

IgE and IL-33–mediated triggering of human basophils inhibits TLR4–induced monocyte activation

Felice Rivellesse^{1,2}, Jolien Suurmond¹, Amato de Paulis², Gianni Marone²,
Tom W.J. Huizinga¹ and René E.M. Toes¹

¹ Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

² Department of Translational Medical Sciences and Center for Basic and Clinical Immunology Research (CISI), University of Naples Federico II, Naples, Italy

Basophils are circulating granulocytes, best known as effector cells in allergic reactions. Recent studies in mice suggest that they might also participate in the suppression of chronic inflammation. The aim of this study was to assess the ability of purified human basophils to modulate monocyte responses upon IL-33 and IgE triggering. Activation of human basophils with IL-33 induced the production of IL-4 and the release of histamine, and enhanced their IgE-mediated activation. In addition, basophils triggered with IL-33 and anti-IgE significantly suppressed the LPS-induced production of the proinflammatory cytokine TNF- α and the upregulation of the costimulatory molecule CD80 by monocytes. These effects were mainly explained by the release of histamine, as they could be inhibited by the histamine receptor 2 antagonist ranitidine, with a smaller contribution of IL-4. In contrast, basophil-derived IL-4 and histamine had opposing effects on the expression of the inhibitory Fc γ receptor IIb and the production of IL-10 by monocytes. Our data show that basophils can influence monocyte activation and suggest a previously unrecognized role for human basophils in the modulation of monocyte-mediated immune responses, through the balanced secretion of histamine and IL-4.

Keywords: Basophils • Cellular activation • Histamine • Immune regulation • Monocytes



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Introduction

Basophils are circulating granulocytes, representing less than 1% of peripheral blood leukocytes. They are classically known for their involvement as effector cells in allergic reactions [1–3]. Because of their paucity and the difficulty in isolating them, there is still relatively little information available on their functions and potential immunomodulatory effects [4]. Studies in mice suggest a possible role for basophils in the induction of adaptive immunity in the context of Th2 responses, where they have been shown to be the major producers of IL-4 [5], although their function as

antigen presenting cells and their dispensability in the induction of Th2 responses are still under debate [6, 7]. In addition, recent experimental data showed how these rare leukocytes might also exert anti-inflammatory effects in the context of autoimmune and allergic inflammation. In a mouse model of arthritis, basophils have been implicated in the immunosuppressive response mounted upon injection of intravenous immunoglobulin (IVIG) [8]. Briefly, the authors proposed the following scenario to elucidate IVIG-mediated immune suppression: myeloid-derived cells, in response to the sialylated fraction of IVIG, release IL-33, which, in turn, activates basophils to produce IL-4. Finally, basophil-derived IL-4 induces the upregulation of the inhibitory Fc γ Receptor (Fc γ RIIb) on inflammatory macrophages, leading to the suppression of antibody-dependent inflammation. Thus, IL-33

Correspondence: Dr. Felice Rivellesse
e-mail: rivelles@gmail.com

was shown to be involved in the suppression of arthritis through its action on basophils. Even though the role of the sialylated fraction of IVIG or basophils in the immunomodulatory effects of IVIG therapy has recently been challenged [9], another study using an experimental model of IgE-mediated chronic allergic inflammation, showed that mouse basophils, through IL-4, drive the differentiation of monocytes into anti-inflammatory macrophages, thereby regulating chronic allergic skin inflammation *in vivo* [10].

Human basophils, which can significantly differ from their mouse counterparts [1, 3, 11], have been known for a long time to produce IL-4 [12], and have been recently shown to induce Th2 skewing of naïve helper T cells *in vitro* [13, 14]. On the other hand, the function as antigen presenting cells, still debated in mice, has been ruled-out for human basophils by several groups [15–17]. In addition to well-known basophil triggers like IgE or IL-3, IL-33 also activates human basophils, inducing the production of several cytokines, including IL-4 [18–20]. IL-33 is a member of the IL-1 cytokine family that plays an important role in the induction and effector phases of type 2 immune responses [21], suspected to be involved in the pathogenesis of several allergic and autoimmune diseases [22], where it can have both protective and deleterious effects in different settings [23]. For example, in the aforementioned arthritis model [8], IL-33 production was critically involved in the suppression of arthritis upon IVIG administration, specifically through its effects on basophils. Since IL-33, as an alarm in [22], is released in any inflamed tissue and reaches high levels locally but also in the sera of patients with several allergic and autoimmune conditions, such basophil-driven anti-inflammatory network could be active, independently from exogenous stimuli like IVIG, in any condition in which IL-33 is released upon cell necrosis.

Therefore, it would be of invaluable interest to verify whether IL-33 is able to induce anti-inflammatory responses through the activation of human basophils.

To this end, we investigated the interaction of IL-33-stimulated human basophils with monocytes. We found that purified human basophils, triggered via FcεRI ligation and IL-33, suppressed LPS-induced monocyte activation. Unexpectedly, only little of this effect could be attributed to IL-4. Instead, basophil-derived histamine was found to be the main factor involved. Overall, our data show that human basophils are able to influence monocyte activation via the balanced secretion of IL-4 and histamine, and suggest that they play an important role in the regulation of the immune responses.

Results

IL-33 activates human basophils

To determine basophil activation upon IL-33 triggering, we performed *in vitro* stimulation of purified human basophils. Basophils were identified by the expression of CD123 (IL-3 receptor), FcεRI (Fig. 1A), and CD203c (Fig. 1B). To exclude contamination with plasmacytoid dendritic cells (pDCs) (CD123⁺, BDCA-2⁺), also known to express low levels of FcεRI [24], we verified

that the isolated fractions, while positive for the basophil marker CD203c, did not contain BDCA2⁺ cell (Fig. 1B).

IL-4 levels were measured after 24 h of stimulation with different doses of IL-33 or with two classical basophil stimuli, i.e. IgE-mediated cross-linking of the FcεRI and IL-3 (Fig. 1C). In addition, IL-4 production upon combined stimulation of basophils with IL-33 and anti-IgE or IL-3 was evaluated (Fig. 1D). These data show that IL-33 alone induced low but detectable amounts of IL-4, which is in line with previous literature [20]. Additionally, as shown in Figure 1D, IL-33, at 100 ng/mL, significantly increased IL-4 production upon IgE or IL-3 triggering.

To further study basophil activation, we measured the membrane expression of CD63, as a marker of degranulation [25]. IL-33, as expected, did not induce degranulation when used alone, but significantly enhanced IgE-mediated degranulation of basophils (Fig. 1E and 1F). In addition, histamine release was evaluated (Fig. 1G). In line with CD63 expression, histamine was released by basophils upon IgE cross-linking. In addition to a spontaneous secretion of histamine in unstimulated basophils, we observed a significant induction of histamine release upon IL-33 triggering. Such histamine release was independent from CD63, which is possibly in line with previous reports of spontaneous and degranulation-independent release of histamine by human basophils [26, 27].

These data indicate that IL-33 can activate human basophils, inducing the production of IL-4 and the release of histamine. Furthermore, IL-33 can enhance IgE-mediated basophil degranulation, histamine release and cytokine production.

Activated human basophils modulate monocyte activation

To evaluate whether human basophils can modulate monocyte activation, monocytes were incubated with supernatants of basophils or control media (containing the stimuli originally used to trigger basophils) and activated with LPS. As shown in Figure 2A, TNF-α production was significantly suppressed when monocytes were conditioned with basophils supernatants. Unstimulated basophils partially inhibited TNF-α production by monocytes, but a significantly stronger inhibition of TNF-α production by monocytes was induced by supernatants of basophils triggered with anti-IgE together with IL-33. IL-10 production by monocytes was not influenced, indicating that monocytes were still equally able to produce cytokines, and that they were still viable (Fig. 2B). Indeed, the percentages of live monocytes were similar in all conditions, confirming that the observed reduction of TNF-α was not due to impaired survival of monocytes (data not shown), but rather to a suppression of TNF-α production upon exposure to basophil supernatants. To confirm that TNF-α and IL-10 were derived from monocytes, the cytokine levels were also measured in basophil supernatants, and were below detection limit (data not shown).

