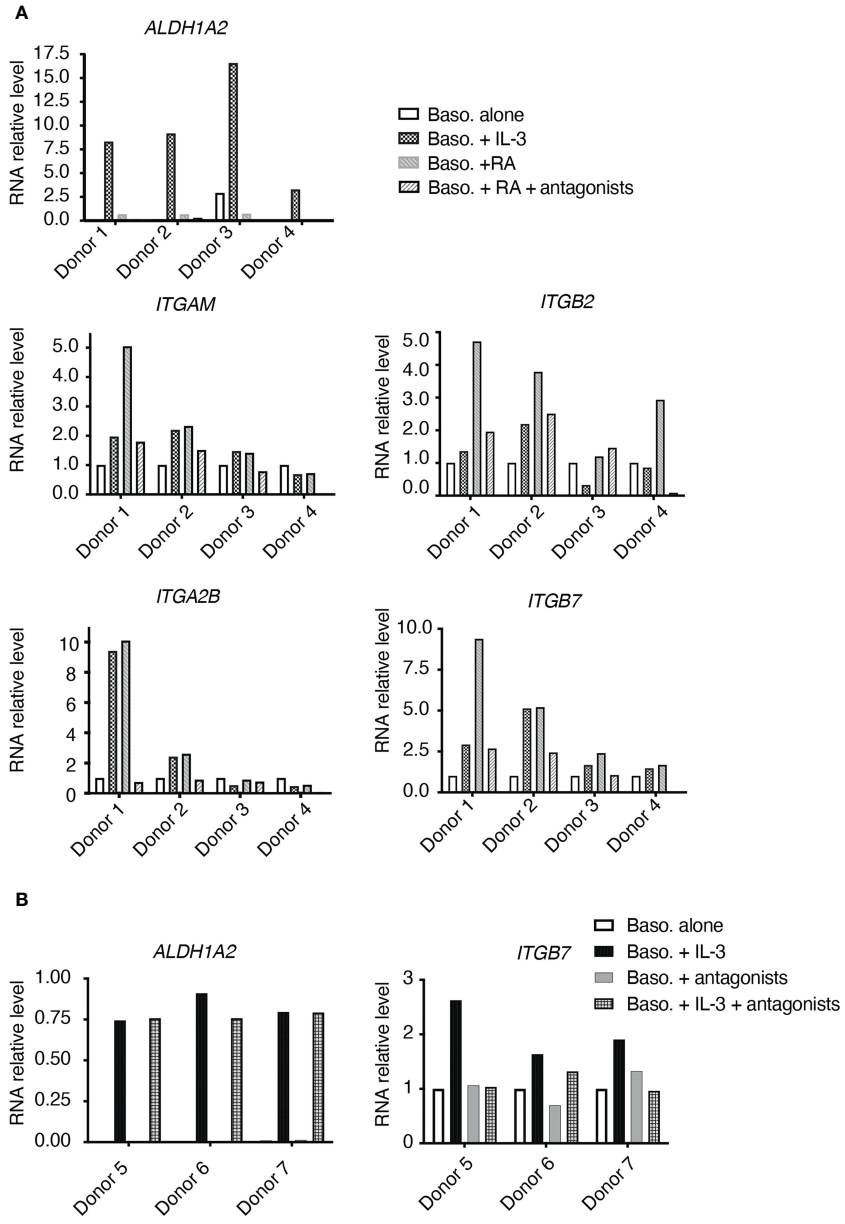

FIGURE 7
RT-qPCR analyses of in vitro cultured human basophils. **(A)** Basophils isolated from 4 donors (Donor 1, 2, 3, 4) were cultured alone, or in the presence of IL-3, all-trans retinoic acid (RA), or RA+RAR antagonists. **(B)** Basophils isolated from 3 donors (Donor 5, 6, 7) were cultured alone, or in the presence of IL-3, RAR antagonist, or IL-3+RAR antagonists.

and to test their effects on blocking the recruitment of basophils to inflamed skin, thereby modulating the established inflammation.

While IL-3 could exert multiple functions, our data revealed that IL-3 signalling on basophils was crucial for these cells to upregulate their RNA expression of integrins including *Itgam*, *Itgb2*, *Itga2b* and *Itgb7*. Indeed, the upregulation of *Itgam* was previously reported for mouse basophils stimulated *in vitro* with IL-3 (34), and for human basophils (23), which enhances their adhesiveness to ECs. In addition, *in vitro* studies suggested that IL-3 could stimulate human basophil rolling and adhesion to ECs, and blocking Abs against ITGB1, ITGB2, ITGAM and ITGAL inhibited basophil rolling and adhesion to ECs (21–23). Here, our data identified that in addition to *Itgam* and *Itgb2* as previously reported, *Itgb7* and *Itga2b* were also regulated by IL-3 signaling. Particularly, *Itgb7* and *Itga2b* are highly expressed by basophils compared neutrophils, eosinophils and T cells from FITC-treated wildtype mice (Figure 4), and moreover, in our tests with human primary basophils stimulated with IL-3, *ITGB7* induction was most reproducible, suggesting a potential role of ITGB7 in basophil extravasation, which deserves further investigation.

Our data suggest a possible IL-3-RA axis through ALDH1A2 expression to regulate the gene expression of integrins in basophils. First, we showed that in FITC-treated mice, *Aldh1a2* expression by basophils is diminished in IL-3-KO mice, while in human primary

basophils, IL-3 stimulation induces *ALDH1A2* expression, suggesting a conserved regulation of ALDH1A2 by IL-3 from mouse to human. These data are in good agreement with previous reports, which show that IL-3 induces ALDH1A2 expression and RA production by basophils (43, 44). It should be noted that genetic polymorphism in human IL-3Rα has been documented (46, 47), and our previous data have also revealed variations among healthy donors in their response to IL-3 (48), thus pointing towards polymorphism in IL-3Rα as one potential factor, which determines response of basophils to IL-3 and as a consequence, induction of ALDH1A2. This could explain the difference in the induction of ALDH1A2 in IL-3 stimulated basophils from different donors in our human experiment (Figure 7A; donor 4 had much lower ALDHL1A2 expression compared with other 3 donors). In addition, genetic polymorphism of RAR/RXR (receptors for RA) or RA response elements can impact the transcriptional regulation of ITGs by RA signaling, which may also explain the differential induction of ITG in IL-3-stimulated basophils among the donors (Figure 7A). Interestingly, it was previously proposed that RA produced by basophils promotes the expression of ITGA4/ITGB7 heterodimer on T cells in a paracrine manner thus influencing T cell polarisation (43). In contrast, our study provides evidence that RA promotes the expression of integrins particularly *ITGB7* in human basophils, and IL-3-induced *ITGB7* could be suppressed by RAR antagonists. Moreover, at-RA administration could restore at least partially basophil extravasation to the skin in *Il3*$^{-/-}$ mice. Thus, RA produced by basophils may act in an autocrine manner to regulate the expression of integrins implicated in basophil extravasation.

Based on these data, we propose a model illustrated in Figure 8: upon hapten sensitization and challenge, T cells secrete IL-3, which binds to IL-3 receptor complex on basophils and induces expression of ALDH1A2, resulting in the production of RA by basophils; in turn, RA activates RAR/RXR receptor heterodimer in basophils in an autocrine manner, and thereby upregulates the expression of integrins ITGAM, ITGB2, ITGA2B, and ITGB7, promotes the interaction between basophils and ECs, and eventually permits basophil extravasation to ACD skin. To fully determine the role of IL-3-RA axis in basophil extravasation process, mice in which *Aldh1a2* is conditionally knocked out in basophils (breeding *Aldh1a2*$^{L2/L2}$ mice with *Mcpt8*^{Cre}) will be useful to provide evidence on whether this enzyme and RA production are crucial for basophil extravasation. One might also envisage to use RARE (RAR responding element) reporter mice to track RA production and activity in basophils during inflammatory processes. Moreover, it will be also interesting to test whether RAR antagonists could block basophil recruitment to inflamed skin site. We could not provide data at this stage with the *in vivo* administration of RAR antagonists (CD2665 and BMS614) due to their toxicity in mice (data not shown), but further investigation on the possible strategies to block RA synthesis and signaling in basophils, as well as to target key molecular players including integrins (e.g. using blockade antibodies), should be further tested. Finally, it will be interesting to examine whether the proposed mechanism applies generally to basophil-related skin pathologies (1), such as allergen (e.g. house dust mite)-induced atopic dermatitis or urticaria, which will help to develop strategies for treating these diseases.

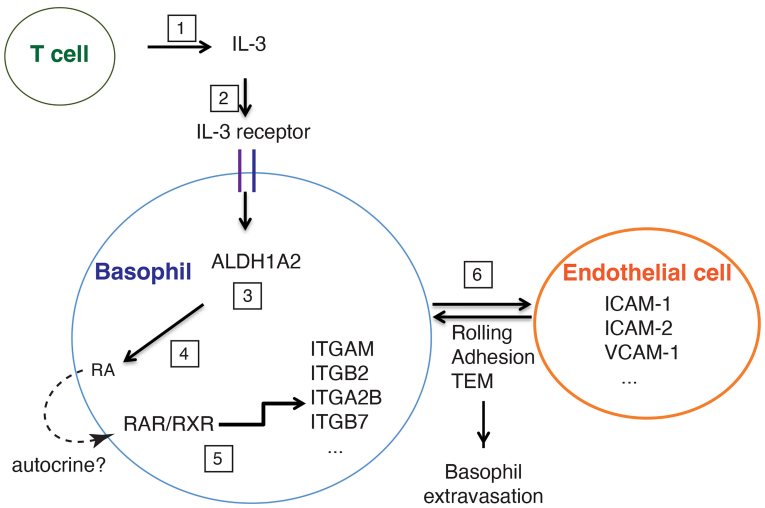

FIGURE 8
A schematic representation of the role of T cell-derived IL-3 in mediating FITC-induced basophil extravasation to ACD skin. Upon FITC treatment, T cells secrete IL-3 (1), which binds to IL-3 receptor complex on basophils (2), and induces expression of ALDH1A2 (3), leading to the production of retinoic acid (RA) by basophils (4). In turn, RA activates RAR/RXR receptor heterodimer in an autocrine manner, which upregulates the expression of integrins such as ITGAM, ITGB2, ITGA2B, ITGB7 by basophils (5). The interaction between integrins (expressed by basophils) and their ligands (expressed by endothelial cells, such as ICAM-1, ICAM-2, VCAM-1) is crucial for baso- phil extravasation to ACD skin through rolling, adhesion and trans-endothelial migration (TEM) (6).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved (18/EFS/041) by the ethical committee blood collection centres (EFS)–INSERM, Paris. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements (but patients/participants provided written informed consent at the source of EFS). Breeding and maintenance of mice were performed under institutional guidelines, and all of the animal studies and experimental protocols were approved by the animal care and ethics committee of animal experimentation of the IGBMC n°017 and by the Ministère de l'enseignement supérieur, de la recherche et de l'innovation.

Author contributions

CH and LM conceived and designed mouse study, AK, SB and JB conceived and designed human primary basophil study. CH initiated this study and conducted most experiments and acquired data. PMa contributed to the characterization of Il3-knockout and Il3-conditional knockout mouse lines, and the analyses of IL-3 expression by intracellular staining. PH established FITC model and conducted RA+FITC treatment mouse experiments. AK and SB performed human primary basophil culture and treatment, RNA extraction and cDNA preparation. PMe and EF performed qPCR analyses of human basophils. M-CB contributed to the design and the generation of Il3-knockout and -conditional knockout mouse lines. CH, PMa, PH, AK, SB, PMe, EF, JB and LM analyzed and interpreted data. CH, JB and LM wrote and revised the manuscript. LM directed the study and supervised the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1151468/full#supplementary-material

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Basophils beyond allergic and parasitic diseases

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Basophils bind IgE *via* FcεRI-$\alpha\beta\gamma_2$, which they uniquely share only with mast cells. In doing so, they can rapidly release mediators that are hallmark of allergic disease. This fundamental similarity, along with some morphological features shared by the two cell types, has long brought into question the biological significance that basophils mediate beyond that of mast cells. Unlike mast cells, which mature and reside in tissues, basophils are released into circulation from the bone marrow (constituting 1% of leukocytes), only to infiltrate tissues under specific inflammatory conditions. Evidence is emerging that basophils mediate non-redundant roles in allergic disease and, unsuspectingly, are implicated in a variety of other pathologies [e.g., myocardial infarction, autoimmunity, chronic obstructive pulmonary disease, fibrosis, cancer, etc.]. Recent findings strengthen the notion that these cells mediate protection from parasitic infections, whereas related studies implicate basophils promoting wound healing. Central to these functions is the substantial evidence that human and mouse basophils are increasingly implicated as important sources of IL-4 and IL-13. Nonetheless, much remains unclear regarding the role of basophils in pathology *vs.* homeostasis. In this review, we discuss the dichotomous (protective and/or harmful) roles of basophils in a wide spectrum of non-allergic disorders.

