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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 ($0.1x10^6$ cells/200 µl/well) in X-vivo-10% human AB serum and IL-2 (100 IU/$0.5x10^6$ cells, ImmunoTools, Friesoythe, Germany) either alone; or with basophils in the presence of IL-3 (100 ng/$1x10^6$ cells, Miltenyi Biotec) or IL-3 and monoclonal anti-human IgE (10 ng/$0.1x10^6$ cells, clone GE1, Sigma-Aldrich, Saint Quentin Fallavier, France); or with peptidoglycan-stimulated monocytes (5 µg/0.5 $x10^6$ cells, Invivogen, Toulouse, France); or with peptidoglycan-stimulated monocytes and IL-3-primed basophils; or with peptidoglycan-stimulated monocytes and IL-3-anti-IgE-

122 treated basophils. The activation of basophils was analyzed by the expression of
123 CD63 by using fluorescence-conjugated mAb (BD Bioscience). Monocytes and
124 basophils were stimulated in the co-culture and were not pre-activated. The ratio of
125 memory $CD4^+$ T cells and monocytes and/or basophils was maintained at 5:1. After
126 five days of culture, the cells were harvested and cell-free culture supernatants were
127 collected for the analysis of IL-17A and IL-17F. The cells were processed for staining
128 and flow cytometry as described below.
129
130 *2.4. Intracellular staining and flow cytometry*
131 The harvested cells were re-stimulated with phorbol 12-myristate 13-
132 acetate/ionomycin (Sigma-Aldrich) for 6 hours, with GolgiStop (BD Biosciences)
133 during last 3 hours. Surface staining was done with fluorescence-conjugated CD4
134 mAb (BD Biosciences) and fixable viability dye (eBioscience), in order to gate and
135 analyze viable $CD4^+$ T cells. Further, cells were fixed, permeabilized (Fix/Perm;
136 eBioscience), and incubated at 4°C with fluorochrome-conjugated mAbs to IFN-γ, IL-
137 4 (BD Biosciences) and IL-17A (eBioscience). The stained cells were subjected to
138 flow cytometry (BD LSR II). Ten thousand cells were acquired for each sample and
139 data were processed by using FACS DIVA software (BD Biosciences).
140
141 *2.5. Cytokines analysis*
142 Levels of IL-17A (DuoSet ELISA kits, R&D Systems), IL-17F and IL-6 (ELISA
143 Ready-SET-Go, eBioscience) in cell-free culture supernatants were quantified by
144 ELISA. The detection limits were 15 pg/mL for IL-17A, 16 pg/mL for IL-17F and 2
145 pg/mL for IL-6.
146

147 *2.6. Measurement of plasma IgE*

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

152

153 *2.7. Statistical analysis*

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

157

158 **3. Results**

159

160 *3.1. Activated human basophils lack the capacity to promote Th17 expansion*

161 We investigated the direct effect of basophils on the expansion of Th17 cells. As
162 stimulated basophils are known to secrete variety of cytokines and other chemical
163 mediators, we also examined if enhanced degranulation of basophils through FcεRI
164 cross-linking would augment Th17 responses. IL-3-primed basophils were co-
165 cultured with $CD4^+CD45RO^+$ memory T cells either in the presence or absence of
166 FcεRI cross-linking. To avoid nonspecific stimulatory effects of xeno-proteins in the
167 fetal calf serum, we utilized X-vivo medium-containing 10% human AB serum for the
168 experiments. Also, survival of basophils in the co-cultures was ensured by the
169 addition of IL-3 at the time of co-culture of cells. As activated $CD4^+$ T cells produce
170 IL-3, this will further ensure the survival of basophils [13, 14].

171

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.