Together, these results indicate that basophils significantly suppress LPS-induced TNF-α, without affecting the production of IL-10, thereby modulating the proinflammatory responses of monocytes.

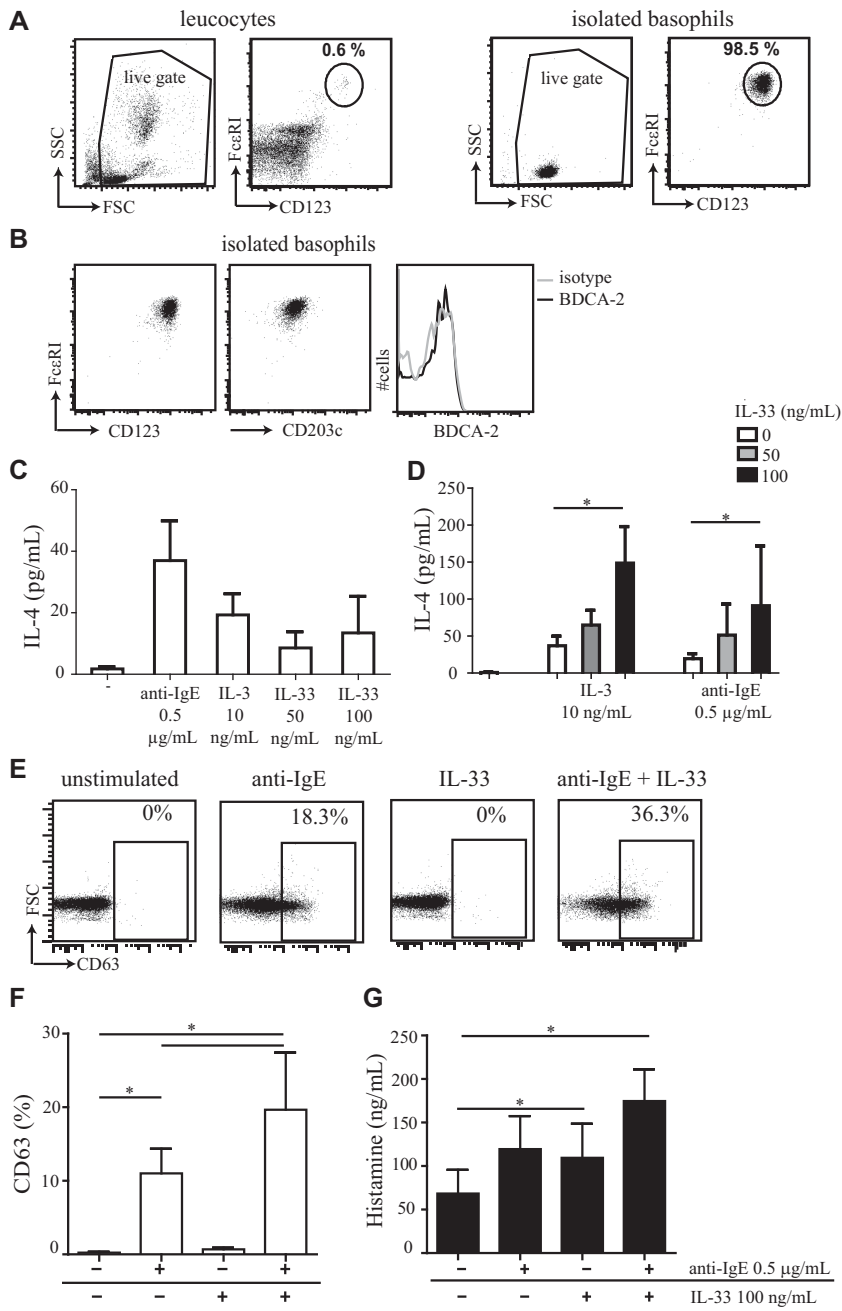


Figure 1. Activation of human basophils. (A) Basophils were isolated from peripheral blood leucocytes of healthy donors by immunomagnetic negative selection. Cells were analyzed by FACS to verify purity. A representative example is shown, with total leucocytes (left), and isolated basophils (right). After gating out debris using FSC/SSC, basophils were identified as CD123⁺FcεRI⁺⁺ cells. (B) Isolated basophils were analyzed for the expression of CD203c and BDCA2. Total cells from the live gate are shown. (C) Isolated basophils were stimulated with the indicated concentrations of anti-IgE, IL-3, or IL-33 for 24 h and IL-4 production was measured by ELISA in the supernatants after harvesting. (D) IL-4 production after 24 h of incubation of isolated basophils with IL-3 or anti-IgE + indicated concentrations of IL-33. (E, F) Flow cytometric expression of CD63 by isolated basophils after 24 h of stimulation with anti-IgE (0.5 μg/mL), IL-33 (100 ng/mL), or both. A representative example is shown in (E) and the summary of all donors in (F). (G) Histamine release, measured by ELISA, after 24 h of incubation with the indicated stimuli. (A, B, and E) Data shown are representative of five independent experiments performed. (C, D, F, and G) Data are shown as mean + SEM and are pooled from (C, D) three independent experiments ($n = 3$ basophil donors) or (F, G) five independent experiments ($n = 5$ donors). * $p < 0.05$ determined by ANOVA with Bonferroni's post-test.

To further study monocytes activation, we analyzed surface markers by flow cytometry. While the expression of several markers, including activating and inhibitory Fcγ receptors (CD14, HLA-DR, CD16, CD64, CD32a, CD32b), were not significantly influenced (data not shown), basophils supernatants reduced the percentage of monocytes expressing the costimulatory molecule CD80 upon LPS stimulation (Fig. 2C and D). Although this effect was statistically significant only for the combined activation of basophils with IgE and IL-33, these data further confirm the ability of basophils to modulate monocyte function, by influencing their expression of costimulatory molecules.

Together, these data show that basophil supernatants significantly modulate monocyte proinflammatory activation, by inhibiting the LPS-induced production of TNF-α and upregulation of CD80.

Basophil-derived IL-4 and histamine modulate cytokine production by monocytes

We next sought to identify the mechanism by which basophils affected monocyte cytokine production and speculated that

