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Abbreviated Title: basophils and human Th17 response
Abstract

Basophils are the rare granulocytes and play an important role in the polarization of Th2 responses and protection against helminth parasites. In addition, basophils contribute to the pathogenesis of several diseases such as asthma, chronic allergy and lupus. Notably, Th17 cells are also implicated in the pathogenesis of these diseases suggesting that basophils support the activation and expansion of this subset of CD4⁺ T cells. Therefore, we explored whether basophils promote the expansion of human Th17 cells. We show that basophils lack the capacity to expand Th17 cells and to induce the secretion of Th17 cytokines either directly or indirectly via antigen presenting cells such as monocytes. As human basophils lack HLA-DR and co-stimulatory molecules, their inability to confer T cell receptor- and co-stimulatory molecule-mediated signals to CD4⁺ T cells might explain the lack of Th17 responses when memory CD4⁺ T cells were co-cultured with basophils.

Key words: basophils; IL-17; Th17; IL-22; monocytes
1. Introduction

Basophils are the rare granulocytes and represent less than 1% of circulating leukocytes. They play an important role in the polarization of Th2 responses and in the protection against helminth parasites [1-5]. Recent studies have identified several surface markers of human and mouse basophils that could be used for the identification and isolation of these cells. These markers include CD49b (DX5), CD123 (IL-3 receptor α chain), CD200R3 (a disulfide-linked dimeric CD200R-like receptor belonging to the immunoglobulin superfamily), CD203c, 2B4 (or CD244, a 66-kDa protein from the CD2 family), CCR2, CCR3, CD45R (intermediate level of expression) and FcεRI. Further, in contrary to mast cells, basophils are c-Kit− (CD117) and this marker could be used to discriminate basophils from mast cells in the tissues [2].

Since long time, basophils have been neglected in immunology due to their low number in the circulation and their shared features with tissue-resident mast cells. However, recent studies indicate that basophils have a major impact on the immune responses and diverse roles of these cells in autoimmune and inflammatory diseases are emerging. Because basophils express several sensing molecules including FcεRI, toll-like receptors (TLRs such as TLR2 and TLR4) and receptors for various cytokines including IL-3, IL-33 and IL-25, basophils can readily respond to various stimuli and release immune modulators such as cytokines, chemokines, histamine and lipid mediators [2]. Therefore, a higher number of activated basophils could tilt the homeostatic balance of the immune system leading to inflammatory conditions.
Activated basophils act as accessory cells to provide Th2 environment and enhance dendritic cell-mediated Th2 responses. In fact, recent reports indicate that the function of basophils in the polarization of Th2 responses is not only important for the protection against helminth parasites but it can also contribute to the pathogenesis of asthma, allergy and autoimmune diseases such as systemic lupus erythematosus [1, 2, 6-8].

A newly identified subset of CD4⁺ T cells namely Th17 cells are also implicated in the pathogenesis of asthma, chronic allergy and lupus suggesting that basophils might support the activation and expansion of this subset of CD4⁺ T cells [9, 10]. Th17 cells express lineage specific transcription factor RORC and IL-17A is the prototype cytokine of these cells. In addition, Th17 cells secrete other inflammatory mediators such as IL-17F and IL-22 [9]. As basophils have an important role in the regulation of immune responses such as T and B cell responses, we explored whether basophils promote the expansion of human Th17 cells.

2. Materials and Methods

2.1. Isolation of circulating human basophils and monocytes

Buffy coats of healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France upon ethical committee permission (N°12/EFS/079). Basophils from the buffy coats were isolated by two-step process. By percoll density gradient centrifugation, we first obtained peripheral blood mononuclear cells (PBMCs). These PBMCs were subjected to MicroBead-based negative isolation of basophils by using basophil isolation kit II (Miltenyi Biotec, Paris, France) [11]. Monocytes from PBMCs were purified by using CD14
MicroBeads (Miltenyi Biotec). The purity of basophils as well as that of monocytes was in the range of 94±5% as analyzed by flow cytometry (BD LSR II, BD Biosciences, Le Pont de Claix, France). Basophils were analyzed by using fluorochrome-conjugated mAbs to CD203c (eBioscience, Paris, France) FcεRI and CD123 (both from Miltenyi Biotec) [12] while monocytes were monitored by using fluorochrome-conjugated mAb to CD14 (BD Biosciences).