197 *3.3. Human basophils are inapt at promoting antigen presenting cell-mediated Th17*
198 *expansion*
199 It is known that basophils secrete various inflammatory mediators and hence could
200 influence the activation of other immune cells [2, 15]. Therefore, by mimicking
201 closely the tissue microenvironment i.e. in the presence of activated antigen
202 presenting cells (APCs, TLR2-activated monocytes in our experiments) that would
203 provide all different signals required for $CD4^+$ T cell activation, we investigated the
204 effect of activated basophils on APC-mediated Th17 responses.
205
206 In line with previous reports, we found that IL-$17A^+$ Th17 cells were significantly
207 enhanced when memory $CD4^+$ T cells were co-cultured with monocytes, thus
208 confirming the ability of activated APCs to expand Th17 cells [9, 10, 16]. Whereas,
209 IL-3 treated basophils did not further amplify monocyte-mediated Th17 responses
210 (Fig. 1B and 1C). The proportion of IL-$17A^+$/IFN-γ^- and IL-$17A^+$/IFN-γ^+ T cells was
211 not significantly altered in the presence of IL-3-primed basophils with monocytes
212 (Fig. 1B and 1C). Interestingly, similar results were also obtained in the presence of
213 FcεRI-activated-basophils. These flow-cytometry results were further confirmed by
214 the analysis of secretion of Th-17-derived cytokines. Monocytes significantly
215 enhanced the production of IL-17A and IL-17F by ten to fifteen times (Fig. 2A and
216 2B). Although, there was a slight increase in the production of these cytokines in the
217 presence of basophils, the values were not statistically significant (Fig. 2A and 2B).
218 We have recently demonstrated that basophils also lack the capacity to modulate
219 another Th17 cytokine IL-22 from $CD4^+$ T cells [17]. Together, these results thus
220 provide a pointer that circulating human basophils lack the capacity to enhance APC-
221 mediated Th17 responses.

3.4. 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

247 expression to a smaller extent in some donors, these cells did not express co-
248 stimulatory molecules [33]. Thus, the inability of human basophils to confer T-cell
249 receptor- and co-stimulatory molecule-mediated signals to $CD4^+$ T cells might explain
250 the lack of Th17 responses when $CD4^+$ T cells were co-cultured with basophils.
251
252 Recently Wakahara et al., demonstrated that human basophils enhance Th17
253 responses upon interaction with memory $CD4^+$ T cells [34]. The reasons for the
254 discrepancies in the results are not clear. Differences in the type of serum used and
255 stimulatory conditions could be the possible reasons. Based on their results and the
256 presence of basophils in the inflamed mucosal tissues, Wakahara et al., also suggested
257 a role for basophils in the pathogenesis of inflammatory bowel disease [34]. However,
258 on the contrary, a recent report demonstrates that basophils limit the disease severity
259 in experimental murine colitis model [35]. Also, a recent randomized, double-blind
260 placebo-controlled clinical trial failed to demonstrate effectiveness of a human anti-
261 IL-17A monoclonal antibody Secukinumab for moderate to severe Crohn's disease
262 [36]. Therefore, the pathogenic role of Th17 cells in inflammatory bowel disease
263 remains controversial.
264
265 To conclude, our results indicate that basophils lack the ability to augment Th17 cell
266 responses either directly or via APCs. Therefore, we suggest that increased activation
267 and accumulation of Th17 cells in various inflammatory diseases such as asthma,
268 chronic allergy and lupus are under the control of innate cells such as monocytes,
269 macrophages or DCs but not basophils.
270

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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.