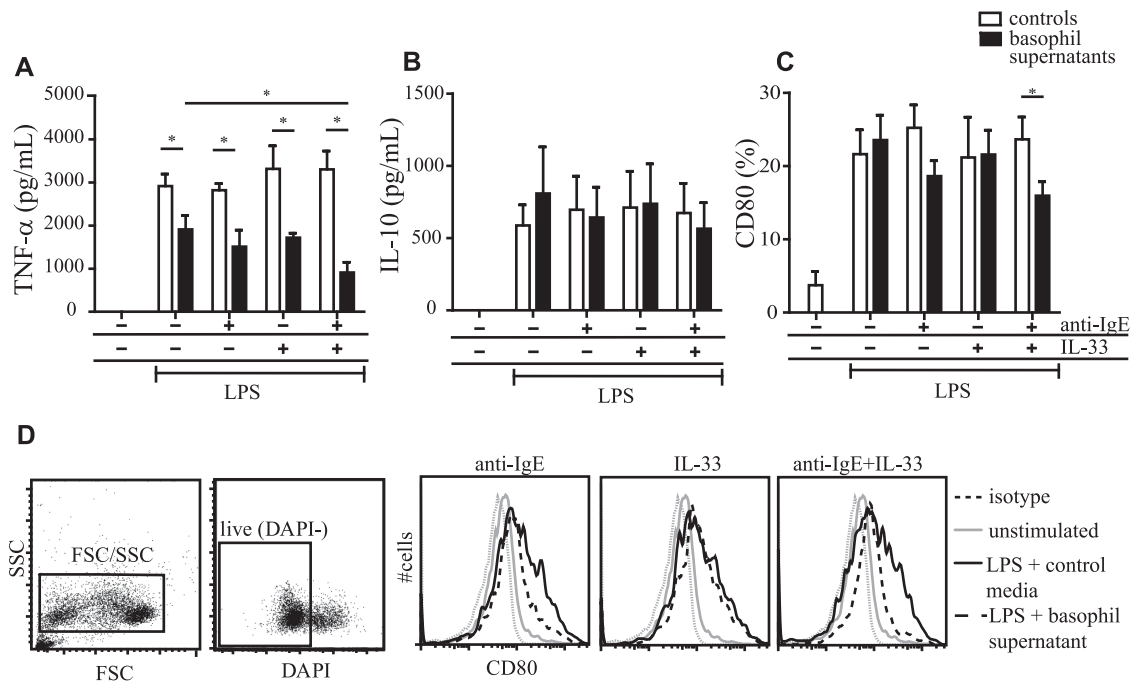


Figure 2. Effects of basophil supernatants on LPS-induced activation of monocytes. (A–C) Monocytes were isolated by positive selection using CD14 immunomagnetic beads, incubated with the supernatants of basophils (black bars) or with control media (white bars) and activated with LPS (5 ng/mL). The stimuli used to activate basophils and also present in the control media are indicated on the x axis. (A) TNF- α and (B) IL-10 were measured by ELISA in the supernatants of LPS-stimulated monocytes after overnight incubation (18 h). (C) Monocyte expression of CD80 was evaluated by (D) flow cytometry after FSC/SSC gating and exclusion of dead cells with DAPI. (A–C) Data are shown as mean + SEM from five independent experiments ($n = 5$ monocyte donors). (D) Data shown are representative of five independent experiments performed. * $p < 0.05$; for comparison of each basophil supernatant with its own control, Student t-test was performed. For multiple comparisons between basophil supernatants ANOVA with Bonferroni's post-test was performed.

IL-4 might play a role in this process. Indeed, IL-4 did inhibit the LPS-elicited TNF- α production by monocytes to a similar extent as activated basophil supernatants (Fig. 3A). Nonetheless, IL-4 blocking in the supernatants of anti-IgE and IL-33 activated basophils only slightly reverted the inhibition of TNF- α (Fig. 3B), indicating that this effect was mainly mediated by other factors. Among other mediators produced by basophils, histamine has been shown to modulate monocytes responses to LPS [28]. Accordingly, exogenously added histamine could suppress LPS-induced TNF- α production and, when used together with IL-4, the suppression of TNF- α was almost complete, as shown in Figure 3A. Additional experiments (not shown) confirmed that this effect of histamine is specifically mediated by the interaction with histamine 2 receptor (H2R) as it could be inhibited by H2R antagonist ranitidine and not by H1R antagonist cetirizine or H3–4R antagonist thioperamide (at doses from 10^{-6} to 10^{-4} M). In line with these observations, preincubation of monocytes with ranitidine significantly prevented the inhibitory effects of activated basophil supernatants, while concomitant blocking of IL-4 in the supernatants showed almost no additional effect (Fig. 3B). These data indicate that basophil-derived histamine is predominantly responsible for suppressing LPS-induced TNF- α production by monocytes.

We next analyzed the influence of exogenous histamine and IL-4 on LPS-induced IL-10 production and, intriguingly, observed

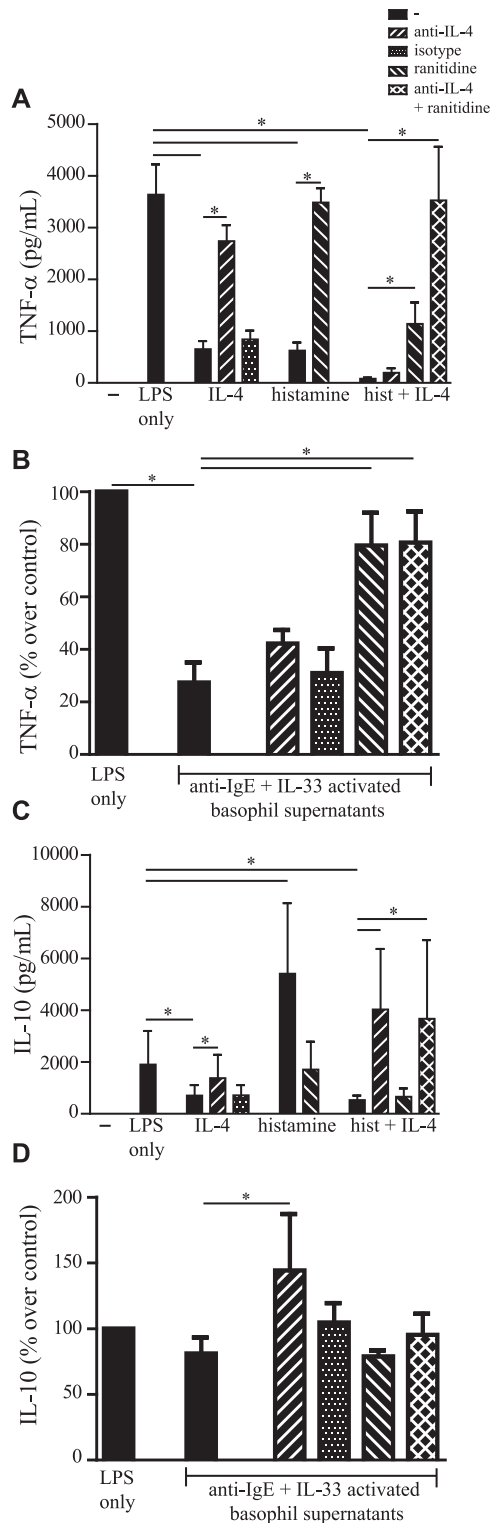
opposite effects, as shown in Figure 3C. Consequently, only when IL-4 was blocked in basophil supernatants, the IL-10 inducing effects of histamine could be revealed. The enhanced IL-10 production observed after blocking IL-4 could be reverted by the addition of ranitidine, showing it was mediated by histamine via H2R (Fig. 3D). Together, these data indicate that IL-4 and histamine in basophils supernatants have opposite effects with respect to IL-10 production.

In order to further confirm that basophil derived histamine and IL-4, at the levels found in the supernatants, are relevant mediators involved in the inhibitory effect on TNF- α production, we performed a titration experiment (Supporting Information Fig. 1). Both histamine and IL-4 suppressed LPS-induced TNF- α at very low concentrations (as low as 1×10^{-9} M of histamine and 1 pg/mL of IL-4). Moreover, additional suppression of TNF- α could be obtained by combining histamine and IL-4 at these low concentrations (data not shown). This confirms that low levels of histamine and IL-4 can modulate monocyte responses to LPS triggering.

Altogether, these results show that basophil-derived IL-4 and histamine can differently modulate the response of human monocytes to TLR4-triggering, where opposing effects are observed regarding IL-10-production. In contrast, both molecules, but mainly histamine, contribute to the suppression of LPS-induced TNF- α production by monocytes.

IL-4 and histamine suppress CD80 but have opposite effects on the expression of FcγRIIb by monocytes

Having established that IL-4 and histamine can have different effects on the modulation of cytokine production by monocytes,



we next studied their influence on the expression of monocyte surface markers by flow cytometry. As expected, IL-4 induced the expression of the inhibitory FcγRIIb in monocytes (Fig. 4A). Intriguingly, when histamine was added to IL-4 this effect was lost. Therefore, we speculated that histamine could be responsible for inhibiting the upregulation of FcγRIIb by basophil supernatants. Accordingly, when histamine effects were blocked with ranitidine, basophil supernatants did induce an upregulation of FcγRIIb on monocytes (Fig. 4B). The upregulation of FcγRIIb was lost when IL-4 present in the basophil supernatant was blocked (data not shown), confirming our hypothesis that basophil-derived histamine masked the FcγRIIb induction by basophil-derived IL-4.