KEYWORDS

alarmins, allergy, autoimmunity, basophil, cancer, COVID-19, myocardial infarction

1 Basic concepts of basophils

Basophils are rare blood cells, accounting for 1% or less of the circulating leukocytes-a feature evident both in humans and mice. Basophils share several morphological and functional characteristics with tissue-resident mast cells. Most recognized are the cytoplasmic granules that each cell possesses and that stain so predominantly with basic stains. Phenotypically, both cell types s uniquely express the $\alpha\beta\gamma_2$ structure of the high-

affinity receptor (FcεRI) for IgE, which enables both cells to rapidly release pre-formed histamine and newly generated cysteinyl leukotriene C_4 (LTC_4), upon encountering relevant allergen (1, 2). Accordingly, basophils were initially viewed, incorrectly, as blood-circulating mast cells, which prompted the notion of using them as surrogates to study tissue mast cells, which proved far more difficult to obtain (2). However, it is now widely accepted that basophils and mast cells profoundly differ in several fundamental aspects (3). For example, the lifespan of basophils (~days) is much shorter than the months estimated for mast cells (4). Transcriptionally, basophils are more closely related to eosinophils than mast cells (5, 6). These differences (among many more discussed elsewhere (7) suggest that basophils have unique pathophysiological roles different from those of mast cells.

IL-3 is central to the growth, differentiation, priming, and overall activation of both human and mouse basophils (8, 9). It does so by binding, with high-affinity, to the α subunit of its receptor (IL-3Rα/CD123) highly expressed on basophils (10). Many cell types are implicated in producing the IL-3 that impacts basophil development and function, including T cells (11, 12), B cells (13), human eosinophils and neutrophils (14), but also mast cells and even basophils (15, 16). Although the IL-3 receptor is highly expressed on basophils (17–28), mice incapable of producing IL-3 and/or deficient in IL-3Rα/CD123 reportedly develop all blood lineages, including basophils and mast cells (29–31). In this regard, thymic stromal lymphopoietin (TSLP) is also reported to regulate mouse basophil development (32, 33) and activation (9) *in vivo* and may therefore represent an important early growth factor for these cells. In contrast, numerous studies show that IL-3 is quite sufficient in promoting the *in vitro* growth of functional human and mouse basophil-like cells from progenitors. TSLP is reported to activate human basophils from asthmatic subjects by promoting histamine release and cytokine secretion, along with inducing cell surface expression of CD203c and IL-3Rα (34). In contrast, several other investigators have since reported that TSLP does not activate human basophils isolated from healthy subjects or allergic patients (9, 10, 35). In light of the latter findings, TSLP may have very different effects on human *versus* mouse basophils (9). Finally, IL-3 is well known for its capacity to mediate synergistic (or priming) effects when combined with a diverse array of co-stimuli (9, 36–40).

It has been shown in mice that basophils originate from hematopoietic stem cells (HSCs) in the bone marrow (41, 42). So-called granulocyte-macrophage progenitors (GMPs), which develop later than the HSCs giving rise to most of the common myeloid progenitors, are thought to be the relevant basophil progenitors (BaPs (43). Common basophil-mast cell progenitors are also present in the spleen (43, 44). Single-cell transcriptomic analyses have highlighted the differentiation pathways of various cell lineages in mice (45–47). Single-cell culture of mouse bone marrow progenitors generated $FcεRI^+$ basophils and erythroid cells (48). The erythroid trajectory is close to that of basophils/mast cells, both in mice (49) and humans (50–53). Human $CD131^+$ CMP progenitors in the bone marrow can differentiate into basophil/mast cell/eosinophil and erythroid/megakaryocyte populations (51). Likewise, studies of human bone marrow cells using single-cell transcriptome analysis found the basophil trajectory to be more linked with that of the megakaryocyte and erythroid lineages, rather than those of granulocytes/monocytes (52). It is likely that the differentiation pathways of basophils and mast cells are more closely linked to those of the erythroid/megakaryocyte lineages, rather than to granulocytes/monocytes, both in mice and humans.

Several analytical tools for the study of mouse basophil biology have been developed in recent years. In particular, the use of antibodies capable of depleting basophils *in vivo* (54, 55) as well as mice that are genetically altered to be deficient of basophils (56–61), which includes reporter mouse models (58, 61), and basophil-specific Cre-expressing mice (58, 62, 63). The results obtained with these different models have demonstrated non-redundant roles of basophils in experimental Th2-type inflammation, comprising certain aspects of various allergic responses (3, 64, 65). Likewise, these models have substantiated the long-held belief that basophils help mediate immunity against parasitic infections (66–69). Some of these analytical tools have been employed to evaluate the role of mouse basophils in myocardial infarction (MI) (70), renal fibrosis (71), cancer (72–75), autoimmune disorders (76, 77), and chronic obstructive pulmonary disease (COPD) (62). Table 1 lists the antibody-mediated and genetic models for analyzing the *in vivo* contribution of mouse basophils in various pathophysiological conditions.

Several outstanding reviews have discussed the roles of mouse and human basophils in allergic disorders (1, 64, 74, 81, 82) and parasitic infections (66–68). Increasing evidences indicate that basophils also play relevant roles in several other types of responses, including autoimmunity (83, 84), myocardial infarction (70), fibrosis (70, 71, 85), cancer (86–88), and COVID-19 (89). In this review, we discuss the recent basophil contribution to the pathogenesis of several non-allergic inflammatory diseases.

Abbreviations: ACPA, anticitrullinated protein antibody; AIP, autoimmune pancreatitis; AM, alveolar macrophage; BAFF, B cell activating factor; BAP, basophil progenitor; CAF, cancer-associated fibroblast; CD, Crohn disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CMP, common myeloid progenitor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; CT, cholera toxin; CXCR4, CX-C motif chemokine receptor 4; DC, dendritic cell; DMBA, 7,12-dimethylbenz[a] anthracene; dsDNA, double-stranded DNA; DT, diphtheria toxin; EAE, experimental autoimmune encephalomyelitis; EGPA, eosinophil granulomatosis with polyangitis; EoE, eosinophilic esophagitis; FcεRI, high affinity IgE receptor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; IBD, inflammatory bowel disease; IFN-γ, interferon- γ; IgG4-RD, IgG_4-related disease; IL, interleukin; IM, interstitial macrophages; LTC_4, cysteinyl leukotriene C_4; MI, myocardial infarction; MCTD, mixed connective tissue disease; MRI, magnetic resonance imaging; MS, multiple sclerosis; NK cell, natural killer cell; NSCLC, non-small cell lung cancer; OVA, ovalbumin; PAD, peptidyl arginine deiminase; PDAC, ductal adenocarcinoma; PDGFB, platelet derived growth factor subunit B; PDGFBR, platelet derived growth factor subunit B receptor; PGD_2, prostaglandin D_2; PT, proximal tubular cell; RA, rheumatoid arthritis; RBL, rat basophil cell; SLE, systemic lupus erythematosus; TDLN, tumor-draining lymph node; TGF-β, transforming growth factor-β; TME, tumormicroenvironment; TPA, tetradecanoylphorbol-13-acetate; Treg cell, T regulatory cell; TSLP, thymic stromal lymphopoietin; UC, ulcerative colitis; UUO, unilateral ureter obstruction; WT, wild type.

TABLE 1 Antibody-mediated and genetic depletion models for the *in vivo* study of basophils in different pathological conditions.

Methods to deplete basophils	Examined pathological conditions	References
Antibody-mediated		
Monoclonal antibody (mAb) anti-FcεRI (MAR-1)	IgE-mediated chronic allergic dermatitis (IgE-CAI)	(54)
mAb anti-CD200R3 (Ba103)	Description of the mAb	(78)
mAb MAR-1	Allergic inflammation	(79)
mAb MAR-1	Myocardial infarction (MI)	(70)
mAb anti-CD2003 (Ba103)	Emphysema	(62)
mAb MAR-1	Kidney fibrosis	(71)
Genetically engineered mice		
Mcpt8^{Cre} mice	*N. brasiliensis* infection IgE-CAI Systemic anaphylaxis	(57)
Mcpt8^{DTR} mice	Tick-borne disease	(56)
Runx1	IgE-CAI *Strongyloides* infection	(59)
BasTRECK	IgE-CAI	(59)
BasoDTR mice	IgE-CAI	(60)
Basoph8xiDTR mice	Skin allergic inflammation	(61)
Mcpt8^{Cre}/DTR mice	Kidney fibrosis	(71)
Mcpt8^{DTR} mice	Emphysema	(62)
Mcpt8^{iCreERT2}*Stim1*$^{fl/fl}$	IgE-CAI	(63)
Mcpt8^{Cre} mice	MI	(70)
CT-M8 mice	Systemic Lupus Erythematous	(80)

DTR, diphtheria toxin receptor; IgE-CAI, IgE-mediated chronic allergic dermatitis; mAb, monoclonal antibody; MI, myocardial infarction.

2 Basophils in myocardial infarction

Myocardial infarction (MI) occurs when coronary arteries that supply oxygen and nutrients to the heart become obstructed by atherosclerotic arterial walls (90). The consequence is an ischemic injury that mobilizes a repertoire of innate and adaptive immune cells (91, 92). Shortly, after ischemic occurs, resident cardiac mast cells release their preformed mediators (93), resident macrophages and cardiomyocytes produce cytokines and chemokines (94, 95), fibroblasts release growth factors (96) and endothelial cells are activated. These events typically cause an influx of various immune cells, including neutrophils, monocytes, macrophages (92, 97), and mast cells (98, 99).

The inflammatory response following MI deeply affects subsequent cardiac remodeling and fibrosis (100, 101). The composition of immune cell types identified in the infarcted myocardium consists mostly of macrophages, monocytes, neutrophils, DCs, B and T cells, and NK cells (70, 97). Using a mouse model, Sicklinger and coworkers demonstrated that basophils infiltrate infarcted hearts, reaching a peak between days 3 and 7 and reverting to baseline on day 14 (70). The administration of the monoclonal antibody (mAb) anti-FcεRI (MAR-1) depleted basophils in the heart, peripheral blood, and spleen. In contrast, mast cells and a subset of DCs expressing FcεRI were not altered following MAR-1 administration. Depletion of basophils reduced left ventricular ejection fraction 4 weeks after MI and increased heart weight compared to control. Moreover, basophil-depleted mice showed reduced scar thickness.

Sicklinger et al. also studied the inflammatory response after MI in Mcpt8-Cre-transgenic (Baso-KO) mice constitutively deficient in basophils (57). In this model, the infarct size did not differ between Baso-KO compared to WT mice. However, 28 days after inducing the MI, the basophil-deficient mice developed cardiac dysfunction and increased heart weight compared to their WT littermates. Finally, Baso-KO mice showed increased scar thinning compared to controls. MI in genetic basophil ablation mice was associated with an altered cellular inflammatory response in infarcted hearts. Four days after MI, there was a change in the composition of monocyte subpopulations in the infarcted myocardium of the basophil-depleted mice, namely a shift from reparative Ly6C^{lo} macrophages toward inflammatory Ly6C^{hi} monocytes. This proinflammatory response could be reversed by the adoptive transfer of basophils into the basophil-deficient mice. The absence of basophils was associated with lower concentrations of cardiac IL-4 and IL-13, two cytokines typically released by mouse (9, 57, 102–105) and human basophils (9, 16, 36–38, 106–108). The authors

concluded that the IL-4/IL-13 secreted by basophils infiltrating these lesions is critical in the transition from inflammatory monocytes to reparative macrophages (81, 109) Figure 1 illustrates the proposed mechanisms by which basophils influence the inflammatory response following myocardial infarction.

The authors also evaluated the cytokines produced in the heart 3 days after the MI event, both in the Baso-KO and WT mice. Among the cytokines commonly reported to be produced by mouse basophils (IL-4, IL-13, IL-6, TNF-α), there was a reduction only of IL-4 in the injured heart tissue of the basophil-deficient mice. Mice-deficient in IL-4/IL-13 showed a higher proportion of inflammatory Ly6C^{hi} monocytes and worsened cardiac function following MI. In contrast, the increased release of IL-4 by basophils following the administration of the glycoprotein IPSE/α-1 (a known stimulus of these cytokines from basophils) resulted in enhanced post-MI healing. The authors concluded that myocardial basophils are activated to produce IL-4 following MI and that this response is critical in healing the damaged myocardium (70). What currently remains unknown, however, is the exact mode of stimulation in the myocardium responsible for inducing basophils to produce IL-4.

These experimental results were supported by observations that human subjects presented with decreased blood basophil numbers within the first week following an MI event, and that this basopenia associated with an increased scar size, as measured by late gadolinium enhancement cardiac MRI after one year of follow-up (70). Importantly, this correlation persisted after the adjustment of possible confounders (e.g., initial infarct size, systemic inflammation, cardiovascular risk factors). The authors suggested that basophils may also influence cardiac remodeling after MI in humans.

These studies, emphasizing the protective role of basophils following MI, might have translational relevance. For example, a growing number of allergic patients (e.g., asthma, atopic dermatitis) are being treated with biologics that block the IL-4/IL-13 axis (e.g., dupilumab, an anti-IL-4Rα mAb) (82, 120). Thus, the possible protective role of basophil-derived IL-4/IL-13 in MI should stimulate further mechanistic studies to investigate possible links between these therapies and whether they might impact myocardial healing following MI.