2.2. Isolation of memory CD4⁺ T cells

To isolate memory CD4⁺ T cells, untouched total CD4⁺ T cells were first purified from PBMCs by using CD4⁺ T-cell isolation kit II (Miltenyi Biotec). Further, by using CD45RA MicroBeads (Miltenyi Biotec), naïve CD4⁺CD45RA⁺ T cells were depleted from total CD4⁺ T cells. Finally, CD4⁺CD45RO⁺CD25⁻ memory T cells were obtained by depleting CD25⁺ cells with CD25 MicroBeads (Miltenyi Biotec). The purity of isolated cells was in the range of 95±4%.

2.3. Co-culture of basophils and monocytes with CD4⁺CD45RO⁺CD25⁻ memory T cells

Allogeneic memory CD4⁺ T cells were cultured in U-bottomed 96 wells plate (0.1x10⁶ cells/200 µl/well) in X-vivo-10% human AB serum and IL-2 (100 IU/0.5x10⁶ cells, ImmunoTools, Friesoythe, Germany) either alone; or with basophils in the presence of IL-3 (100 ng/1x10⁶ cells, Miltenyi Biotec) or IL-3 and monoclonal anti-human IgE (10 ng/0.1x10⁶ cells, clone GE1, Sigma-Aldrich, Saint Quentin Fallavier, France); or with peptidoglycan-stimulated monocytes (5 µg/0.5 x10⁶ cells, Invivogen, Toulouse, France); or with peptidoglycan-stimulated monocytes and IL-3-primed basophils; or with peptidoglycan-stimulated monocytes and IL-3-anti-IgE-
treated basophils. The activation of basophils was analyzed by the expression of CD63 by using fluorescence-conjugated mAb (BD Bioscience). Monocytes and basophils were stimulated in the co-culture and were not pre-activated. The ratio of memory CD4\(^+\) T cells and monocytes and/or basophils was maintained at 5:1. After five days of culture, the cells were harvested and cell-free culture supernatants were collected for the analysis of IL-17A and IL-17F. The cells were processed for staining and flow cytometry as described below.

2.4. Intracellular staining and flow cytometry

The harvested cells were re-stimulated with phorbol 12-myristate 13-acetate/ionomycin (Sigma-Aldrich) for 6 hours, with GolgiStop (BD Biosciences) during last 3 hours. Surface staining was done with fluorescence-conjugated CD4 mAb (BD Biosciences) and fixable viability dye (eBioscience), in order to gate and analyze viable CD4\(^+\) T cells. Further, cells were fixed, permeabilized (Fix/Perm; eBioscience), and incubated at 4°C with fluorochrome-conjugated mAbs to IFN-γ, IL-4 (BD Biosciences) and IL-17A (eBioscience). The stained cells were subjected to flow cytometry (BD LSR II). Ten thousand cells were acquired for each sample and data were processed by using FACS DIVA software (BD Biosciences).

2.5. Cytokines analysis

Levels of IL-17A (DuoSet ELISA kits, R&D Systems), IL-17F and IL-6 (ELISA Ready-SET-Go, eBioscience) in cell-free culture supernatants were quantified by ELISA. The detection limits were 15 pg/mL for IL-17A, 16 pg/mL for IL-17F and 21 pg/mL for IL-6.
2.6. Measurement of plasma IgE

The IgE in the plasma of healthy donors was measured by an automated classical sandwich immunoassay by ImmunoCap technology (Thermo Fischer, Phadia SAS, St Quentin Yvelines, France). Results are expressed in kU/L and the admitted correspondence is 2.4 ng/ml per kU/L.

2.7. Statistical analysis

Statistical analysis was done by one-Way ANOVA (Friedman test) or two-tailed Student’s-t-test using Prism 5 software (GraphPad softwares). Values of P < 0.05 were considered as statistically correlated.