In contrast, exogenously added histamine and IL-4 synergized in the inhibition of the LPS-induced CD80-expression on monocytes (Fig. 4C). Accordingly, inhibition of basophil supernatants with ranitidine and anti-IL-4 significantly reverted the inhibitory effect of basophil supernatants on CD80 expression, although the contribution of basophil-derived histamine appeared to be prevalent (Fig. 4D).

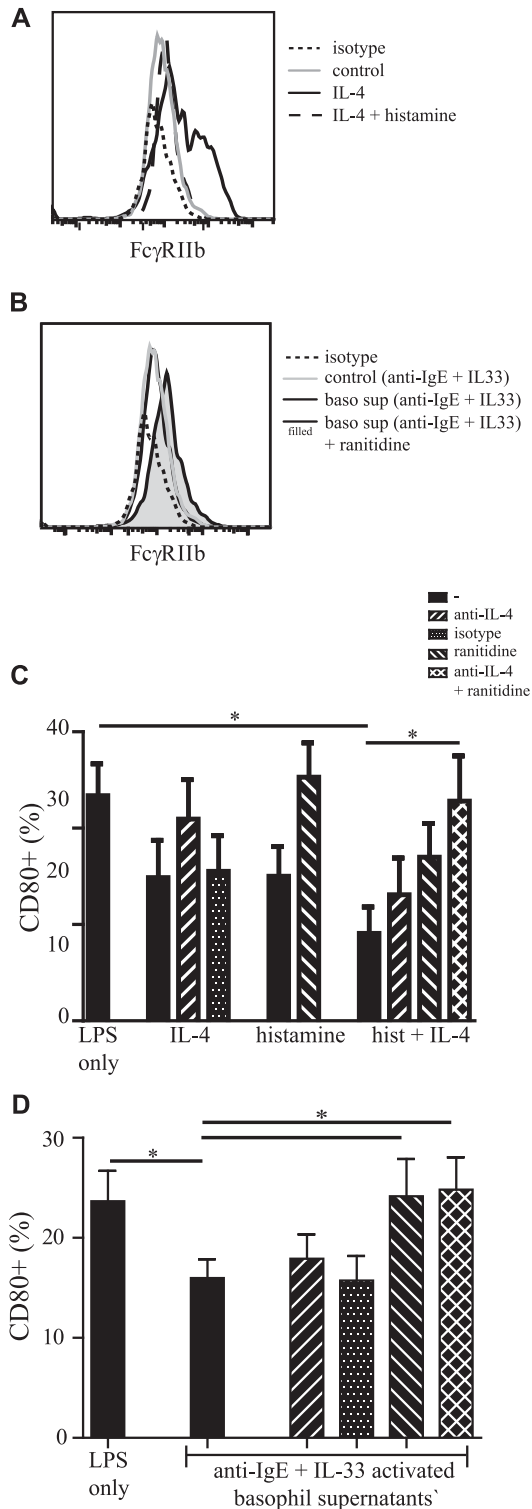
Taken together, these results indicate that basophil derived IL-4 and histamine can have combined effects, as shown for the inhibition of the costimulatory molecule CD80, but also contrasting effects. Remarkably, histamine was shown to modulate the ability of basophil-derived IL-4 to upregulate the inhibitory FcγRIIb on monocytes.

Basophils directly inhibit monocyte activation in coculture

We showed how basophil supernatants inhibit monocyte activation, with a contribution of IL-4 and histamine, two of the main mediators produced by basophils. Nonetheless, the release of mediators by basophils is known to be differentially regulated over time, with an immediate release of high amounts of granule-stored mediators (including histamine) upon IgE cross-linking, followed by an active production of cytokines and other mediators in the later phase of basophil activation. Therefore, to better reproduce this physiological time course, and to verify possible additional

Figure 3. Basophil-derived mediators and the modulation of LPS-induced cytokine production by monocytes. (A) TNF-α in the supernatants of monocytes conditioned with IL-4 and histamine. Briefly, isolated monocytes were stimulated with LPS (5 ng/mL) with or without IL-4 (10 ng/mL) and/or histamine (10⁻⁵ M). Blocking was performed by preincubation with anti-IL-4 antibody and matching isotype control or with histamine receptor 2 antagonist ranitidine. (B) Blocking experiments, as described above, were also performed using anti-IgE + IL-33 activated basophil supernatants. TNF-α produced by basophil-conditioned monocytes was compared with the amount produced by monocytes incubated with control media, and is shown as percentage over control. Monocytes stimulated with LPS alone correspond to 100% of TNF-α production. Direct effects of anti-IL-4 and ranitidine on monocytes were excluded by adding them to the respective control media. (C, D) IL-10 concentration was measured in the supernatants, as described in (A, B). (A–D) Data are shown as mean + SEM pooled from five independent experiments (n = 5 monocyte donors). * *p* < 0.05 determined by ANOVA with Bonferroni's post-test.

effects dependent on cell-to-cell contact, we performed experiments in which basophils and monocytes were cocultured. LPS-induced TNF- α production by monocytes was inhibited in the presence of basophils (Fig. 5A). Further activation of basophils with anti-IgE and IL-33 significantly enhanced the inhibition of TNF- α



production by monocytes. This effect could be reverted by preincubation of monocytes with ranitidine and only partially by blocking IL-4, again pointing toward a prevalent action of basophil-derived histamine. Similar to what was observed for supernatants, LPS-induced IL-10 was significantly upregulated only when IL-4 was blocked (Fig. 5B). Control conditions with basophils alone did not produce TNF- α or IL-10, suggesting that the cytokines in the coculture were derived from monocytes (data not shown). Cell viability, as measured by DAPI staining, was comparable in all conditions (data not shown).

Furthermore, activated basophils in coculture with monocytes significantly inhibited LPS-induced CD80 expression with a contribution of both histamine and IL-4 (Fig. 5C and F). On the other hand, the expression of Fc γ RIIb was not influenced by the presence and activation of basophils, unless histamine effects were blocked by ranitidine (Fig. 5D).

These data are similar to the results obtained with supernatants, as we could not observe, for the analyzed parameters, additional or different effects possibly due to cell-to-cell contact.

However, these results indicate that human basophils can influence monocyte responses when cells are being exposed to the proinflammatory stimulus at the same time, further confirming the immunomodulatory properties of basophil-derived soluble mediators.

Discussion

Here, we show that human basophils, in addition to their known effector functions [29], can participate in immune regulation, by suppressing monocyte-mediated proinflammatory responses. In particular, purified human basophils, activated via Fc ϵ RI ligation and IL-33, inhibited the LPS-induced production of the prototypical proinflammatory cytokine TNF- α and the expression of the costimulatory molecule CD80 by monocytes. In other words, monocyte activation via a strong proinflammatory stimulus such as LPS was dampened when they were exposed to basophil supernatants or cocultured with basophils. Similar results were obtained using basophil supernatants or coculturing basophils and monocytes, suggesting that these effects do not require cell-to-cell

Figure 4. Fc γ RIIb and CD80 modulation by basophil supernatants. After 18 h of incubation with LPS (5 ng/mL) monocytes were analyzed by flow cytometry for the expression of surface markers. (A) Fc γ RIIb expressed by monocytes stimulated with rIL-4 (10 ng/mL), histamine (10⁻⁵ M), or both. (B) Fc γ RIIb expressed by monocytes conditioned with anti-IgE + IL-33 triggered basophil supernatants and blocked with H2R antagonist ranitidine. Histograms from a single experiment representative of five independent experiments are shown. (C–D) CD80 expression by monocytes incubated with (C) rIL-4 (10 ng/mL), and histamine (10⁻⁵ M) and with (D) anti-IgE + IL-33 triggered basophil supernatants. Blocking was performed by preincubation with anti-IL-4 antibody and matching isotype control or with histamine receptor 2 antagonist ranitidine. DAPI⁺ cells were gated out to exclude dead cells from the analysis as shown in Figure 2D. (C, D) Data are shown as mean + SEM of percentages of CD80⁺ cells and are pooled from five independent experiments (n = 5 monocyte donors) * p < 0.05 determined by ANOVA with Bonferroni's post-test.