3 Basophils in kidney fibrosis

Chronic kidney disease (CKD) is a final manifestation of renal fibrosis and its incidence is increasing (121). Various inflammatory stimuli, including chronic infections, tissue injury, autoimmune disorders, chemical insults, and radiation result in kidney fibrosis (117, 122). Chronic low-grade inflammation is a crucial promoter of fibrosis (117, 123), but immune pathways orchestrating kidney fibrosis are largely unknown. Doke and collaborators investigated the interactions between altered renal tubules and basophils in a mouse model of kidney fibrosis by employing single-cell RNA-seq analysis (71). In this model of CKD, mice experienced either a sham operation or underwent unilateral ureter obstruction (UUO)

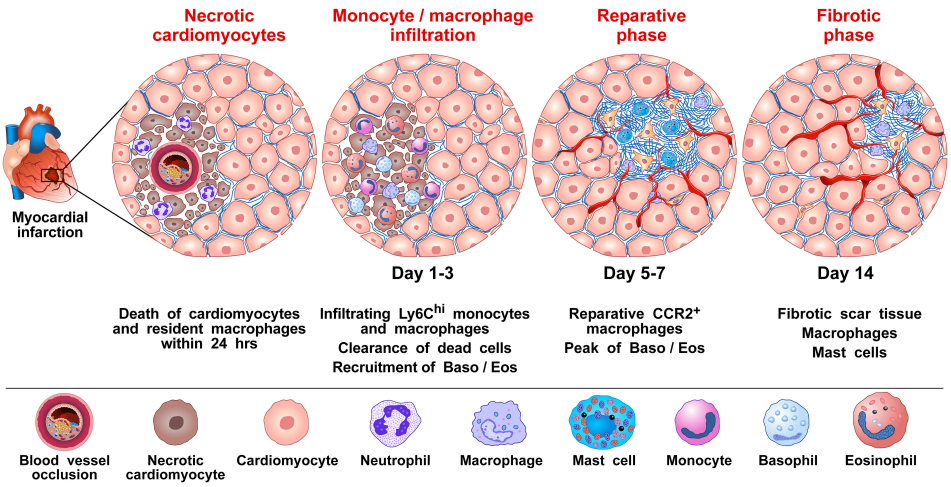

FIGURE 1
Proposed mechanism by which basophils influence the inflammatory response to promote wound healing and tissue repair following myocardial infarction (MI). MI is caused by the rupture of an atherosclerotic plaque causing the occlusion of a coronary artery, which then results in cardiac tissue damage due to ischemia (90). It has been shown in mice that several immune cells [e.g., monocytes/macrophages, neutrophils, dendritic cells (DCs), B and T cells, and natural killer (NK) cells, basophils and eosinophils infiltrate the heart after experimental MI (70, 97, 110). For basophils, this infiltration into the heart is evident 3 days following MI and peaks 7 days after the MI event (70). Monocytes/macrophages represent the most prevalent immune cells after MI. Cardiac resident macrophages contribute to the initial neutrophil infiltration into the ischemic area (111). Resident macrophages are reduced in murine models 1 day post-infarction (112). Within 1-3 days infiltrating bone marrow- and spleen-derived Ly6C^{hi} monocytes are recruited into the injured cardiac tissue and differentiate to Ly6C^{low} macrophages facilitating clearance of necrotic cardiomyocytes. At approximately 5-7 days post MI, macrophages adopt a reparative phenotype, contributing to the resolution of inflammation and fibrotic tissue formation (70). By day 3, infiltrating basophils into the injured cardiac tissue release IL-4 and IL-13, which induce phenotypical and functional changes within macrophages expressing anti-inflammatory and tissue repair genes (70). Formation of neovessels in the healing infarct play an important role in repairing the infarcted myocardium (113). Basophils (114), macrophages (115–117), and cardiac mast cells (118, 119), are major sources of angiogenic factors. Collectively, results in mice models of MI indicate that basophils infiltrating infarcted heart promote resolution of cardiac inflammation and scar formation.

surgery. Injured tubular cells (PTs) expressed several cytokines and chemokines known to induce the recruitment of basophils and other immune cells. PTs also released platelet-derived growth factor B (PDGFB), which upon binding to its receptor (PDGFBR) on fibroblasts induces these cells to release TGF-β. CXCL1, secreted by profibrotic tubules, recruited $CXCR2^+$ basophils. The density of basophils ($FcεRI^+CD200R3^+CD49b^+$ cells) was markedly increased in UUO kidneys compared to sham operation. Using antibody-mediated and genetic approaches to delete basophils, the authors explored the role of these cells in this model. In the latter model, injection of diphtheria toxin (DT) into $Mcpt8^{Cre}$/DTR mice induced depletion of basophils in the kidney and mitigated fibrosis in UUO kidney. Single-cell analysis and *in situ* hybridization demonstrated overexpression of *Il6* by basophils in UUO kidneys, indicating that mouse basophils are a source of this cytokine in UUO kidneys. In the other model, basophil depletion was mediated by MAR-1 administration into WT mice, followed by UUO surgery and kidney examination 7 days later. MAR-1-treated mice showed a reduction of the fibrosis markers induced by the UUO surgery. These results from two complementary models of basophil depletion highlight the importance of these cells in the development of experimental kidney fibrosis.

There is evidence that T_H17 cells contribute to renal fibrosis (124). For example, basophils were shown to directly interact with T_H17 cells and macrophages (104, 125). Both T_H17 cell number and IL-17A expression were increased in UUO, but they were lower in UUO kidneys of basophil-depleted mice. Single-cell RNA-seq analysis indicated a shift toward T_H17 cells in fibrosis. Basophil-derived IL-6 contributed to enhanced T_H17 cell differentiation from $CD4^+$ T cells in UUO kidney (126). Moreover, the expression of *Il17a* and *Tgfb1* were higher in UUO kidneys and were lower in UUO kidneys of basophil-depleted mice. Mice treated with an anti-IL-6R antibody were partially protected from renal fibrosis.

To evaluate the relevance of the above experimental findings to human kidney fibrosis, Doke and collaborators examined human kidneys, comparing those from healthy controls and CKD subjects using single-cell RNA-seq (71). They found that basophil numbers were increased in the kidney of patients with CKD, compared to healthy controls. Moreover, a correlation between renal fibrosis and basophil density was evident in the kidneys of CKD patients. There was also a positive correlation between *IL6* expression and the severity of renal fibrosis, which further showed a negative correlation between *IL6* and kidney function. Moreover, renal *IL6* correlated with CKD severity. Collectively, the above results reveal several correlations between both basophil density and their function and renal fibrosis. Figure 2 schematically illustrates the contribution of basophil-derived cytokines and T_H17 as downstream mediators in kidney fibrosis.

4 Basophils in cancer

There is mounting evidence showing that basophils are an important component within the tumor microenvironment (TME) of several human (72, 88, 131, 132) and mouse experimental cancers (72, 73, 132, 133). Moreover, these studies indicate that basophils may play an active role in the onset and development of both solid and hematologic tumors (74, 86, 134). The results from these studies reveal that basophils can have both pro-tumor and antitumor effects depending on the context and type of tumor.

In particular, immune profiling studies show that basophils constitute a portion, albeit small, of the immune landscape in human non-small cell lung cancer (NSCLC) tumors (131) and in the immune infiltrate seen in the early stage of lung adenocarcinoma (132). Several studies additionally show that mouse and human basophils support the development and expansion of M2-like monocytes/macrophages (127–130), which are often prevalent in the TME favoring tumorigenesis. An *in vivo* study in mice points to the importance of IL-4/IL-13, promoting carcinogenesis by reducing Th1-like immunity (72). Likewise, basophils are known to secrete vascular endothelial growth factor-A (VEGF-A) (114) and cysteinyl leukotriene C_4 (LTC_4) (18, 19) with the latter more recently implicated in tumorigenesis and metastasis formation (135). In particular, both tumor growth and metastases were reduced in mice deficient in the cysteinyl leukotriene 2 receptor ($CysLT_2R$). Moreover, administration of a $CysLT_2R$ antagonist reduced tumor growth and metastases in WT mice (135).

In exploring the immune cells involved in human pancreatic cancer (PC), *IL4*-expressing basophils were identified in the tumor-draining lymph nodes (TDLNs). Moreover, their presence was a negative prognostic marker of patient survival (72). To further investigate the underlying mechanisms of this association, the $Mcpt8^{Cre}$ basophil deficient mouse strain (57) and WT mice were implanted with PC cells. Strikingly, 80% of the WT mice developed PC-like cancer, but this was not observed in the basophil-deficient mice (72). The authors reported that TSLP released from basophils and cancer-associated fibroblasts (CAFs) within TDLNs activated $CD4^+$ T cells to produce IL-3. CCL7, derived from DCs and monocytes, promoted basophil recruitment into TDLNs. IL-3-activated basophils exerted a pro-tumorigenic role by secreting IL-4, which induced the polarization of Th2 and M2 cells. Thus, these results not only confirmed/supported the notion that basophil-derived IL-4/IL-13 promote Th2 and M2-like cells, but also demonstrated that these cells actively participate in promoting PC.

With the concept that various basophil-derived products (e.g., IL-4, IL-13, VEGF-A, LTC_4) promote tumorigenesis, an equally important issue pertains to the stimuli mediating their release. Schroeder and colleagues have shown that human basophils release copious amounts of histamine, IL-4 and IL-13 when co-cultured with the human lung adenocarcinoma cell line A549 (16). These responses were dependent on basophils expressing IgE, since removal/depletion of this immunoglobulin prevented basophil activation. Since pharmacologic inhibitors of FcεRI signaling also suppressed these responses, it seemed clear that basophils were being activated *via* IgE/FcεRI crosslinking to secrete these cytokines. Importantly, direct contact between basophils and A549 was necessary and occurred even if the adenocarcinoma cells were fixed with paraformaldehyde prior to co-culture. In a

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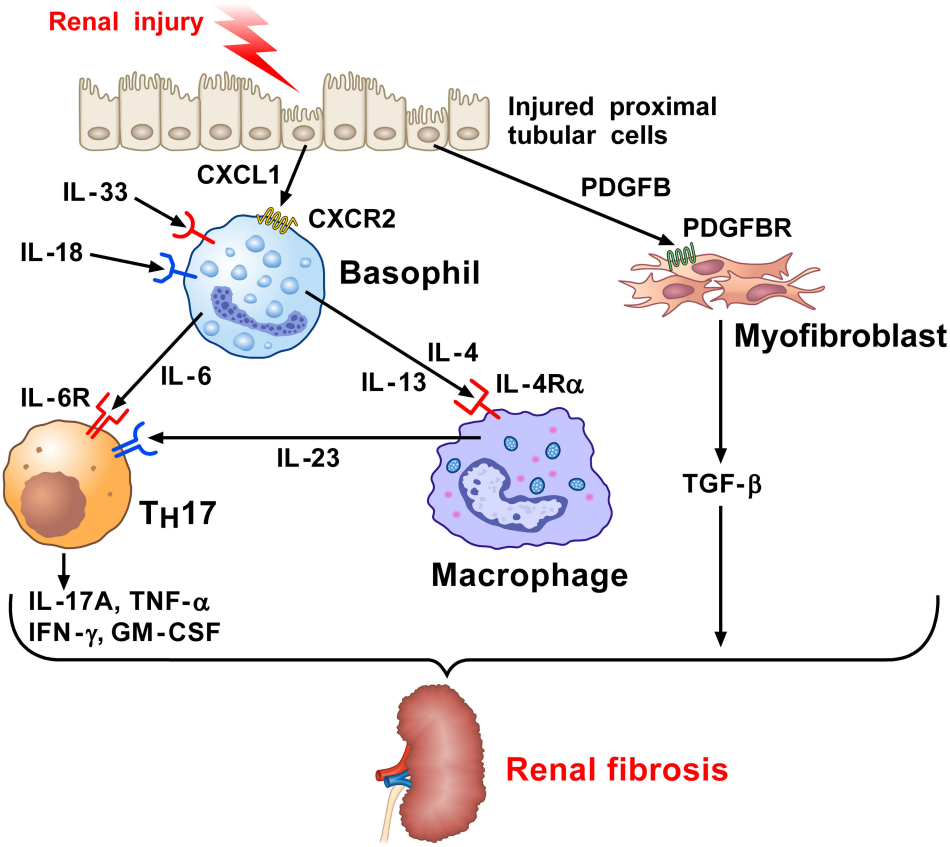

FIGURE 2
Kidney and wild-type mice subjected to unilateral ureter obstruction (UUO) surgery revealed the presence of neutrophils, monocytes/macrophages, dendritic cells (DCs), and basophils (71). Injured proximal tubular cells (PTs) in UUO kidney express *Il34*, *Cxcl10*, and the key profibrotic factor (71), platelet derived growth factor subunit B (PDGFB). PDGFB released by injured tubular activates the PDGFB receptor (PDGFBR) on fibroblasts to release TGF-β. Profibrotic PT cells participate in the recruitment of myeloid and lymphoid cells and the local fibroblast activation. CXCL1 released from PT cells induces the recruitment of basophils through the engagement of CXCR2. Basophils in UUO kidney can be activated by IL-33 and IL-18 released from the stroma to secrete IL-6. This cytokine favors T_H17 differentiation from $CD4^+$ T cells in UUO kidneys. IL-17A and TGF-β released from T_H17 cells contribute to renal fibrosis. IL-4 and IL-13 released from activated basophils can contribute to macrophage activation (127–130). PDGF released from injured PT cells activates the PDGFR on myofibroblasts causing the release of TGF-β. Macrophages are also a major source of IL-6. Collectively, these findings indicate that basophils and their mediators contribute to kidney fibrosis.

follow-up study, the IgE-binding lectin, galectin-3 (Gal-3) expressed on the A549 cells, proved crucial for basophil activation in these co-cultures, as A549 clones lacking Gal-3 failed to activate basophils (136). Gal-3 is widely implicated in various cancers and is a marker of chronic inflammation (137). These findings reveal a potentially new mechanism by which Gal-3 expressed by human lung adenocarcinoma cells can activate basophils to release cytokines and pro-inflammatory mediators that promote tumorigenesis. Additional investigations are required to fully understand all aspects of this mechanism and how it might be targeted for therapeutic intervention.