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Figure Legends

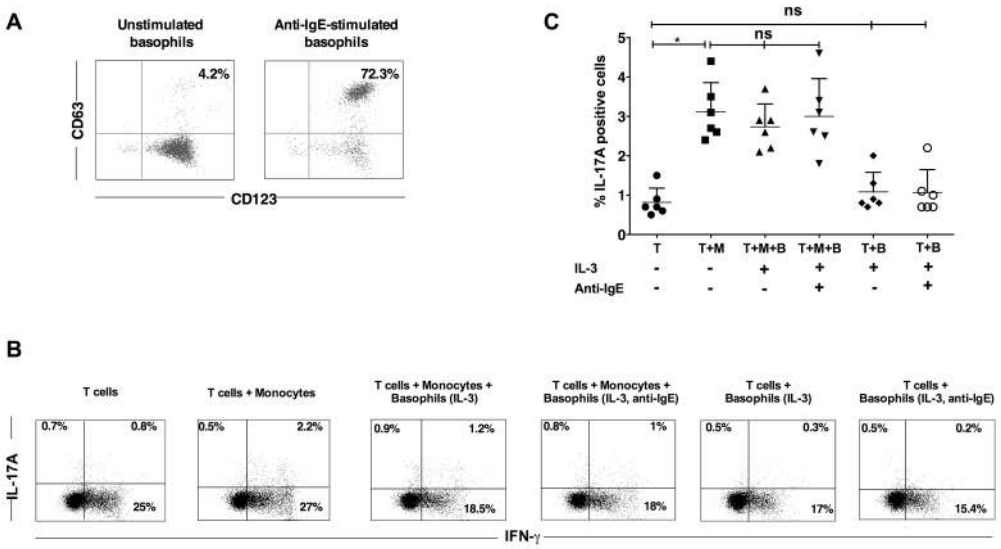
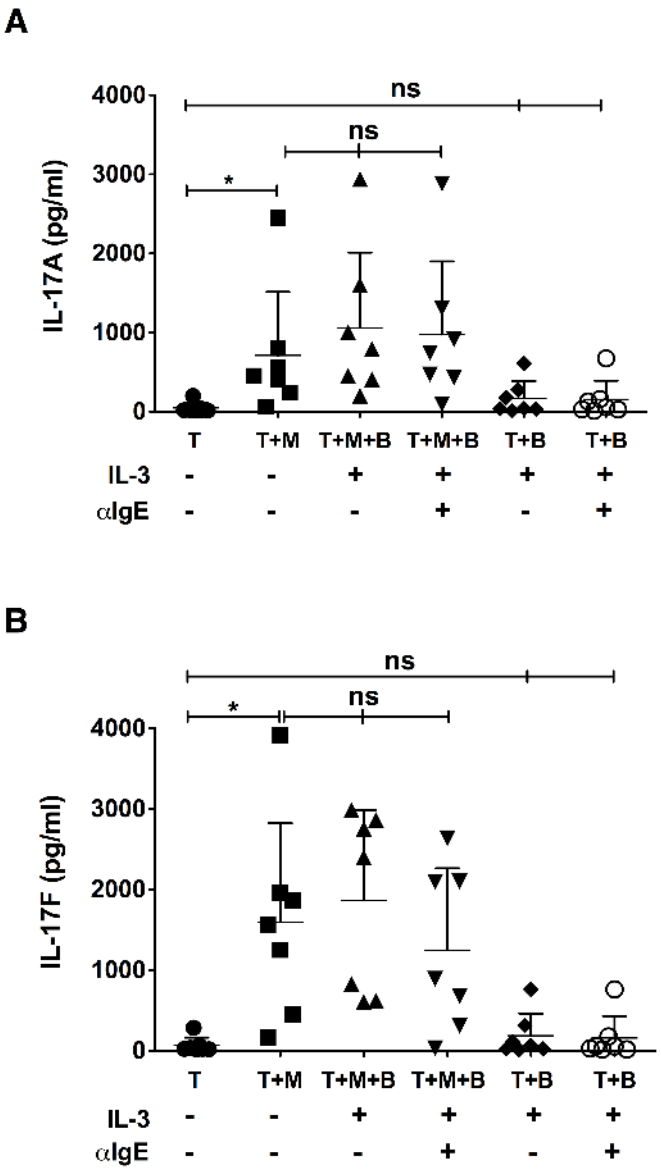


Fig 1. Human basophils are mute-spectators in Th17 expansion. (A) The expression of CD63 on the surface of unstimulated and anti-IgE stimulated basophils. (B and C) Memory $CD4^+$ T cells were cultured alone with IL-2 (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated 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 $IL\text{-}17A^+$ (n=6) were shown. *, $P<0.05$; ns, not-significant as analyzed by one-way ANOVA test.



425

426 **Fig 2.** Human basophils do not promote Th17 cytokine secretion. (A-B) The amount
427 of secretion (pg/ml) of (A) IL-17A and (B) IL-17F in the culture supernatants of
428 memory $CD4^+$ T cells that were cultured alone with IL-2 (T) or with basophils (T+B)
429 or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated
430 monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or
431 combination of IL-3 and anti-IgE. The cytokines were measured by ELISA. The data
432 represent mean±SD from six independent experiments using cells from different
433 donors. *, $P<0.05$; ns, not-significant as analyzed by one-way ANOVA test.