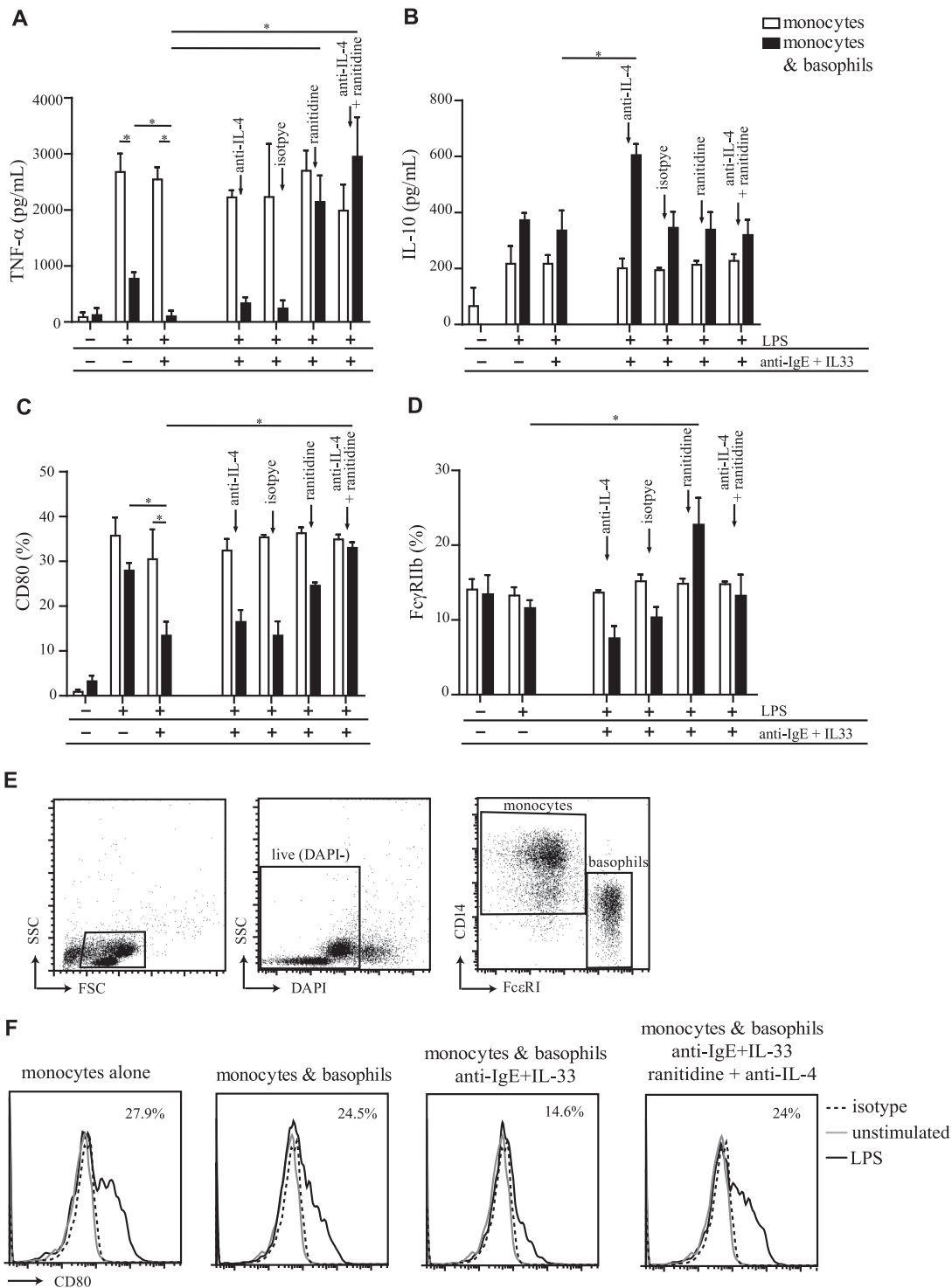


Figure 5. Monocytes and basophils coculture. Isolated autologous basophils and monocytes were cultured alone or together, in the presence or absence of LPS (5 ng/mL) and anti-IgE (0.5 μ g/mL) + IL-33 (100 ng/mL) for 18 h. The concentration of (A) TNF- α and (B) IL-10 in the supernatants of monocytes cocultured with basophils (black bars) or monocytes alone (white bars), followed by the activation with LPS and blocking by preincubation of cells with anti-IL4 antibody, matching isotype control, and ranitidine, was measured by ELISA. The cytokines in conditions with basophils alone were below detection limit and are not shown. (C, D) The percentages of (C) CD80 and (D) Fc γ RIIb in monocytes and monocytes cocultured with basophils are shown, as described in (A and B). (E) Example of gating strategy. (F) Histograms of CD80 expression by monocytes. Data from one donor representative of three independent experiments. (A–D) Data are shown as mean $\pm$ SEM ($n = 3$ donors) and are pooled from three independent experiments. * $p < 0.05$; for comparison between two groups, Student t test was performed. For multiple comparison of coculture conditions, ANOVA was used with Bonferroni's post-test.

contact and are mainly dependent on soluble mediators. Indeed, blocking experiments showed that basophil-derived IL-4 and, most of all, histamine, were responsible for the modulation of monocyte activity.

These data are in line with recent findings in a mouse model of allergic inflammation, in which basophils, via IL-4, were shown to induce the differentiation of inflammatory monocytes into anti-inflammatory macrophages [10]. Likewise, in a mouse model of arthritis, basophils, by producing IL-4 in response to IL-33, were essential to mediate the immunosuppressive effects of IVIG [8]. We now provide evidence, for the first time, that basophils can mediate similar immunomodulatory effects in human settings.

Because of the aforementioned studies in mice [8, 10], we expected basophil-derived IL-4 to be the main factor responsible for the anti-inflammatory effects of human basophils. Instead, our results show that histamine, specifically via H2R, is also involved in the modulation of the monocyte response. The interaction of basophil-derived histamine and IL-4 proved to be more complex regarding the modulation of the expression of the inhibitory FcγRIIb and the production of IL-10, which were differently regulated by histamine and IL-4.

In particular, histamine dampened the upregulation of the FcγRIIb by basophil-derived IL-4. A very recent publication offers an intriguing *in vivo* model to reproduce these findings in humans [30]. Studying patients with primary immune deficiency, it was found that IVIG therapy suppresses dendritic cell function via IL-33-mediated induction of IL-4 and IL-13. Similar to our *in vitro* findings with monocytes, the authors did not observe an upregulation of the inhibitory FcγRIIb on dendritic cells, but on the contrary, an inhibition of the activating FcγRIIa. Our data, although not directly related to IVIG treatment, provides additional immunological insights and an alternative mechanism, suggesting that basophil-derived histamine is able to inhibit the IL-4 dependent upregulation of FcγRIIb, without influencing, in the short term, the expression of FcγRIIa.

Overall, the involvement of histamine in the immunomodulatory ability of basophils has been apparently underestimated by previous studies, both in mice and humans, and most definitely warrants more attention.