By utilizing a model whereby the skin of mice were topically exposed to the proinflammatory 12-0-tetradecanoylphorbol-13-acetate (TPA), Hayes et al. showed that serum IgE increased in these animals, which was accompanied by increased numbers of IgE-bearing basophils that promoted skin tumorigenesis (73). In a similar model of epithelial carcinogenesis involving the use of [7,12-dimyethylbenz(a)anthracene (DMBA) and subsequent exposure to TPA], mice lacking IgE (*Igh7*$^{-/-}$) developed less tumors compared to WT mice. The influx of basophils into skin was promoted by CXCR4, TSLP and IL-3. IgE-signaling played a key role in basophil activation and infiltrating tissue basophils expressed *Cxcr2, Cxcr4*, and *Ptgdr2* (CRTH2, the PGD_2 receptor). Tumor development was markedly reduced when conducting the same experiment in *Mcpt8*$^{Cre/+}$ mice, which were made deficient in basophils but retained normal mast cell numbers (57). Collectively, these *in vivo* results further indicate that FcεRI-signaling in basophils promotes inflammation-driven epithelial hyperplasia and tumor growth. While the role of galectin-3 in this tumorigenesis was not investigated, it seems worthy of future investigation, as mechanisms of this response are further elucidated.

In contrast to the belief that basophils contribute to tumorigenesis, association studies have shown evidence that higher expression of basophils (i.e., $CD123^+$, $CCR3^+$, $FcεRI^+$) in tumors correlated with better overall survival (88). In particular, increased basophil numbers are associated with beneficial outcomes

in several cancers, including sarcoma, lung, and breast. While several additional markers (e.g., CD63, CD203c) indicated that these tumor-associated basophils were, indeed, activated, relevant mediators commonly released by these cells (histamine, LTC_4, IL-4, IL-13) were not investigated. Thus, the exact contribution of basophils in the increased survival rates remains challenging to interpret at this time. Likewise, the same group has reported evidence that the *in vitro* responses of peripheral blood basophils from cancer patients can predict survival rates. While such correlations are intriguing, the exact mechanisms by which basophils contribute to increased survival rates is an area requiring further elucidation.

In agreement with the concept that basophils mediate a beneficial role in cancer, evidence from a mouse melanoma model showed that basophils released CCL3 and CCL4, which induced $CD8^+$ T cell recruitment and promoted tumor rejection (75). MAR-1 administration in these melanoma-bearing mice depleted basophils and prevented melanoma rejection. However, it is important to note that basophil depletion using the MAR-1 is also reported to deplete/activate other immune cells expressing FcεRI, including mast cells, monocytes and DCs (138, 139). Whether these cells were also depleted and possibly involved in tumoricidal activity remains unclear.

IL-33 has been shown to promote tumoricidal activity mediated by eosinophils (140, 141), possibly by upregulating granzyme B (142). As noted, this cytokine also activates both human and mouse basophils (9, 36, 38, 143–145). Hence, IL-33-activated basophils co-cultured with B16.F10 melanoma cells were shown to inhibit tumor growth compared to melanoma cells co-cultured with un-stimulated basophils (142).

Overall, there are several studies indicating that basophils promote tumorigenesis (72, 74). In this instance, the tumor cells cause basophils to release cytokines/chemokines that may facilitate the development of protumorigenic TME (Figure 3). Interestingly, many of the same TME elements involved in this activity (e.g., IL-4, IL-13, galectin-3, VEGF-A, M2 and Th2 cells) are also implicated in promoting wound healing. Conversely, in certain tumors (e.g., melanoma), basophils mediate anti-tumor effects (75, 88, 154) (Figure 4). The mechanisms underlying the protective effects of basophils remain largely unknown. It has been suggested that certain mediators (e.g., TNF-α and granzyme B) released by basophils exert tumoricidal effect. In addition, other molecules (e.g., CCL3 and CCL4) can favor the recruitment of cytotoxic $CD8^+$ T cells (74). Collectively, these findings highlight some apparently conflicting results regarding the role that basophils potentially exert in different models of tumorigenesis, and thus warrant further investigation.

5 Basophils in autoimmune disorders

5.1 Systemic lupus erythematosus

With the discovery of IgE (168, 169), immunologists focused their attention on understanding its relevance for allergic disorders and host defense against parasitic infestations (2, 64, 81, 170).

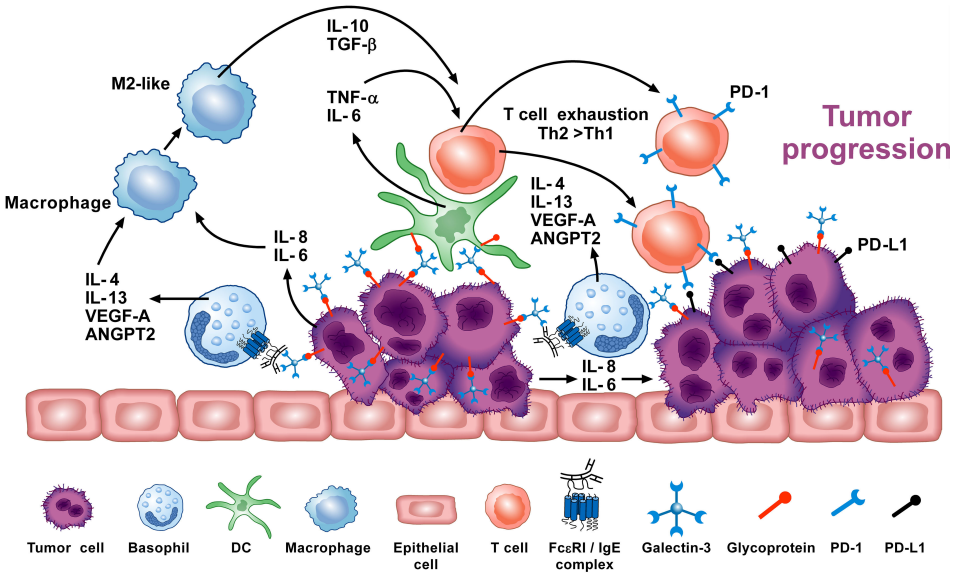

FIGURE 3
Basophils can promote tumor progression through different mechanisms. Galectin-3 (Gal-3) is a lectin expressed by several cancer cells (137), including the A549 adenocarcinoma cell line (EC-Gal-3). Gal-3 activates human basophils to release IL-4 and IL-13 (16, 136), which are widely known to promote M2-like macrophages, the major players in the TME (127–130). IL-4^+ basophils have been found in the TME of human and experimental pancreatic cancer (72). Human and mouse basophils also secrete VEGF-A and angiopoietin 2 (ANGPT2) that can promote tumor angiogenesis (114, 146–148). Basophils can promote IL-6 and IL-8 release from epithelial cell lines through a mechanism requiring cell-to-cell contact (149) (JTS, unpublished). Tumor cell-derived IL-6/IL-8 play a critical role in metastasis formation (150). Dendritic cells and monocytes activated by EC-Gal-3 release TNF-α and IL-6 *in vitro* (151). These cytokines, combined with M2 cell-derived IL-10 and TGF-β induce T-cell exhaustion by up-regulating checkpoint inhibitors (i.e., PD-1), which interact with tumor cell-associated PD-L1 to decrease cytotoxic T cell activity (152, 153). These results suggest that basophils can promote tumorigenesis in certain experimental and clinical conditions. Adapted from Poto *et al.* (74).

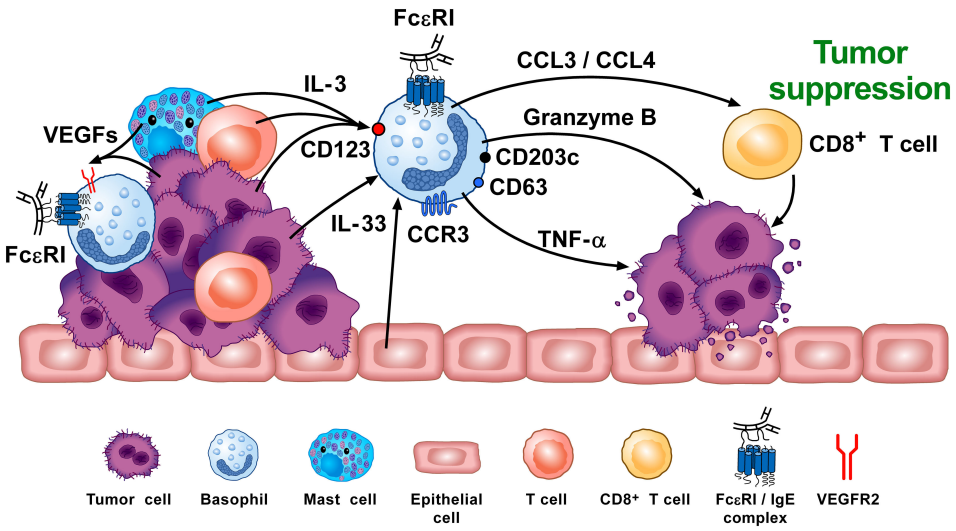

FIGURE 4
Basophils can promote tumor suppression through different mechanisms. Vascular endothelial growth factors (VEGFs) released by tumor and immune cells in the TME (e.g., macrophages, mast cells) (155–159) induce basophil recruitment via the activation of VEGFR2 on these cells (155). IL-3, released from intratumoral lymphocytes, mast cells and tumor cells (10, 160, 161), is the major growth, differentiation, priming and activating factor for both human and mouse basophils via the activation of the IL-3 receptor (IL-3Rα/CD123) (8–10). Intratumoral basophils secrete CCL3 and CCL4 which favor $CD8^+$ T cell infiltration in TME, favoring melanoma rejection in mice (75). IL-33 produced by epithelial and tumor cells, plays a critical role in tumorigenesis (162) by upregulating granzyme B mRNA and the surface expression of CD63 in basophils. Mouse basophils activated by IL-33 cause melanoma cell death *in vitro* (142). Mouse (104, 163) and, in certain conditions, human basophils (164, 165) release TNF-α and granzyme B (142, 166), which exerts cytotoxic activity on cancer cells (102, 167). Tumor resident basophils overexpressing CD123, CCR3, CD63, CD203c mRNAs are associated with improved outcome in ovarian cancer (88, 154). These findings indicate that, under specific experimental and clinical circumstances, basophils can play an anti-tumorigenic role. Adapted from Poto *et al.* (74).

However, circulating IgE autoantibodies in rheumatoid arthritis and SLE patients had been reported as early as the late 70's (171). While these early studies were conducted mostly using small cohorts of patients, they did confound the thought at the time that atopy was generally limited to patients suffering from allergic disease and/or parasitic infestations.

Systemic lupus erythematosus (SLE) is an autoimmune disorder associated with circulating self-reactive antibodies (172) (i.e., IgG anti-double-stranded DNA: anti-dsDNA). Several studies reported increased serum IgE in SLE, which correlated with severe disease manifestations (76, 173–175). A portion of the circulating IgE in these SLE patients was determined to be self-reactive, binding to nucleic acids, as was often the case for most IgG autoantibodies (176). In fact, several studies identified IgE against at least one autoantigen in SLE patients (171, 173, 177–182). Importantly, IgE anti-dsDNA antibodies are associated with disease activity and hypocomplementemia (177). Moreover, the levels of IgE anti-dsDNA proved to be an independent risk factor for SLE activity, even after excluding the levels of IgG anti-dsDNA (178). One study reported that IgE anti-dsDNA antibodies are found in ~ 70% of lupus patients, and are possibly linked to kidney damage (178). In a Franco-American cohort, IgE anti-dsDNA antibodies did associate with lupus nephritis, whereas IgE against other nucleic acid–containing autoantigens (Sm, SS-A/Ro, and SS-B/La) did not associate with disease (177). These findings suggested that IgE autoantibodies could play a role in the pathophysiologic mechanisms of lupus nephritis. The French-American collaborative study identified IgE autoantibodies against three new autoantigens: APEX nuclease 1, N-methylpurine DNA glycosylase and CAP-Gly domain-containing linker protein family member 4. These autoantigens specifically elicited IgE autoantibodies but not IgG autoantibodies (177). Collectively, these results indicate that IgE autoantibodies are prevalent in lupus nephritis patients and are associated with disease activity. Likewise, these findings provided the impetus for treating SLE patients in a randomized clinical trial using anti-IgE mAb (omalizumab) (NCT01716312).