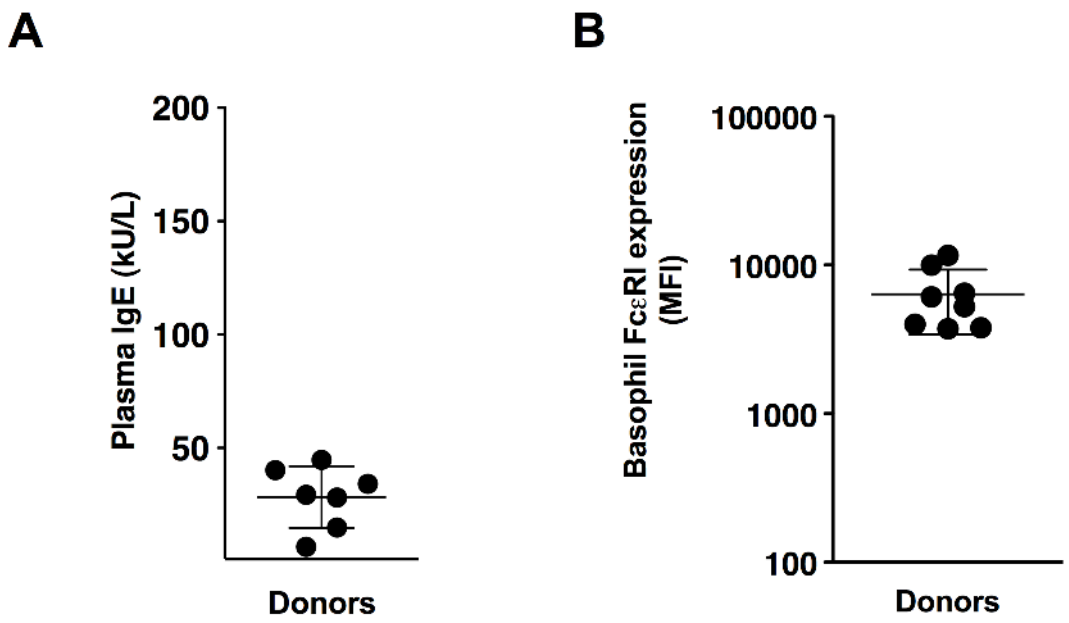


Fig 3. FcεRI-mediated activation of basophils is not influenced by the level of plasma IgE and the expression of FcεRI on the basophils. (A) The level of IgE (kU/L) in the plasma of healthy donors (n=7). (B) The expression (MFI) of FcεRI on the basophils of healthy donors (n=8). The lines represent mean and SD values.

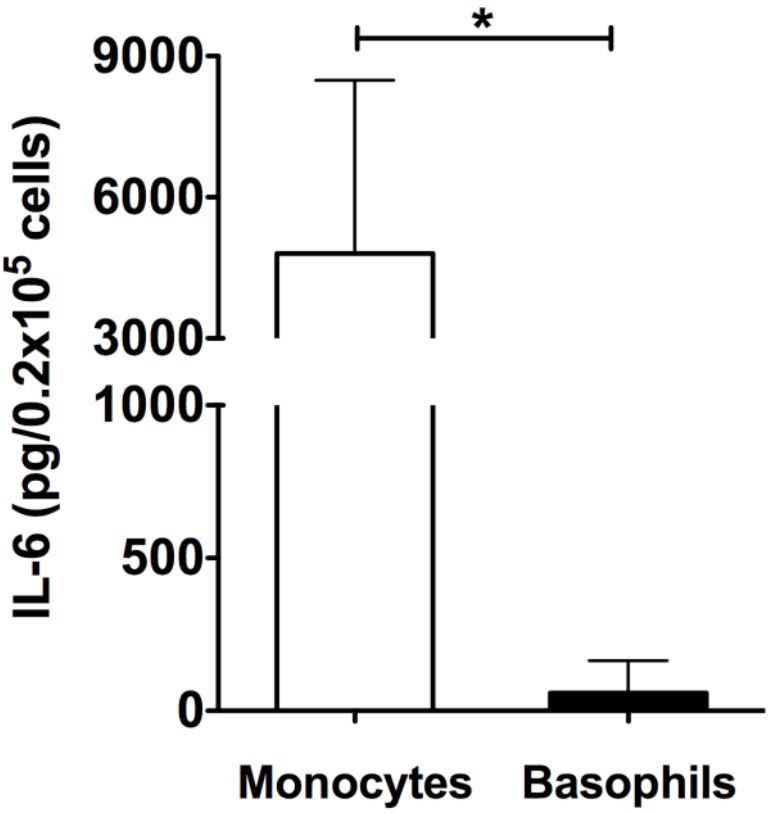


Fig 4. Human basophils produce minute amounts of IL-6. Basophils were stimulated with a combination IL-3 and anti-IgE for 24 hours. Monocytes were activated with peptidoglycan. IL-6 in the culture supernatants was quantified (pg/0.2x10^5 cells) by ELISA. The results are mean±SD from four donors. *, $P<0.05$ as analyzed by two-tailed Student's -t- test.