To further show the relevance of histamine-mediated immune modulation, we provide evidence that very low concentrations of histamine still influence LPS-induced cytokine production by monocytes (Supporting Information Fig. 1). Accordingly, very low amounts of IL-4 (as low as 1 pg/mL) could further modulate the histamine-induced responses (and vice versa), suggesting a balance between these two mediators in tuning monocyte responses.

The immunomodulatory properties of histamine have been investigated over the past years, showing both pro- and anti-inflammatory effects [31]. As an example, in line with our observations, histamine via H2R, has been shown to inhibit the cytokine response to microbial products of monocytes [28, 32] and dendritic cells [33]. Differentiation of monocytes into macrophages, on the other hand, induces the preferential expression of H1R over H2R [34], leading to increased production of

IL-8 upon exposure to histamine [35]. Therefore, the response to histamine can greatly vary among different cells, depending on expression profile of histamine receptors and among different individuals, possibly depending on genetic variation of histamine receptors [36].

Our data further underline the relevance of histamine in immune regulation, showing that, among basophil-derived mediators, histamine represents a prominent factor responsible for the modulation of monocyte activation.

As for the intracellular mechanisms involved in the influence of basophil-derived histamine on TLR4 signaling, cAMP, induced upon histamine triggering of H2R [31], is known to inhibit NF-κB and therefore TNF-α production [37], while enhancing IL-10 [38]. IL-4, on the other hand, via STAT-6, is able to inhibit both NF-κB activation [39] and IL-10 production [40]. Overall, these intracellular pathways explain the effects on TLR4 signaling we observed when monocytes were exposed simultaneously to basophil-derived histamine and IL-4. While extensively studied independently, very little is known about the combined effects of these mediators on immune cells. In particular, our findings suggest for the first time histamine as an important player, which contribution has been so far underestimated, in the cross-talk between basophils and monocytes.

Although difficult to directly show in a human setting, our data argue for a basophil- and histamine-dependent contribution to immunomodulation *in vivo*. Plasma levels of histamine in healthy subjects are known to be less than 1×10^{-8} M [41]. We found that the suppressive effects of histamine on LPS-induced TNF-α production by monocytes were present up to a histamine concentration of 10^{-9} M. Although other cells are able to secrete histamine, in the peripheral blood the amounts produced by basophils are estimated to be 100× to 1000× higher than the amounts produced by other leukocytes [42], suggesting that, even with their relatively low percentage, most of the circulating histamine is likely to originate from basophils.

Basophils can also infiltrate tissues, as shown in several inflammatory conditions [43, 44]. Upon activation by local stimuli (exogenous and endogenous TLRs ligands, IL-33 etc.) basophil could, potentially, influence monocyte activation locally, as was shown in the previously discussed mouse model of allergic inflammation [10].

Unexpectedly, we observed the presence of histamine in basophil cultures in which the basophils remained unstimulated or were stimulated with IL-33 only. Although we can't exclude that histamine release is partially due to *in vitro* culture conditions, these findings are in line with previous studies showing histamine release by a mechanism defined as "piecemeal" degranulation [45]. While it would be tempting to speculate that the described IL-33-induced histamine release by human basophils and subsequent immune regulation are also operative in IgE-independent inflammatory conditions, further studies will be needed to address the relevance of the IgE-independent histamine release by basophils.

In conclusion, our data show that IL-33 significantly enhances IgE-mediated activation of basophils, inducing the production of

IL-4 and the release of histamine. These basophil-derived mediators, in turn, were found to modulate monocyte responses, by suppressing their LPS-induced activation. Histamine, in particular, played a central role in basophil-mediated immune modulation. Overall, our observations show that human basophils are able to inhibit monocyte proinflammatory activation and point to a previously unrecognized role for human basophils, through the balanced secretion of IL-4 and histamine, in the regulation of the immune response.

Materials and methods

Cell isolation

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, NL). For isolation of basophils, leukocyte-rich plasma was first prepared with HetaSep (StemCell Technologies) and basophils were then selected by negative magnetic bead isolation using the EasySep™ Human Basophil Enrichment Kit (StemCell Technologies), according to the manufacturers' instructions. The purity of enriched basophils (mean 98%) was determined by flow cytometric analysis of FcεRI, CD123, and CD203c, using BDCA-2 to exclude the presence of contaminating pDC. For monocyte isolation, PBMCs were isolated from buffy coats and CD14⁺ monocytes were selected using magnetic-labeled anti-CD14 beads (Miltenyi Biotec), according to the manufacturer's instructions (mean purity 96%).

Basophil activation

After isolation, basophils were cultured in RPMI-1640 medium containing 10% fetal calf serum, glutamine, penicillin, and streptomycin (Invitrogen) at 37°C in 5% CO₂ atmosphere at a concentration of 1×10^6 cells/mL. Basophils were stimulated using 0.5 µg/mL of goat-anti-human IgE (Nordic-MUBio), 100 ng/mL of recombinant human IL-33 (PeproTech), or both. After 24 h, cells were harvested, supernatants were collected and stored at –20°C and cells were used for flow cytometric analysis.

Monocyte stimulation

After isolation, monocytes were cultured in the same medium under the same conditions as basophils. Monocytes were incubated with basophil supernatants (diluted 1:4 in normal medium) or control media (containing the same stimuli used for basophil activation to exclude their direct effects on monocytes). In parallel, monocytes were incubated with recombinant human IL-4 (PeproTech) at a concentration of 10 ng/mL and histamine (Sigma-Aldrich) at a concentration of 10^{-5} M. Monocytes were stimulated with LPS from *Salmonella typhosa* (Sigma-Aldrich) at a concentration of 5 ng/mL. After overnight (18 h) incubation cells were harvested, supernatants collected and stored at –20°C

for further analysis and cells were resuspended in PBS with 2 mM EDTA (Sigma-Aldrich) for 15 min on ice. Cells were detached by vigorously pipetting, washed, and resuspended in PBS 0.5% BSA for flow cytometric analysis. For blocking experiments, supernatants of anti-IgE and IL-33 activated basophils were used. They were preincubated with anti-IL-4 antibody or matching isotype control rat IgG1 (both from eBioscience) at a concentration of 20 µg/mL for 30 min at 37°C in 5% CO₂ atmosphere, prior to addition to the monocytes. For inhibition of histamine, monocytes were preincubated for 30 min at 37°C with histamine receptor 2 antagonist ranitidine at a concentration of 10^{-4} M. For blocking experiments with exogenous histamine in addition to ranitidine, histamine receptor 1 antagonist cetirizine and histamine receptor 3/4 antagonist thioperamide (Sigma-Aldrich) were used, at concentrations from 10^{-6} to 10^{-4} M.

Basophils and monocytes coculture

Isolated autologous basophils and monocytes were cultured alone or together at 1×10^6 cells/mL in a ratio 1:2, in the presence or absence of LPS and anti-IgE/IL-33 at the same concentrations mentioned above, for 16–18 h.

Supernatants were collected and stored at –20°C for further analysis, monocytes were detached with PBS 2 mM EDTA as previously indicated and cells were used for flow cytometric analysis.

Flow cytometry

For standard surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0.5% BSA at 4°C for 30 min. After washing, cells were suspended in PBS 0.5% BSA. To exclude dead cells, cells were suspended in PBS/0.5%BSA and just prior to flow cytometric acquisition, 0.2 µM DAPI (Invitrogen) was added.