Charles et al. first demonstrated mechanistic evidence that basophils are implicated in the pathobiology of lupus nephritis by using a spontaneous murine model of SLE ($Lyn^{-/-}$ mice) (76). This observation was subsequently confirmed using a model of pristane-induced lupus-like nephritis (183) as well as in a cohort of SLE patients (181). Basophils from SLE patients express significantly higher levels of the basophil activation marker, CD203c, compared to healthy controls (76). It was also found that the basophil density in both lymph nodes and spleen of SLE patients was higher than controls. Basophil-derived IL-4 reportedly induced B cell class switching toward IgE, and the autoreactive IgE produced was determined to be a relevant inducer of lupus (177, 178, 181, 184). Basophils from human patients with SLE and from two different lupus-like mouse models, overexpress both PGD_2 receptors (PTGDR-1 and PTGDR-2) and CXCR4, the receptor for CXCL12 (185). Basophils seemingly contribute to SLE pathobiology by

migrating to secondary lymphoid organs in a prostaglandin D_2 (PGD_2)- and CXCL12-dependent manner (185). These basophils can then support plasma cell functions by amplifying the production of autoantibodies and circulating immune complexes (76, 183, 185). Figure 5 schematically illustrates the mechanisms presumably linking IgE and basophils to SLE.

5.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease primarily involving inflammation of the joints (187). On a genetic background (i.e., HLA-DR4 found in ~ 70% of RA patients compared to 30% of controls), post-translational citrullination of several self-proteins generates altered self-antigens that activate $CD4^+$ T cell responses in RA patients. Citrullination occurs *via* the conversion of arginine into citrulline by peptidyl arginine deiminases (PADs). Anti-citrullinated protein antibodies (ACPAs) are specific and predictive for RA and are implicated in the pathogenesis of RA (187).

IgE antibodies against citrullinated fibrinogen were detected in the serum of ~ 60% of $ACPA^+$ RA patients (188). These authors reported that basophils from $ACPA^+$ RA patients can be activated by citrullinated protein, whereas basophils from healthy controls were not activated. Serum from IgE-$ACPA^+$ RA patients passively sensitized human $Fc\varepsilon RI^+$ expressing rat basophil cells (RBL) for activation by citrullinated proteins. These finding indicate that basophils from IgE-$ACPA^+$ RA patients can be activated by citrullinated antigens. The results of this original study deserve to be extended using citrullinated proteins specific for RA patients.

5.3 Autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an animal model widely used to investigate the mechanisms underlying multiple sclerosis (MS) (189). EAE differs from MS in needing to be induced rather than occurring spontaneously, although recent transgenic mouse models have indicated spontaneous development of EAE (189, 190). However, inoculation with central nervous system antigens and adjuvant or passive transfer of lymphocytes reactive with these antigens are often employed to induce EAE in many animal strains (189).

Yuk and collaborators have investigated the mechanisms by which basophils can contribute to T_H17 differentiation and EAE pathogenesis (126). For example, IL-17 is highly expressed in MS lesions (191) and T_H17 cells mediate blood-brain barrier disruption and the expression of IL-17 and IL-22 (192). T_H17 differentiation requires IL-6 and TGF-β (193), yet whether basophils promote T_H17 induction in EAE had remained unknown. To address this possibility, Yuk and coworkers demonstrated that IgE cross-linking, or the use of cholera toxin (CT), induced the release of IL-6 and IL-4 from bone marrow-derived basophils (126). Moreover, they found that basophils mediate T_H17 differentiation through IL-6 secretion. The authors also examined whether basophils contribute to T_H17 polarization *in vivo*. WT and IL-6-deficient mice were challenged with CT plus antigen. IL-17A producing $CD4^+$ T cells were reduced in IL-6 deficient animals, suggesting that IL-6 is critical for the antigen-induced T_H17 response. The role of basophils was also examined in basophil-deficient mice. The authors found that basophil-derived IL-6 cooperates with DCs to promote the differentiation of CD4 T cells into T_H17 cells. T_H17 responses

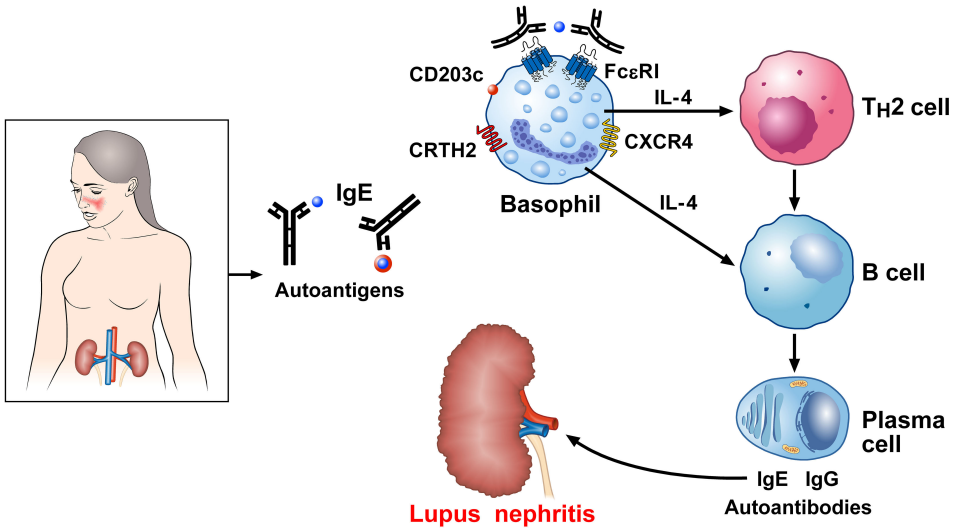

FIGURE 5
Proposed mechanism linking IgE basophils to autoimmunity in systemic lupus erythematosus (SLE). Serum IgE levels are increased in SLE and correlate with severe disease manifestations (76, 173–175). IgE against several autoantigens have been reported in SLE (171, 173, 177–182). Basophils from SLE patients show an activated phenotype in overexpressing CD203c (76), the prostaglandin D_2 (PGD_2) receptor [chemoattractant receptor-homologous molecule (CRTH2) expressed on Th2 cells], and CXCR4, the receptor for CXCL12 (185). Once recruited to the secondary lymphoid organs, activated basophils release IL-4, which drives B cell isotype switching toward IgE and autoreactive IgE (177, 181). Dendritic cells (DCs) in lymph nodes also act on B cells, triggering their differentiation into plasma cells and potentiating the formation of self-reactive autoantibodies (186). IgE immune complexes contribute to basophil activation. Deposits of IgG and IgE autoantibodies in the kidney play a major role in lupus nephritis.

were reduced in the absence of basophils or IL-6. Collectively, these findings suggest that basophil-derived mediators (e.g., IL-6) are involved in T_H17 cell differentiation, allowing T_H17 cells to migrate to the site of inflammation mediating pathogenic functions in EAE. These studies identify basophils and their mediators as candidates for investigating pathogenic mechanisms in MS patients. It should be noted that EAE pathology is not driven exclusively by T_H17 and IL-17; other cells (e.g., $CD8^+$, T cells, γδ T cells) and cytokines may also be involved (194).

5.4 Mixed connective tissue disease

Mixed connective tissue disease (MCTD) is a rare systemic autoimmune disease (incidence ~ 2 per 100,000 adults) affecting mainly women (~ 90%) (195). Its clinical manifestations often overlap with other connective tissue disorders, including SLE, systemic sclerosis, or myositis (196). The defining immunological feature of MCTD is the presence of autoantibodies recognizing the 70-kDa subunit of the U1 small nuclear ribonucleoprotein (U1-snRNP 70k) in the absence of IgG against dsDNA or to Sm, two SLE hallmarks (197). The pathophysiology underlying MCTD remains poorly understood, but posttranslational modifications of U1-snRNP are known to generate neoepitopes that may contribute to the disease (198). These neoepitopes can result in T cells recognizing U1-snRNP, which ultimately lead to the induction and proliferation of autoreactive B cells synthesizing autoantibodies (199). Immune complexes made of anti-U1snRNP antibodies and their antigen can activate endothelium and immune cell *via* a variety of receptors (e.g., Fc, complement, and Toll-like receptors, TLR), resulting in vascular disease and tissue injury (200–203). Pulmonary involvement characterizes more than 70% of MCTD patients (197). A mouse model has been described whereby mice immunized with human U1-snRNP develop a MCTD-like lung disorder (204).

Lamri and collaborators observed that basophils from patients with MCTD present an activated phenotype (77), sharing some features with basophils from SLE patients (i.e., overexpression of CD203c, CXCR4) (76, 185). In addition, basophils from MCTD expressed increased surface markers such as CCR3, yet unchanged expression levels of CD62L (77). A similar basophil phenotype was found in a MCTD-like mouse model in which activated basophils infiltrated in the lungs and lymph nodes. To study the contribution of basophils in the development of lung pathology in this model, basophils were depleted through the injection of DT in female $Bcpt8^{DTR}$ mice. Basophil depletion reduced the cellular infiltrates (e.g., $CD4^+$ T cells) in the lungs. The authors also examined the MCTD-like lung disease in IgE-deficient mice (*Igh7*$^{-/-}$). Similar to that seen with basophil depletion, IgE deficiency also protected mice from developing immune cell infiltration and lung fibrosis. These results indicate that basophils play a major effector role in inducing lung fibrosis *via* an IgE-dependent mechanism. The authors suggested that basophils, activated by the U1-snRNP antibodies complex, accumulate in the airways, where they release IL-4 contributing to lung fibrosis development. In this scenario, IgE-mediated basophil activation may play both immunoregulatory and effector roles in the development of MCTD lung disease. These mouse models identify basophils, and IgE as candidates for investigating pathogenic mechanisms in patients with MCTD.

6 Basophils in IgG4-related disease

IgG4-related disease (IgG4-RD) is a rare multi-organ disorder characterized by lympho-plasmacytic infiltration, fibrosis, and obliterative phlebitis (205, 206). This condition is characterized by $IgG4^+$ plasma cell infiltration in different organs (e.g., biliary tree, pancreas, retroperitoneum, salivary and lacrimal glands, and lymph nodes) (207, 208). The disease was first described in 2003 in a cohort of seven patients with a diagnosis of autoimmune pancreatitis (AIP) associated with $IgG4^+$ plasma cell infiltration (209). Although the pathogenic mechanisms underlying IgG4-RD remain elusive (206), an increased production of Th2 cytokines (IL-4, IL-5, IL-13) has been identified in IgG4-related cholangitis and pancreatitis (210). These cytokines favor IgE production and eosinophil recruitment. It has also been reported that in patients with IgG4-RD, there is an accumulation of T regulatory cells (Tregs) in the blood, along with evidence that these cells infiltrate affected tissues, showing overexpression of IL-10 and TGF-β (211, 212). TGF-β released from Tregs can stimulate fibroblasts to produce collagen. IL-10 produced by Tregs can also stimulate secretion of IgG4 from plasma cells. The involvement of IL-10 and TGF-β secreting basophils has been suggested in patients with IgG4-related submandibular gland disease (213). B cell activating factor (BAFF) and APRIL, in combination with IL-21, can promote the expansion of IgG4-committed B cells (214, 215).

Two studies performed by different investigators in Japan proposed a possible mechanism whereby basophils are stimulated *via* a TLR-dependent activation involving IgG4-RD (214, 216). When activated by TLR2/TLR4 agonists, basophils from healthy donors induced B cells to produce IgG4 and IgG1 (214). TLR4 activation of basophils induced the release of IL-13 and BAFF. Basophils from IgG4-RD patients, upon activation with TLR2 and TLR4 ligands, induced more IgG4 than IgG1 when co-cultured with B cells. The authors suggested that the activation of TLRs in basophils play a role in IgG4-RD development (214).

Another study examined the role of basophils from peripheral blood and pancreatic tissue in patients with autoimmune pancreatitis (AIP) (216). AIP is a manifestation of IgG4-RD (208). Basophil density in the pancreas of AIP patients was higher than in alcoholic pancreatitis (216). In some of these patients, peripheral blood and intrapancreatic basophils were TLR2 or TLR4 positive. The authors suggested that basophils activated by TLRs could play a role in AIP. At present, the possible involvement of basophils and their mediators in the pathogenesis of different localizations of IgG4-RD remains unknown.

7 Basophils in chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a primary cause of morbidity and mortality worldwide (217). COPD is characterized by chronic inflammation, progressive airflow limitation and emphysema. Relative to asthma, the cellular and molecular mechanisms of COPD remain ill defined (117). It also differs in being characterized by a non-reversible airway obstruction (82, 218).

Shibata and collaborators elegantly investigated the potential role of basophils and their mediators in an elastase-induced murine model of COPD (62). Intranasal elastase elicited the recruitment of monocytes to the lung, followed by differentiation into interstitial macrophages (IMs) rather than alveolar macrophages (AMs). Matrix metalloproteinase-12 (MMP-12) played a key role in developing elastase-induced emphysema and was mainly expressed by IMs. The expression of *Il4*, but not *Il10*, *Il13*, or *Tgfb* was upregulated in the lung after the instillation of elastase. Expression of *Il4* mRNA was detected mainly in basophils, which accumulated in the lung. The authors used two complementary methods to deplete basophils *in vivo*, namely: diphtheria toxin (DT) treatment of $Mcpt8^{DTR}$ mice and anti-CD200R3 antibody treatment of WT mice. Using these models, they demonstrated impaired emphysema formation in basophil-depleted mice. They suggested that basophil-derived IL-4 promoted the differentiation of infiltrating monocytes into MMP-12–producing IMs that caused the alveolar wall destruction and emphysema formation. The authors concluded that the basophil-derived IL-4/monocyte–derived IM/MMP-12 axis plays a role in emphysema development. They also proposed that this novel cellular and humoral axis may be a potential target for COPD treatment.