3. Results

3.1. Activated human basophils lack the capacity to promote Th17 expansion

We investigated the direct effect of basophils on the expansion of Th17 cells. As stimulated basophils are known to secrete variety of cytokines and other chemical mediators, we also examined if enhanced degranulation of basophils through FcεRI cross-linking would augment Th17 responses. IL-3-primed basophils were co-cultured with CD4^+CD45RO^+ memory T cells either in the presence or absence of FcεRI cross-linking. To avoid nonspecific stimulatory effects of xeno-proteins in the fetal calf serum, we utilized X-vivo medium-containing 10% human AB serum for the experiments. Also, survival of basophils in the co-cultures was ensured by the addition of IL-3 at the time of co-culture of cells. As activated CD4^+ T cells produce IL-3, this will further ensure the survival of basophils [13, 14].
FcεRI cross-linking led to activation of basophils as analyzed by the expression of CD63 (Fig. 1A). We observed that neither IL-3-primed nor FcεRI-activated basophils could amplify IL-17A⁺ Th17 cells from memory CD4⁺ T cells (Fig. 1B and 1C). The percentage of IL-17A⁺/IFN-γ⁺ and IL-17A⁺/IFN-γ⁺ T cells remained unaltered in the presence of either IL-3-primed or FcεRI-activated basophils. In addition, basophils did not activate Th17 cells to secrete Th-17-derived cytokines. Only marginal changes in the secretion pattern of IL-17A and IL-17F were observed (Fig. 2A and 2B). Thus, our results imply that basophils alone are poor inducers of Th17 cell expansion and hence ruled out the possibility of the direct association of basophils in the development of Th17 responses. We also analyzed the proportion of IFNγ⁺CD4⁺ T cells and IL-4⁺CD4⁺ T cells among CD4⁺ T cells that were co-cultured with basophils. We observed an increased tendency of Th2 response and decreased Th1 response. However, results were statistically non-significant due to variations among the individual donors (data not shown).

3.2. Activation of basophils is not influenced by the donor-dependent variations in the level of plasma IgE and the expression of FcεRI on the basophils

We examined whether the concentration of IgE in the plasma of healthy donors and the expression of FcεRI on the basophils influence the activation of basophils. We found that donors had uniform level of plasma IgE (28.25±5.1 kU/L, n=7) (Fig. 3A) and the expression of FcεRI on the basophils (mean fluorescence intensity: 6367±1045, n=8) (Fig. 3B). These data thus ruled out the possibility of significant donor-dependent variations in basophil stimulation due to plasma IgE and FcεRI expression on the basophils.
3.3. *Human basophils are inapt at promoting antigen presenting cell-mediated Th17 expansion*

It is known that basophils secrete various inflammatory mediators and hence could influence the activation of other immune cells [2, 15]. Therefore, by mimicking closely the tissue microenvironment i.e. in the presence of activated antigen presenting cells (APCs, TLR2-activated monocytes in our experiments) that would provide all different signals required for CD4$^+$ T cell activation, we investigated the effect of activated basophils on APC-mediated Th17 responses.

In line with previous reports, we found that IL-17A$^+$ Th17 cells were significantly enhanced when memory CD4$^+$ T cells were co-cultured with monocytes, thus confirming the ability of activated APCs to expand Th17 cells [9, 10, 16]. Whereas, IL-3 treated basophils did not further amplify monocyte-mediated Th17 responses (Fig. 1B and 1C). The proportion of IL-17A$^+$/IFN-γ and IL-17A$^+$/IFN-γ$^+$ T cells was not significantly altered in the presence of IL-3-primed basophils with monocytes (Fig. 1B and 1C). Interestingly, similar results were also obtained in the presence of FcεRI-activated-basophils. These flow-cytometry results were further confirmed by the analysis of secretion of Th-17-derived cytokines. Monocyes significantly enhanced the production of IL-17A and IL-17F by ten to fifteen times (Fig. 2A and 2B). Although, there was a slight increase in the production of these cytokines in the presence of basophils, the values were not statistically significant (Fig. 2A and 2B).

We have recently demonstrated that basophils also lack the capacity to modulate another Th17 cytokine IL-22 from CD4$^+$ T cells [17]. Together, these results thus provide a pointer that circulating human basophils lack the capacity to enhance APC-mediated Th17 responses.
Human basophils produce minute amounts of IL-6 following activation

A slender increase in the production of monocyte-mediated Th17 cytokines in the presence of activated basophils suggest that basophils secrete cytokines or soluble factors that stimulate Th17 cytokines. However, human basophils produce undetectable levels of other Th17 propagating cytokines such as IL-23 and PGE$_2$[18]. On the other hand, basophils have been shown to secrete small amounts of IL-6 that could explain marginal increase in the level of Th17 cytokines. In fact, IL-3 and FcεRI-activated-basophils (0.2x10$^5$ cells) produced 57.4±52.8 pg (n=4) of IL-6. However, equivalent number of TLR2-activated monocytes produced 4829.5±1426.3 pg (n=4) of IL-6 (Fig. 4). As activated innate cells such as monocyte, macrophages and dendritic cells (DCs) secrete massive quantities of Th17-amplifying cytokines [19, 20], the basophil-secreted IL-6 effect would be nullified.