Cells were acquired on a LSR-II (BD Biosciences) and analysis was performed using FlowJo software (Tree Star, Inc). The following antibodies (clone) were used: CD123-PerCPy5.5 (clone 7G3), CD63-PE (H5C6), HLA DR-FITC (L243), CD80-PE (L307.4), CD16-FITC (3G8), CD64 PE (10.1), mIgG2b-FITC (27–35), IgG2ak-FITC (X39). IgG2ak-allophycocyanin (MOPC-21) from BD Biosciences; FcεRI-FITC (AER-37), CD14-PerCP-Cy5.5 (61D3), mIgG1-PE (P3.6.2.8.1), mIgG2b-PE (eBMG2b), mIgG2b-PerCP-Cy5.5 (MOPC-21) from eBioscience; BDCA2-PE (AC144) and CD203c (FR 3–16A11) from Miltenyi and CD32-FITC (IV.3) from Stemcell. AlexaFluor488-CD32b (ch2b6-N297Q) and corresponding isotype control (ch4420-N297Q) were generously provided by MacroGenics [46, 47].

Measurement of cytokines

IL-4 was measured using the IL-4 Human PeliPair™ ELISA kit (Sanquin Reagents), TNF-α and IL-10 were measured using the

Human TNF ELISA set (BD Biosciences) and the IL-10 Human PeliPair™ ELISA kit (Sanquin Reagents), respectively. Histamine was measured using the Histamine ELISA kit (Neogen). All kits were used according to manufacturer's instructions.

Statistical analysis

Data are expressed as mean + SEM. For comparisons of two groups, paired Student's *t*-test was performed. For comparisons of multiple groups, ANOVA was performed with Bonferroni's post-test to determine statistical significance between each group. The number of independent experiments is indicated in each figure legend and each independent experiment represents a different donor. *p*-values <0.05 were considered significant and are shown in the figures. Analyses were performed using GraphPad Prism 5 Software (GraphPad Inc Software).

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

References

- Schroeder, J. T., Basophils: emerging roles in the pathogenesis of allergic disease. *Immunol. Rev.* 2011. **242**: 144–160.
- Siracusa, M. C., Kim, B. S., Spergel, J. M. and Artis, D., Basophils and allergic inflammation. *J. Allergy Clin. Immunol.* 2013. **132**: 789–801.
- Borriello, F., Granata, F. and Marone, G., Basophils and skin disorders. *J. Invest. Dermatol.* 2014. **134**: 1202–1210.
- Karasuyama, H., Mukai, K., Obata, K., Tsujimura, Y. and Wada, T., Nonredundant roles of basophils in immunity. *Annu. Rev. Immunol.* 2011. **29**: 45–69.
- van Panhuys, N., Prout, M., Forbes, E., Min, B., Paul, W. E. and Le Gros, G., Basophils are the major producers of IL-4 during primary helminth infection. *J. Immunol.* 2011. **186**: 2719–2728.
- Otsuka, A., Nakajima, S., Kubo, M., Egawa, G., Honda, T., Kitoh, A., Nomura, T. et al., Basophils are required for the induction of Th2 immunity to haptens and peptide antigens. *Nat. Commun.* 2013. **4**: 1739.
- Hammad, H., Plantinga, M., Deswarte, K., Pouliot, P., Willart, M. A., Kool, M., Muskens, F. and Lambrecht, B. N., Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 2010. **207**: 2097–2111.
- Anthony, R. M., Kobayashi, T., Wermeling, F. and Ravetch, J. V., Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature* 2011. **475**: 110–113.
- Campbell, I. K., Miescher, S., Branch, D. R., Mott, P. J., Lazarus, A. H., Han, D., Maraskovsky, E. et al., Therapeutic effect of IVIG on inflammatory arthritis in mice is dependent on the Fc portion and independent of sialylation or basophils. *J. Immunol.* 2014. **192**: 5031–5038.
- Egawa, M., Mukai, K., Yoshikawa, S., Iki, M., Mukaida, N., Kawano, Y., Minegishi, Y. and Karasuyama, H., Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. *Immunity* 2013. **38**: 570–580.
- Lee, J. J. and McGarry, M. P., When is a mouse basophil not a basophil? *Blood* 2007. **109**: 859–861.
- Brunner, T., Heusser, C. H. and Dahinden, C. A., Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J. Exp. Med.* 1993. **177**: 605–611.
- Wakahara, K., Van, V. Q., Baba, N., Begin, P., Rubio, M., Delespesse, G. and Sarfati, M., Basophils are recruited to inflamed lungs and exacerbate memory Th2 responses in mice and humans. *Allergy* 2013. **68**: 180–189.
- Suurmond, J., Stoop, J. N., Rivellesse, F., Bakker, A. M., Huizinga, T. W. and Toes, R. E., Activation of human basophils by combined toll-like receptor- and FcεRI-triggering can promote Th2 skewing of naive T helper cells. *Eur. J. Immunol.* 2013.
- Sharma, M., Hegde, P., Aimaniananda, V., Beau, R., Senechal, H., Poncet, P., Latge, J. P., Kaveri, S. V. and Bayry, J., Circulating human basophils lack the features of professional antigen presenting cells. *Sci. Rep.* 2013. **3**: 1188.
- Eckl-Dorna, J., Ellinger, A., Blatt, K., Ghanim, V., Steiner, I., Pavelka, M., Valent, P., Valenta, R. and Niederberger, V., Basophils are not the key antigen-presenting cells in allergic patients. *Allergy* 2012. **67**: 601–608.
- Kitzmüller, C., Nagl, B., Deiß, S., Walterskirchen, C., Jahn-Schmid, B., Zlabinger, G. J. and Bohle, B., Human blood basophils do not act as antigen-presenting cells for the major birch pollen allergen Bet v 1. *Allergy* 2012. **67**: 593–600.
- Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. and Dahinden, C. A., Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009. **113**: 1526–1534.
- Smithgall, M. D., Comeau, M. R., Yoon, B. R., Kaufman, D., Armitage, R. and Smith, D. E., IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 2008. **20**: 1019–1030.
- Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S. et al., An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J. Immunol.* 2008. **181**: 5981–5989.
- Mirchandani, A. S., Salmond, R. J. and Liew, F. Y., Interleukin-33 and the function of innate lymphoid cells. *Trends Immunol.* 2012. **33**: 389–396.
- Liew, F. Y., Pitman, N. I. and McInnes, I. B., Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat. Rev. Immunol.* 2010. **10**: 103–110.
- Liew, F. Y., IL-33: a Janus cytokine. *Ann. Rheum. Dis.* 2012. **71** (Suppl 2): i101–i104.