In other findings, both eosinophils and basophils have been detected in several lung compartments of COPD patients, particularly in very severe COPD (219). Eosinophilic infiltration was patchy, and mainly confined eotaxin signatures with $CCL11^+$ fibroblasts and $CCL24^+$ macrophages. Basophils were preferentially localized in lymphoid tissue. These studies identify basophils and perhaps eosinophils as candidates for future investigations on their role in the pathogenic mechanisms of COPD.

8 Basophils in COVID-19

The current COVID-19 pandemic is caused by the novel severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) (220). A dysregulated innate immune response is a key driver of clinical complications culminating in COVID-19 (221, 222). High levels of several cytokines (e.g., IL-1, IL-6, TNF-α, CXCL8) are detected early after viral infection, and many of these mediators are associated with granulocyte activation (223). The recombinant S1 subunit of the SARS-CoV-2 Spike protein activated *in vitro* human peripheral blood monocytes to release several cytokines (e.g., IL-6, IL-1β, TNF-α) and chemokines (e.g., CXCL10/IP-10, CCL3/MIP-1α, CCL4/MIP-1β) linked to COVID-19 (224). In this study, the S1 subunit did not induce any of these cytokines/chemokines from highly purified basophils (224). Another study reported that live SARS-CoV-2 virus induced IL-4 and IL-13 release *in vitro* from unprimed and IL-3-primed basophils (225). Although basophils have been implicated in the host response to other viruses (119, 226–229), the *in vivo* significance of basophil-derived cytokines/chemokines in the pathogenesis of COVID-19 remains unclear.

A detailed analysis at the single-cell resolution of granulocyte diversity in peripheral blood of COVID-19 patients demonstrated an increased level of both mature and immature neutrophils (230). By contrast, decreased basophils and eosinophils are often associated with severe COVID-19 (230, 231). Moreover, the emergence of PD-L1 expression on peripheral blood basophils (as defined as $CD11b^+SS^{low}CrTH2^+$ cells) has been associated with COVID-19 severity (232). It should be pointed out that *in vitro* incubation of live SARS-CoV-2 with basophils purified from normal donors did not induce the expression of PD-L1 (225), whereas INF-γ increased PD-L1 expression on IL-3-primed basophils (233). High basophil counts are associated with a lower risk of developing severe COVID-19 (234). Collectively, these interesting results potentially implicate that basophils and/or their mediators play a protective role in COVID-19.

9 Basophils in inflammatory bowel diseases

Crohn's disease (CD) and ulcerative colitis (UC) are the most common chronic inflammatory bowel disorders (IBDs) (235, 236). The inflammatory infiltrate in IBDs is canonically characterized by activated T cells, macrophages, DCs, neutrophils, and T_H17 cells (236). Basophils were identified in the inflamed mucosa of IBD patients that also expressed IL-33 (125). When activated by IL-3 and IL-33, basophils amplified T_H17 cytokine expression in T cells. Basophils, but not mast cells, accumulated in inflamed CD and UC tissues compared to non-inflamed mucosa (237). No basophils were detected in colons of healthy control donors, indicating selective recruitment and/or survival of these cells at inflamed mucosal sites in patients with IBDs. The accumulation of basophils occurred in colons of untreated patients as well as in patients treated with 5-aminosalicylate acid or immunomodulators (e.g., glucocorticoids and/or immunosuppressive agents and/or biologics). Activated T cells infiltrate inflamed colons and are a major source of IL-3 (10) that may contribute to the infiltration and/or survival of basophils locally (238). Basophils increased IL-17 production and promoted the differentiation of $IL\text{-}17^+$ cells. Collectively, these results demonstrate that basophils accumulate in the inflamed colon in patients with the two most frequent IBDs and may thus contribute to CD and UC pathogenesis. Figure 6 schematically illustrates the potential mechanisms by which basophils, together with other immune cells, contribute to IBD.

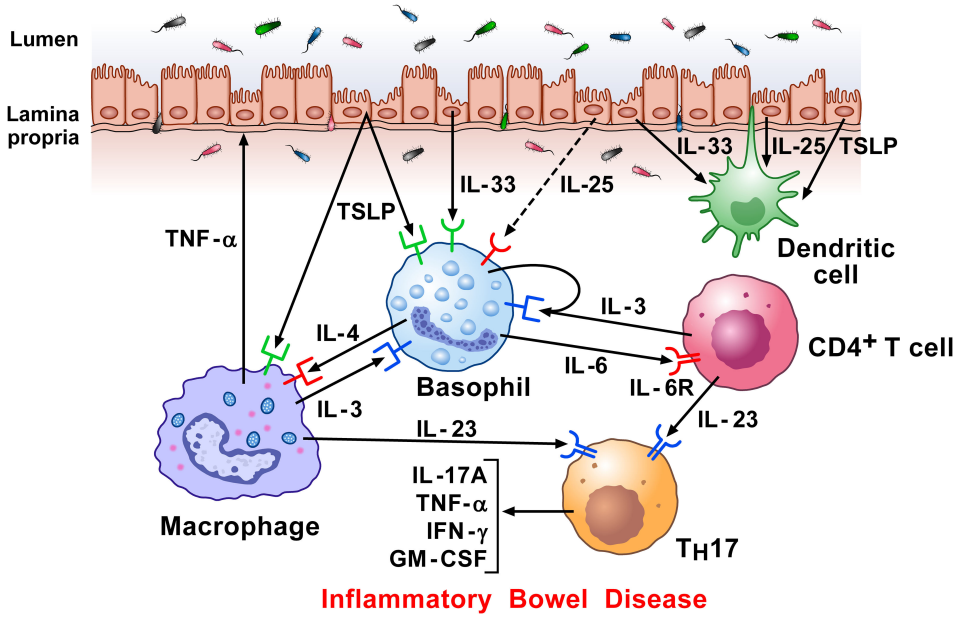

FIGURE 6
Hypothetical mechanisms by which dysregulated epithelial cells and inflammatory signaling by lamina propria immune cells in response to microbiota, contribute to inflammatory bowel disease (IBD) pathogenesis. Intestinal epithelial cells separate the lamina propria and deeper tissues from the luminal environment containing the intestinal microbiota (239). Increased intestinal permeability can potentiate immune-mediated systemic and intestinal inflammation in IBD (240). Damaged epithelial cells release alarmins (IL-33, TSLP, and IL-25) (115, 123, 241), which then regulate underlying immune cells (242), including basophils (9), macrophages (157), and DCs (243). Macrophages can damage epithelial cells directly by TNF-α secretion. Basophils accumulate in inflamed IBD compared to non-inflamed mucosa and to colon of healthy controls (125). Activated T cells infiltrate inflamed colons and release IL-3 which can contribute to the attraction and/or survival of basophils locally (238). Specific components of gut microbiota induce the emergence of intestinal T_H17 cells. Basophils may also promote T_H17 responses (125). Activated T cells release IL-23, which converts homeostatic T_H17 cells to pathogenic T_H17 cells, and play a major role in Crohn's disease (244).

10 Basophils in eosinophilic granulomatosis with polyangiitis

Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare systemic disease characterized by eosinophilic asthma, sinus and pulmonary infiltrates, and eosinophil vasculitis (245). Lung biopsies are rarely done in EGPA and adequate animal models are not currently available. Therefore, the lung immunopathology of this disorder has not been carefully examined. Basophils were detected in four of five EGPA open lung biopsies (246), whereas no basophils were identified in seven control lung biopsies. Mast cell density was increased in EGPA patients compared to the control lungs. These preliminary data show that EGPA lung immunopathology includes infiltrates of eosinophils, basophils, and mast cells. Further studies appear necessary to identify possible interlinks between basophils and IgE and delineate the protective *versus* rather harmful effects of these conditions in EGPA.

Therapeutic management of EGPA is based on glucocorticoids alone and often in combination with immunosuppressive agents (247). Several observational studies have evaluated the role of omalizumab on maintenance therapy in EGPA (247–249). The results of these studies suggest that omalizumab may be clinically beneficial for EGPA patients improving asthma symptoms, lung function, and may have a glucocorticoid-sparing effect (247–249). There is the possibility that the effects of omalizumab in EGPA patients may be related, at least in part, to its effects on human basophils (250).

11 Basophils in eosinophilic esophagitis

Eosinophilic esophagitis (EoE) is a chronic, food-driven allergic disease characterized by esophageal eosinophilia that affects children and adults (251–253). The histopathological and clinical features of EoE have been attributed to overproduction of the type 2 cytokines IL-4, IL-5 and IL-13, which mediate profound alterations in the esophageal epithelium (254–256). The esophageal epithelium likely has an important role in the initiation of EoE *via* production of the epithelium-derived cytokines thymic stromal lymphopoietin (TSLP) and IL-33 (257, 258). EoE is associated with polymorphism in the gene that encodes TSLP in children (259, 260). In a mouse model, EoE-like disease developed independently of IgE, but was dependent on TSLP and basophils (257). Targeting TSLP or basophil depletion during the sensitization phase limited disease and improved established EoE-like disease. Interestingly, increased *TSLP* expression and basophil responses were demonstrated in esophageal biopsies of patients with EoE (257). Collectively, these results suggest that the TSLP-basophil axis contributes to the pathogenesis of EoE.

In another model of EoE-like disease, mice were epicutaneously sensitized with ovalbumin (OVA), followed by intranasal OVA challenge (258). This procedure promoted eosinophilic esophagitis, upregulation of Th2-like cytokines and the IL-33 receptor (ST2). *In vivo* basophil depletion or disruption of the IL-33-ST2 axis mitigated these features. These results suggest that basophils mediate experimental EoE through IL-33-ST2 interaction. These authors also found that pediatric patients with EoE have increased expression of *IL33* and *IL1RL1* (encoding ST2) in esophageal biopsies (258).

Taken together, these studies endorse the paradigm that epithelium-derived cytokines (i.e., TSLP and IL-33) play a role in the pathogenesis of EoE through the activation of basophils and the development of type 2 inflammatory milieu.

12 Concluding remarks and perspectives

Basophils are extremely rare cells, accounting for 1% or less of the circulating blood leukocytes, both in humans and mice. As a result, there was limited capacity to investigate the biology of these immune cells for several decades following their discovery in 1879 (261). However, advances during the past ~30 years have increased interest with compelling new evidence that they represent important effector cells in allergic inflammation (1, 64, 81, 82) and exert a protective role in parasitic infections (66–68). The development of new murine genetic tools and different models of inflammation has also generated novel insight into the potential contribution of basophils to an increasing spectrum of diseases. In particular, basophils and their mediators are now implicated as important participants in pathophysiologic conditions never before considered, including MI (70), kidney fibrosis (71), several autoimmune disorders (76, 77, 126), different cancers (72, 73, 75), COPD (62), and COVID-19 (230–232, 234).

In several pathological conditions, such as kidney fibrosis (71), autoimmune disorders (76, 77, 125, 126), some cancers (72, 73), COPD (62), IgG4-RD (208), IBD (125, 237), and EoE (257, 258) basophils and their mediators play a harmful role. In other inflammatory disorders, such as MI (70), certain cancers (154) (75), and COVID-19 (230–232, 234), basophils appear to play a protective role. The dichotomous pathogenic role of basophils is intriguing and will undoubtedly be the subject of future investigations. There is the possibility that, like mast cells (262–266), macrophages (104, 132, 267, 268), neutrophils (269–272), and eosinophils (273, 274), subpopulations of basophils may also exist. In this regard, distinct phenotypic and functional basophil subpopulations have been described in human peripheral blood (275). Moreover, it has already been demonstrated that tissue-resident basophils differ from circulating basophils in mice (276) and possibly in humans. Finally, basophils might possess a high degree of plasticity and can modify their phenotype and functional characteristics when exposed to different local environments. Whatever the case, the possible existence of basophil subpopulations and the disease-specific heterogeneity of these cells need to be thoroughly and accurately explored in both humans and mice by novel analytical tools (e.g., single-cell RNA seq, CyTOF).

Finally, several biologics have been approved for the treatment of severe allergic disorders and are showing remarkable efficacy (218). Those designed primarily to target mast cells, eosinophils, and Th2 cells (e.g., omalizumab, mepolizumab, benralizumab and dupilumab) also target human basophils and/or their products (250, 277). Thus, there is the possibility that these biologics could prove efficacious in helping to combat other unsuspected conditions/diseases (e.g., cancer, autoimmunity, fibrosis) where basophils are recently implicated. In contrast, with mounting evidence that basophils and their mediators also play critical homeostatic and protective roles (70, 75, 226, 230–232, 234), caution may be warranted when these therapeutic interventions are used.

Author contributions

RP, GM, JS, GV drafted the manuscript and interpreted data; RP, SL, GM, AS, AP, JS, GV edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Basophils in pruritic skin diseases

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Basophils are rare cells in the peripheral blood which have the capability to infiltrate into the skin. Invasion of basophils has been detected in pruritic skin diseases, including atopic dermatitis, bullous pemphigoid, chronic spontaneous urticaria and contact dermatitis. In the skin, basophils are important players of the inflammatory immune response, as they release Th2 cytokines, including interleukin (IL)-4 and IL-13, subsequently inducing the early activation of T-cells. Further, basophils release a multitude of mediators, such as histamine and IL-31, which both play an important role in the initiation of the pruritic response *via* activation of sensory nerves. Chronic pruritus significantly affects the quality of life and the working capability of patients, though its mechanisms are not fully elucidated yet. Since basophils and neurons share many receptors and channels, bidirectional interaction mechanisms, which drive the sensation of itch, are highlighted in this review.