4. Discussion

Various receptor-ligand interactions between APCs and responder CD4$^+$ T cells, and cytokine milieu in the microenvironment determine the activation, polarization and expansion of CD4$^+$ T cells. Previous reports have shown that murine basophils at secondary lymphoid organs display the features of professional APCs and polarize Th2 responses [21-24]. However, these reports are contradictory due to the basophil depletion method employed [25, 26] and also DCs could mediate Th2 polarization independent of IL-4 via Notch ligand Jagged and OX-40 ligand [27, 28]. In contrast to murine basophils, several reports including ours demonstrated that circulating human basophils lack HLA-DR and co-stimulatory molecules CD80 and CD86 and were unable to function as APCs to promote T cell polarization [11, 29-32]. Although, stimulation of basophils with GM-CSF and IFN-γ was shown to induce HLA-DR
expression to a smaller extent in some donors, these cells did not express co-
stimulatory molecules [33]. Thus, the inability of human basophils to confer T-cell
receptor- and co-stimulatory molecule-mediated signals to CD4⁺ T cells might explain
the lack of Th17 responses when CD4⁺ T cells were co-cultured with basophils.

Recently Wakahara et al., demonstrated that human basophils enhance Th17
responses upon interaction with memory CD4⁺ T cells [34]. The reasons for the
discrepancies in the results are not clear. Differences in the type of serum used and
stimulatory conditions could be the possible reasons. Based on their results and the
presence of basophils in the inflamed mucosal tissues, Wakahara et al., also suggested
a role for basophils in the pathogenesis of inflammatory bowel disease [34]. However,
on the contrary, a recent report demonstrates that basophils limit the disease severity
in experimental murine colitis model [35]. Also, a recent randomized, double-blind
placebo-controlled clinical trial failed to demonstrate effectiveness of a human anti-
IL-17A monoclonal antibody Secukinumab for moderate to severe Crohn's disease
[36]. Therefore, the pathogenic role of Th17 cells in inflammatory bowel disease
remains controversial.

To conclude, our results indicate that basophils lack the ability to augment Th17 cell
responses either directly or via APCs. Therefore, we suggest that increased activation
and accumulation of Th17 cells in various inflammatory diseases such as asthma,
chronic allergy and lupus are under the control of innate cells such as monocytes,
macrophages or DCs but not basophils.
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Conflict of interests: The authors declare no competing financial interests.

Author contributions

M.S. performed the experiments, analyzed the data, drawn the figures and wrote the paper.

E.S-V. performed the experiments and analyzed the data.

P.P. performed the experiments and analyzed the data.

S.V.K. analyzed the data.

J.B. analyzed the data, drawn the figures and wrote the paper.

All authors reviewed the manuscript and approved the final version.
References


Figure Legends

**Fig 1.** Human basophils are mute-spectators in Th17 expansion. (A) The expression of CD63 on the surface of unstimulated and anti-IgE stimulated basophils. (B and C) Memory CD4\(^+\) T cells were cultured alone with IL-2 (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or combination of IL-3 and anti-IgE. (B) A representative flow-cytometry analysis of intracellular IL-17A and IFN-γ, and (C) percentage (mean±SD) of CD4\(^+\)CD45RO\(^+\) memory T cells positive for IL-17A\(^+\) (n=6) were shown. *, P<0.05; ns, not-significant as analyzed by one-way ANOVA test.
Fig 2. Human basophils do not promote Th17 cytokine secretion. (A-B) The amount of secretion (pg/ml) of (A) IL-17A and (B) IL-17F in the culture supernatants of memory CD4+ T cells that were cultured alone with IL-2 (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or combination of IL-3 and anti-IgE. The cytokines were measured by ELISA. The data represent mean±SD from six independent experiments using cells from different donors. *, P<0.05; ns, not-significant as analyzed by one-way ANOVA test.
Fig 3. FcεRI-mediated activation of basophils is not influenced by the level of plasma IgE and the expression of FcεRI on the basophils. (A) The level of IgE (kU/L) in the plasma of healthy donors (n=7). (B) The expression (MFI) of FcεRI on the basophils of healthy donors (n=8). The lines represent mean and SD values.
Fig 4. Human basophils produce minute amounts of IL-6. Basophils were stimulated with a combination IL-3 and anti-IgE for 24 hours. Monocytes were activated with peptidoglycan. IL-6 in the culture supernatants was quantified (pg/0.2x10^5 cells) by ELISA. The results are mean±SD from four donors. *, P<0.05 as analyzed by two-tailed Student’s -t- test.