KEYWORDS

basophils, IL-31, atopic dermatitis, neuro-immune interaction, pruritus

Introduction

Basophil granulocytes are named due to their affinity to basic dyes (1). The diameter of basophils is 10 - 14 µm (2) and basophils are the least abundant type of granulocytes in human blood, where they comprise less than 1% of all leucocytes (1). After differentiation from hematopoietic stem cells in the bone marrow, fully matured basophils enter the blood stream (2). Basophils do not proliferate (3) and have a short lifespan of 60 - 70 h in mice (4). In humans, lifespans of up to 11 days have been reported (5). During helminth elimination, basophils are involved in protective mechanisms and also play a significant role in enhancing inflammation (6). Basophils are an important early source of Th2-type cytokines such as interleukin (IL)-4 and IL-13 in inflammation (Figure 1) (7). Moreover, basophils release the pruritic cytokine IL-31, and express its receptor complex consisting of the IL-31 receptor A (IL-31RA), and the oncostatin M receptor β (OSMRβ) (Figure 1, Table 1) (21). Stimulating basophils with IL-31 induces basophil chemotaxis and promotes the secretion of Th2 cytokines (21). Another itch mediator is histamine. The pruritogen is released after

activation of the high-affinity IgE receptor FcεRI (Figure 1) (33). A specific characteristic of human basophils is the potentiation of mediator release after stimulation with priming factors. In the pathogenesis of inflammatory diseases, enhancing factors, such as IL-3, nerve growth factor (NGF), IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF), modulate the functional activity of basophils. IL-3 is the most potent activator of basophils and also promotes basophil differentiation (35). Its receptor α-chain CD123 is expressed by basophils (Figure 1) (13–17). Another priming agent for basophils is the neurotrophin NGF, which induces the release of histamine and the synthesis of leukotriene C4 (LTC4) after stimulation with agonists (Figure 1) (36). NGF has similar effects on basophils as IL-5 and GM-CSF (36). While IL-5 belongs to the group of Th2 cytokines (37), GM-CSF is a monomeric glycoprotein that is present at sites of tissue inflammation (38). Both are produced by basophils and promote inflammation (39). Activation of basophils is associated with upregulation of the cell surface markers CD13, CD45, CD63, CD203c (40), and CD69, for which increased expression is mostly observed after stimulation with IL-3 (41). A method to assess human basophil activation is to determine changes in the amount of these surface proteins. The most reliable activation markers are CD63 and CD203c (40). CD63 is a membrane protein,

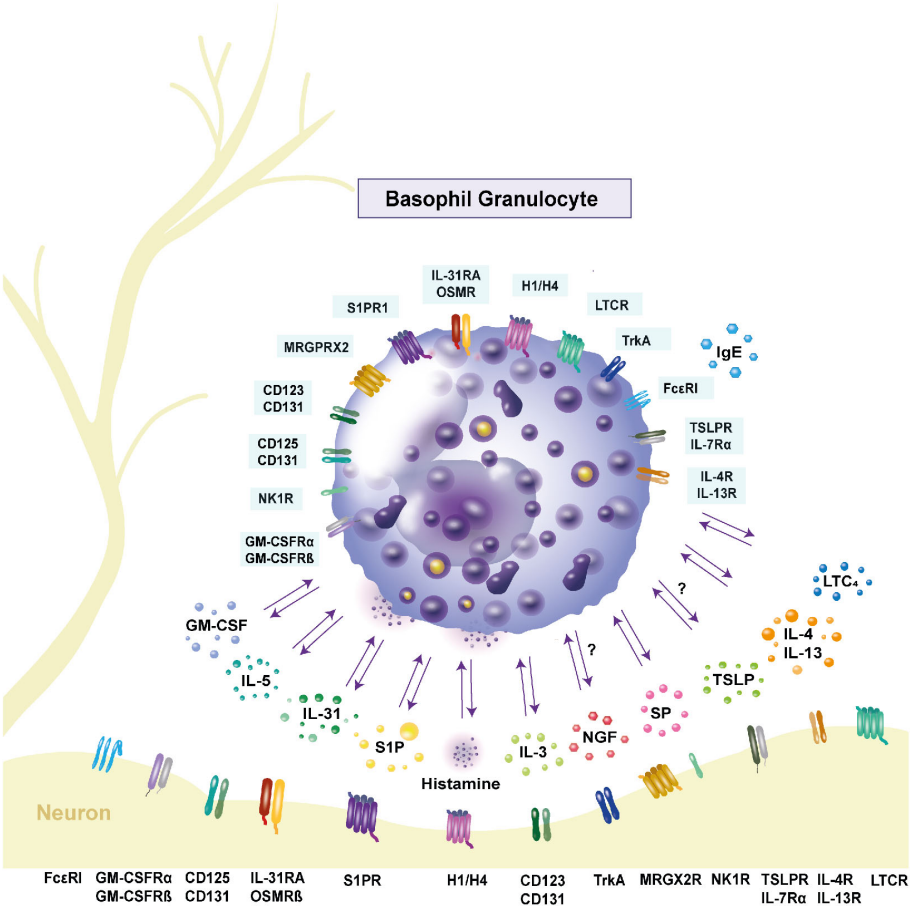

FIGURE 1
Expression of receptors and release of cytokines in human basophils. Basophils interact with other immune cells and neurons through inflammatory mediators and receptor expressions. Interleukin (IL)-31, as well as its receptor complex consisting of the IL-31 receptor A and the oncostatin M receptor β, are expressed by basophils and contribute to pruritus. Stimulation with IL-31 leads to the secretion of the pro-inflammatory cytokines IL-4 and IL-13. Their respective receptors are IL-4R and IL-13R. Basophils express the high-affinity receptor FcεRI. Upon crosslinking of the receptor with IgE, histamine is released, mediating itch. The hormone receptors are present on the cell surface, with the histamine 4 receptor being the most highly expressed. Activation of the neurokinin 1 receptor through substance P (SP) also causes histamine release. Basophils can be primed by IL-5, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF). The respective receptors are CD125 and CD131 for IL-5, CD123 and CD131 for IL-3 and the GM-CSF receptor consists of GM-CSFRα and GM-CSFRβ. Activation of these receptors leads to increased histamine release. Another priming factor is nerve-growth factor, which binds to the tyrosine kinase A receptor on the cell surface. Basophils express the Mas-related G protein-coupled receptor X2 (MRGPRX2), which is part of the signaling cascade in inflammation and serves as a receptor for SP. Another pruritogen is thymic stromal lymphopoietin (TSLP), which binds to the TSLP receptor complex consisting of TSLP receptor and IL-7 receptor α and is proposed to cause itch. Whether basophils respond to TSLP is controversial. The lipid mediator sphingosine-1-phosphate (S1P) is stored in granules and its receptor S1P receptor 1 is expressed on the cell surface. It is proposed to have an anti-inflammatory effect on basophils. The leukotriene C4 (LTC4) is released by basophils and its receptor cysteinyl leukotriene receptor (LTCR) is expressed by basophils. GM-CSF: granulocyte macrophage-colony stimulating factor; GM-CSFRα: GM-CSF receptor α; GM-CSFRβ: GM-CSF receptor β; H1/H4: histamine 1/4 receptor; IL: interleukin; IL-4R: IL-4 receptor; IL-5R: IL-5 receptor; IL-7RA: IL-7 receptor α; IL-13R: IL13 receptor; IL-31RA: IL-31 receptor A; LTC4: leukotriene C4; LTCR: cysteinyl leukotriene receptor; MRGPRX2: Mas-related G protein-coupled receptor X2; NK1R: neurokinin 1 receptor; OSMRβ: oncostatin M receptor β; trkA: tyrosine kinase receptor A; TSLP: thymic stromal lymphopoietin; TSLPR: TSLP receptor; SP: substance P; S1P: sphingosine-1-phosphate; S1PR1: S1P receptor 1.

that is associated with histamine containing granules. After anaphylactic degranulation (42, 43), CD63 is translocated to the cell surface of activated basophils as a result of histamine release (43). The ectoenzyme CD203c (pyrophosphatase/phosphodiesterase) is weakly expressed on resting basophils (44). Whereas CD63 externalization is closely related to basophil degranulation (44). Upon activation, CD203c, which is not associated with mediator release, is upregulated rapidly (43). Basophil infiltration has been observed in atopic dermatitis (AD), bullous pemphigoid (BP), chronic spontaneous urticaria (CSU) and contact dermatitis (7), all of which are pruritic inflammatory skin diseases. The mechanism how basophils are recruited into the skin remains to be fully elucidated. It is assumed that basophils are attracted by a variety of mediators present in the skin, i.e. the chemokines, CCL2, CCL5, CCL11, CXCL12, and prostaglandin D2 (45). Basophils express the respective receptors, CCR4 for CCL2 and CCL5, CCR3 for CCL11, CXCR4 for CXCL12 and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) for prostaglandin D2 (45, 46). CCL11 is produced by dermal fibroblasts and CRTH2 is elevated in AD (46). Other potential chemoattractants of basophils are thymic stromal lymphopoietin (TSLP), IL-3, IL-31, histamine, substance P (SP) and sphingosine-1-phosphate (S1P). TSLP and IL-3 cause the upregulation of CXCR4 and thereby lead to infiltration of basophils into the skin (47). The pruritogen IL-31 has been shown to induce chemotaxis in basophils *in vitro* (21). Upon histamine release from mast cells, murine basophils are recruited to the site of allergen exposure in nasal tissue (48). SP has also been shown to chemoattract basophils, resulting in the infiltration of basophils into the skin of healthy individuals (49). Recently, it was shown that in healthy donors, basophils migrate towards S1P which was observed in an *in vitro* study, while in AD patients a chemorepulsive effect was detected (31). It has however, so far not been described if basophils, that migrated into the skin, return to the blood or travel to draining lymph nodes (45, 50). Pruritus elicits the desire to scratch the skin and is categorized into acute and chronic pruritus. Chronic itch, by definition, lasts longer than 6 weeks, and strongly impairs patients' quality of life. Although its complete mechanism has yet to be elucidated, complex crosstalk between the stratum corneum, keratinocytes, immune cells, and nerve fibers (Figure 1) plays an important role in the initiation and maintenance of pruritus. Itch can originate in the skin or have neuropathic, psychogenic or systemic causes (51). Histamine, IL-31, SP, LTC4, IL-4, IL-13, NGF, brain-derived neurotrophic factor (BDNF), and TSLP, which all are released by or affect basophils (Figure 1), have been reported to cause itch (7, 12, 21, 52–54) and are described in the chapter "Basophils and neuro-immune interactions". Current therapies for itch target different receptors on basophils, such as IL-31RA, neurokinin 1 receptor (NK1R), tropomyosin-receptor kinase A (trkA), or released mediators, i.e. IL-13. The monoclonal IL-31RA antibody nemolizumab binds to IL-31RA and thereby interrupts IL-31 itch signaling in basophils. A trial from Japan in which nemolizumab was administered, found improvements in pruritus and quality of life, leading to the approval of the drug for AD (55). NK1R is expressed in basophils and its antagonists inhibit pruritic signaling and decrease itch in patients. However, the inhibitors are not licensed for use (56). In mice, treatment with signal transducer and activator of transcription 6 (STAT6) inhibitors led to decreased scratching. IL-13 targets STAT6, inducing pruritus (57). Janus kinase (JAK) inhibitors interrupt the JAK-STAT signaling pathway. This disruption, which occurs after treatment with JAK inhibitor upadacitinib, leads to improvement of pruritus in patients (58). Application of the trkA inhibitor CT327 resulted in a significant decrease of pruritus in psoriasis patients (59). The role of basophils as important effector cells in different inflammatory skin diseases and their involvement in pruritus, are described in the following chapters.

Atopic dermatitis

Atopic dermatitis (AD) is an inflammatory skin disease, associated with recurrent dry skin, and the main bothersome symptom, itch (60). In patients with AD, infiltration of basophils into the skin and peripheral blood has been observed, although not in as high numbers as in other skin diseases (46, 61). In one study, significantly less basophil numbers could be detected in peripheral blood of AD patients than in healthy controls (62). Interestingly, increased basophil count is suggested to be a potential causal risk factor for AD (63). Basophils were found to exhibit increased externalization of the activation markers CD63 and CD203c in AD patients (61). This indicates possible involvement of basophils in the pathogenesis of AD. Basophils can be primed by NGF (Figure 1), which is produced by a variety of cells, such as keratinocytes (53), eosinophils (64), T cells (65), and mast cells

TABLE 1 Shared receptors of basophils and neurons with their respective ligand.

Shared receptor	Ligand	References
GM-CSFRα/β	GM-CSF	(8, 9)
H1/H4	Histamine	(10, 11)
IL-4R	IL-4	(12)
IL-3R	IL-3	(13–18)
IL-5R	IL-5	(19, 20)
IL-13R	IL-13	(12)
IL-31 receptor complex	IL-31	(21, 22)
LTCR	LTC4	(23)
MRGPRX2	SP	(24, 25)
NK1R	SP	(12, 26, 27)
trkA	NGF	(28, 29)
TSLP receptor complex	TSLP	(12, 30)
S1PR1	S1P	(31, 32)
FcεRI	IgE	(33, 34)

GM-CSF, granulocyte macrophage-colony stimulating factor; GM-CSFRα/β, GM-CSF receptor α and β; H1/H4, histamine 1/4 receptor; IL, interleukin; IL-3R: IL-3 receptor; IL-4R: IL-4 receptor; IL-5R, IL-5 receptor; IL-7RA, IL-7 receptor α; IL-13R, IL13 receptor; IL-31RA, IL-31 receptor A; LTC4, leukotriene C4; LTCR, cysteinyl leukotriene receptor; MRGPRX2, Mas-related G protein-coupled receptor X2; NK1R, neurokinin 1 receptor; OSMRβ, oncostatin M receptor β; trkA, tyrosine kinase receptor A; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor; SP, substance P; S1P, sphingosine-1-phosphate; S1PR1, S1P receptor 1.

(66). NGF has been shown to be either increased (53), or significantly decreased in AD patients, correlating with disease severity when compared to healthy subjects (67). In lesional skin of subjects with AD, the number of NGF positive nerve fibers is increased (68). Whether basophils are a source of NGF, has yet to be elucidated. In the epidermis, the lipid mediator sphingosine-1-phosphate (S1P) plays an important role regarding structure, lipid signaling and the regulation of keratinocytes. Our group recently discovered that isolated basophils of atopic patients exhibited decreased S1PR1 expression, and possessed intracellular S1P in isolated basophils (31). Furthermore, in the stratum corneum of AD patients, the lipid is decreased, which might alleviate colonization with *Staphylococcus aureus* (69). The lipid, as well as mRNA expression of the S1P receptors (S1PR) S1PR1, S1PR2, S1PR3 and S1PR4, have been observed in human basophils (Figure 1) (31). The presence of S1PR1 was also confirmed at the cell surface (31) (Table 1). S1PR1, S1PR2 and S1PR4 have been detected in the brain (32), indicating another point of neuro-immune crosstalk. Due to the inhibiting effect of the lipid mediator on chemotaxis, S1P is proposed to have an anti-inflammatory effect on basophils (31). In both mice and humans, significant upregulation of FcεRI on basophils during AD has been observed, indicating that IgE might also be an important factor in pruritus (70). The pro-inflammatory effect of basophils in AD might be reduced by treatment with dupilumab. The monoclonal IgG4 antibody, which binds to IL-4Rα, showed success in reducing symptoms, such as itch, of AD patients (71). Since the antibody binds to IL-4Rα, the assumption arises, that the cytokines which contribute to the disease are partially derived from basophils (71). Aside from their pro-inflammatory properties, basophils can aid in the resolution of AD. The expansion of M2-like macrophages was promoted by murine basophils, as well as epidermal repair (72), which additionally affirms the role of basophils in AD.

Bullous pemphigoid

Bullous pemphigoid (BP) is a blistering skin disease, that most commonly occurs in elderly people and only rarely affects adolescents or children. An autoimmune reaction against the hemidesmosomal proteins BP180 and BP230 leads to the formation of blisters (73). A case study showed that basophil infiltration took place in early- as well as late-stage lesions (74). The twofold involvement of basophils in BP was shown by Kimura et al. (75). During the early stage of BP, basophil infiltration was correlated with eosinophil infiltration. Cell-to-cell contact was observed, indicating that Th2 immunity is promoted by eosinophils and basophils (75). A case study detected the colocalization of basophils and eosinophils in urticarial plaques (74). The presence of basophils was also demonstrated, as well as eosinophils, underneath the subepidermal cleft during the late-stage of BP (74). Basophils in BP were shown to be present with a high density, similar to that observed in urticaria, but higher than that in AD (46), and increased compared to skin healthy controls (76). Circulating basophils from untreated BP patients were stimulated with BP180, resulting in significantly higher histamine release than those basophils of treated BP patients or healthy controls (77). This suggests an important role for basophils in the development of BP. The amount of anti-basement membrane zone antibodies was positively correlated with IgE serum levels (78). Treatment with the anti-IgE monoclonal antibody omalizumab resulted in the downregulation of FcεRI on basophils in two cases (79). Activation of basophils was determined through measuring CD203c expression. The expression was evaluated before and after treatment with two doses of prednisolone and three sessions of plasma exchange, and found to be significantly reduced after treatment (74). These observations indicate that basophils play a role in the development of BP. In BP, itch is an important factor, which is confirmed as itch severity correlates with the increased numbers of basophils present in the blisters (76). Thus, basophils seem to play an important role in pruritus, blister development and inflammation in BP.

Chronic spontaneous urticaria

Chronic spontaneous urticaria (CSU) presents in patients as pruritic hives, angioedema or a combination of both (80). Patients suffering from CSU often present with peripheral basopenia, where low amounts of basophils are present in the blood, probably due to the infiltration into the skin (81). An inverse correlation between disease severity and the amount of basophils in the blood has been observed (81). Moreover, significantly more infiltrating basophils are present in lesions of CSU patients than in nonatopic subjects (82). Basophil degranulation has also been observed in the skin of CSU patients. Therefore, the reactivity in CSU seems to be partially regulated by basophils (82). Substance P (SP) was shown to be positively correlated with the number of basophils in the peripheral blood of CSU patients (26). Interestingly, basophil numbers were increased in CSU patients compared to healthy controls, in contrast to findings of other studies. These basophils exhibited higher expression levels of SP, as well as its associated receptor NK1R, than those from healthy controls. When activated by its agonist, NK1R mediated up to 41% net histamine release, which is comparable to that induced by anti-IgE and the chemoattractant N-formylmethionyl-leucyl-phenylalanine (fMLP) (26). A similar effect was confirmed in mice. Blood basophil numbers increased after injection with SP. Sensitization with ovalbumin resulted in elevated basophils numbers as well as increased SP and NK1R expression on basophils (26). As itch is a significant symptom of CSU, its origin is important. One causative factor might be IL-31, which is elevated in this disease (83). Basophils have been reported to be the main source of IL-31 in skin lesions of CSU (Figure 1) (21). In CSU, patients can be categorized in three groups; responders, nonresponders and basopenics, depending on how much histamine is released from basophils after stimulation with anti-IgE (84). Upon application of anti-IgE, basophils of responders release high amounts of histamine and exhibit increased CD63 externalization. Nonresponders are characterized by low histamine secretion and CD63 externalization, while almost no reaction can be observed in basophils of basopenics (84). Responders, those with high histamine release, seem to suffer from CSU longer than the other groups.

However, the number and size of hives, as well as the itch score were highest in basopenics (84). Another study confirmed that the duration of the disease is longer in responders. The same group of patients also reported increased itch (85). Treatment with the anti-IgE monoclonal antibody omalizumab showed a decrease of symptoms in CSU patients (86). Furthermore, the number of peripheral blood basophils increased as a result of treatment with omalizumab (87). Whether the monoclonal antibody inhibits basophil migration into the skin, or promotes the release of new basophils from the bone marrow has yet to be investigated. Basophils of CSU patients exhibited significantly higher amounts of CD63, than those of healthy controls. CD203c expression however was unchanged (88). In contrast, another study revealed no difference of activation marker levels in CSU patients in comparison with healthy subjects. However, histamine release was reported to be higher in patients with CSU than in controls (89). In CSU patients in remission, basophils were more activated, as determined through the presence of CD63 and CD203c, than in healthy control (90). This shows that basophils are crucial in the development of CSU.

Contact dermatitis

Irritant contact dermatitis is characterized by non-allergic, pruritic skin inflammation, where basophils infiltrate into the tissue (91). In human and murine irritant contact dermatitis skin lesions, basophils were located in proximity to eosinophils, which were recruited to the site by the basophils (91). Furthermore, in mice, direct cell-to-cell contact of basophils with eosinophils seems to lead to the activation of eosinophils, enhancing the development of irritant contact dermatitis (91). Allergic contact dermatitis, however, is caused by contact with an allergen, which also induces basophil migration. Interestingly, infiltration lasts for several days, where basophils can be detected after 25 hours and then increase in number in allergic contact dermatitis (92). Basophils represent 16% of the infiltrate in allergic contact dermatitis at day 16, resulting in delayed hypersensitivity (92). In accordance with this finding, degranulation of basophils was observed to occur over 72 hours, where approx. 60% of granules were found to be at least partially depleted (93). Eosinophil infiltration occurs after basophil infiltration, indicating that basophils play a role in eosinophil recruitment in contact dermatitis (92). Thus, basophils play an important role in the aspects of cell infiltration and pruritus during the development of irritant and allergic contact dermatitis.

Basophils and neuro-immune interaction

Interactions between the immune system and the nervous system play an important role in inflammatory skin diseases and pruritus. These neuro-immune interactions stem from intense crosstalk between neurons and immune cells, which are located in close proximity to one another. Upon allergen challenge with the irritant calcipotriol and the allergen ovalbumin, murine basophils migrate into the skin, and are consistently observed to be located in close proximity to sensory nerve fibers (Figure 1), indicating neuroimmune interactions (70). The initiation and maintenance of itch is characterized by many mediators expressed by basophils, including IL-31, SP, LTC4, histamine, IL-4 and IL-13. Other pruritic mediators, such as TSLP, NGF and BDNF also affect basophils. It is assumed, that basophils interact bidirectionally with neurons through cytokines and neurotrophins, as they share various channels and surface receptors (Figure 1, Table 1). While IL-31RA is present on most basophils, OSMRβ can only be found on a small subpopulation (21). IL-31RA is expressed on half of dorsal root ganglia (DRG) with a size up to 30 μM (22), and its ligand can act as a neurotrophin on DRG neurons (94). Through activation of IL-31RA (22) on peripheral nerves, itch signals are transmitted to the central nervous system (21).

The Mas-related G-protein-coupled receptor (MRGPR) X2 is expressed on human basophils (Figure 1, Table 1) (24) and DRG (25), and evokes allergic, as well as nonallergic hypersensitivity (32). In mice, the transient receptor potential ankyrin 1 (TRPA1) channel is necessary for MRGPR- and TSLP-mediated pruritus (95). Upon activation, the channel is opened and induces itch (95).

Basophils release the inflammatory mediator LTC4. Its receptor cysteinyl leukotriene receptor 2 (CysLTR2) is expressed on basophils and DRG (23).

After priming with IL-3, human basophils express the TSLP receptor, while expression of the IL-7 receptor α was not detectable (96). In contrast, mice express TSLPR and IL-7 receptor α on basophils (Figure 1), which together form the TSLP receptor complex (30). Stimulation of basophils with TSLP has been shown to cause histamine release, and increase intracellular IL-4 and IL-13 expression, as well as induce the upregulation of TSLPR in patients with allergic asthma (97). In contrast to this study, Guen et al. reported that basophils from healthy and allergic patients did not respond to TSLP (98). The TSLP receptor complex has also been confirmed in DRG. Observations in mice revealed TSLP secretion from basophils and activation of neurons through the cytokine (12). TSLP activates TRPA1 expressing neurons and causes itch (99). Secretion of TSLP by human basophils has not yet been investigated.

Basophils and peripheral nerve endings express the tachykinin neurotransmitter SP and its receptor NK1R (Figure 1) (12, 26, 27). The neuropeptide is involved in inflammation and itch (12). Furthermore, SP induces histamine release from basophils, indicating possible interactions between the nervous system and the granulocytes (26, 27), as basophils are able to communicate with neurons *via* histamine. Basophils express the histamine-1 receptor and histamine-4 receptor (H4R; Figure 1) (10), which have also been confirmed to be expressed in the central nervous system (11). When H4R is activated on basophils, it mediates chemotaxis. However, activation can also lead to basophil silencing, as CD63 and CD203c surface content has been observed to be suppressed and the production and release of sulfidoleukotrienes reduced (10). In mice, knockout of H4R resulted in reduced inflammation and treatment with H4R antagonists alleviated itch (12).

Secretion of IL-4 and IL-13 from basophils (Figure 1), indicates communication between basophils and neurons in pruritus. Their respective receptor subunits IL-4Rα and IL-13Rα are expressed in basophils, as well as in DRG (12). In a murine model, injection of IL-4 caused scratching, suggesting that IL-4 induces pruritus in mice (12).

Neurotrophins play an important role in the communication between basophils and neurons. Basophils and the central nervous system express tyrosine kinase receptor A (Figure 1) (100, 101), to which NGF binds. NGF is also a priming factor for basophils, demonstrating the influence of the neuronal system on basophils. To conclude, interaction between basophils and the neuro-immune system occurs through a variety of channels and mediators, highlighting the importance of basophils in neuro-immune interaction mechanisms.

Conclusion

Basophils play a crucial role in many pruritic inflammatory skin diseases. In these conditions, basophils are among the first cells to infiltrate into the skin. At this location, basophils secrete Th2 cytokines and are drivers of the inflammation. The pruritic effect is further mediated by IL-4, IL-13, IL-31, histamine, SP, TSLP, BDNF and NGF, of which most are released by basophils. IL-31 is a key mediator in itch, its expression being increased in inflammatory and pruritic skin diseases. Basophils also recruit eosinophils to sites of inflammation in BP and CSU, further increasing the inflammation. Moreover, basophils are able to establish cell-to-cell contact with sensory neurons, and enable neuro-immune interaction through the release of inflammatory mediators, such as IL-31. Thus, basophils seem to be major drivers of inflammation and itch in diseases such as AD, BP, CSU and contact dermatitis, which was summarized in this review.

Author contributions

Conceptualization, DW and UR. Writing—original draft preparation, DW. Writing—review and editing, ML, NG, UR. Illustration, ML. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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