- 24 Frischmeyer-Guerrero, P. A. and Schroeder, J. T., Cellular immune response parameters that influence IgE sensitization. *J. Immunol. Methods* 2012. **383**: 21–29.
- 25 MacGlashan, D. W., Jr., Basophil activation testing. *J. Allergy Clin. Immunol.* 2013. **132**: 777–787.
- 26 Akagi, K. and Townley, R. G., Spontaneous histamine release and histamine content in normal subjects and subjects with asthma. *J. Allergy Clin. Immunol.* 1989. **83**: 742–749.
- 27 MacGlashan, D., Jr., Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. *Clin. Exp. Allergy* 2010. **40**: 1365–1377.
- 28 Elenkov, I. J., Webster, E., Papanicolaou, D. A., Fleisher, T. A., Chrousos, G. P. and Wilder, R. L., Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J. Immunol.* 1998. **161**: 2586–2593.
- 29 Schroeder, J. T., Basophils beyond effector cells of allergic inflammation. *Adv. Immunol.* 2009. **101**: 123–161.
- 30 Tjon, A. S., van Gent, R., Jaadar, H., Martin van Hagen, P., Mancham, S., van der Laan, L. J., Te Boekhorst, P. A. et al., Intravenous immunoglobulin treatment in humans suppresses dendritic cell function via stimulation of IL-4 and IL-13 production. *J. Immunol.* 2014. **192**: 5625–5634.
- 31 O'Mahony, L., Akdis, M. and Akdis, C. A., Regulation of the immune response and inflammation by histamine and histamine receptors. *J. Allergy Clin. Immunol.* 2011. **128**: 1153–1162.
- 32 Morichika, T., Takahashi, H. K., Iwagaki, H., Yoshino, T., Tamura, R., Yokoyama, M., Mori, S. et al., Histamine inhibits lipopolysaccharide-induced tumor necrosis factor- α production in an intercellular adhesion molecule-1- and B7.1-dependent manner. *J. Pharmacol. Exp. Ther.* 2003. **304**: 624–633.
- 33 Mazzoni, A., Young, H. A., Spitzer, J. H., Visintin, A. and Segal, D. M., Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *J. Clin. Invest.* 2001. **108**: 1865–1873.
- 34 Triggiani, M., Petraroli, A., Loffredo, S., Frattini, A., Granata, F., Morabito, P., Staiano, R. I. et al., Differentiation of monocytes into macrophages induces the upregulation of histamine H1 receptor. *J. Allergy Clin. Immunol.* 2007. **119**: 472–481.
- 35 Marone, G., Gentile, M., Petraroli, A., De Rosa, N. and Triggiani, M., Histamine-induced activation of human lung macrophages. *Int. Arch. Allergy Immunol.* 2001. **124**: 249–252.
- 36 Micallef, S., Stark, H. and Sasse, A., Polymorphisms and genetic linkage of histamine receptors. *Life Sci.* 2013. **93**: 487–494.
- 37 Parry, G. C. and Mackman, N., Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF- κ B-mediated transcription. *J. Immunol.* 1997. **159**: 5450–5456.
- 38 Brenner, S., Prosch, S., Schenke-Layland, K., Riese, U., Gausmann, U. and Platzter, C., cAMP-induced interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. *J. Biol. Chem.* 2003. **278**: 5597–5604.
- 39 Ohmori, Y. and Hamilton, T. A., Interleukin-4/STAT6 represses STAT1 and NF- κ B-dependent transcription through distinct mechanisms. *J. Biol. Chem.* 2000. **275**: 38095–38103.
- 40 Yao, Y., Li, W., Kaplan, M. H. and Chang, C. H., Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells. *J. Exp. Med.* 2005. **201**: 1899–1903.
- 41 Lin, R. Y., Schwartz, L. B., Curry, A., Pesola, G. R., Knight, R. J., Lee, H. S., Bakalchuk, L. et al., Histamine and tryptase levels in patients with acute allergic reactions: an emergency department-based study. *J. Allergy Clin. Immunol.* 2000. **106**: 65–71.
- 42 MacGlashan, D., Jr., Histamine: A mediator of inflammation. *J. Allergy Clin. Immunol.* 2003. **112**: S53–S59.
- 43 Ito, Y., Satoh, T., Takayama, K., Miyagishi, C., Walls, A. F. and Yokozeki, H., Basophil recruitment and activation in inflammatory skin diseases. *Allergy* 2011. **66**: 1107–1113.
- 44 de Paulis, A., Prevete, N., Fiorentino, I., Walls, A. F., Curto, M., Petraroli, A., Castaldo, V. et al., Basophils infiltrate human gastric mucosa at sites of *Helicobacter pylori* infection, and exhibit chemotaxis in response to H. pylori-derived peptide Hp(2–20). *J. Immunol.* 2004. **172**: 7734–7743.
- 45 Dvorak, A. M., Piecemeal degranulation of basophils and mast cells is effected by vesicular transport of stored secretory granule contents. *Chem. Immunol. Allergy* 2005. **85**: 135–184.
- 46 Veri, M. C., Gorlatov, S., Li, H., Burke, S., Johnson, S., Stavenhagen, J., Stein, K. E. et al., Monoclonal antibodies capable of discriminating the human inhibitory Fc γ receptor IIB (CD32B) from the activating Fc γ receptor IIA (CD32A): biochemical, biological and functional characterization. *Immunology* 2007. **121**: 392–404.
- 47 Cassard, L., Jonsson, F., Arnaud, S. and Daeron, M., Fc γ receptors inhibit mouse and human basophil activation. *J. Immunol.* 2012. **189**: 2995–3006.

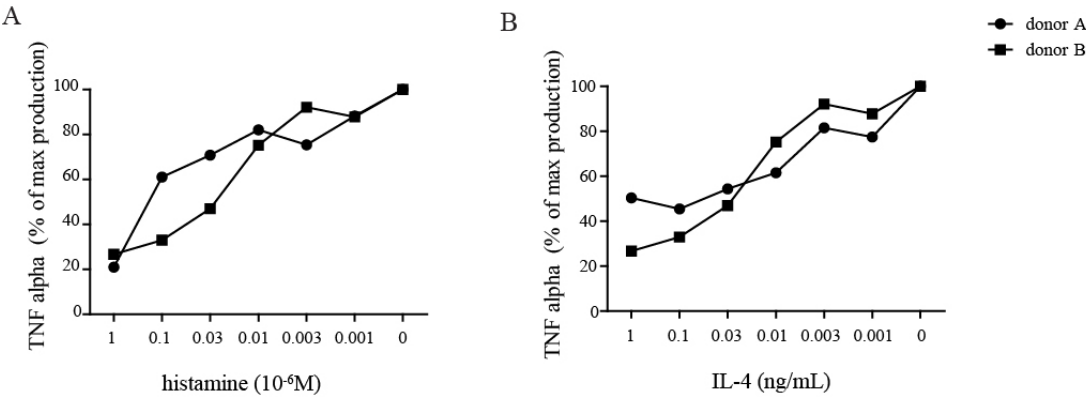
Abbreviations: H1R, H2R, H3R, H4R: histamine receptor 1–4 · IVIG: intravenous immunoglobulin

Full correspondence: Dr. Felice Rivellesse, Leiden University Medical Center, PO BOX 9600, Albinusdreef 2 – C1-R, 2300 RC Leiden, The Netherlands
 Fax: +31-71-5266752
 e-mail: rivellesse@gmail.com

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Supplementary figure 1. Titration of IL-4 and histamine effects on LPS-induced activation of monocytes.

Isolated monocytes were activated with LPS together with decreasing amount of IL-4 (A) or histamine (B). After over-night incubation, TNF-alpha was measured by ELISA in the supernatants. Results are expressed as percentages of maximal TNF-alpha production (with the amount of TNF-alpha produced by monocytes stimulated with LPS alone corresponding to 100% production). Results from 2 independent experiments (2 donors) are shown.



IgE- and IL-33-mediated triggering of human basophils inhibits TLR4-induced monocyte activation

Felice Rivellese, Jolien Suurmond, Amato de Paulis, Gianni Marone, Tom W.J. Huizinga and René E.M. Toes

Correspondence: Dr. Felice Rivelles, Leiden University Medical Center, Leiden, The Netherlands

Review Timeline:	Submission date:	9 April 2014
	First Editorial decision:	3 May 2014
	First Revision received:	17 June 2014
	Accepted:	25 July 2014

Handling Executive Committee member: Dr. Silvano Sozzani

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 3 May 2014

Dear Dr. Rivellese,

Manuscript ID eji.201444731 entitled "IgE and IL-33-mediated triggering of human basophils inhibits TLR4-induced pro-inflammatory activation of monocytes" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. You will see that referee 2 feels that the data presented in your paper have largely been presented in the literature and has some suggestions for enhancing the novelty and therefore impact of your paper. We therefore encourage you to fully address the concerns of both referees in your revision.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Dr. Silvano Sozzani

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

Reviewer: 1

Comments to the Author

The authors report that human blood basophils (isolated by negative selection using magnetic beads) stimulated with IL-33 together with anti-IgE inhibit dose-dependently TNF α production and CD80 expression by LPS-stimulated monocytes. These effects are largely mediated by histamine through H2R. Basophil-derived IL-4 and histamine have antagonistic effects on the production of IL-10 and the expression of Fc γ RIIb by LPS-stimulated monocytes.

Although the concept that basophils may inhibit the pro-inflammatory activity of macrophages has been previously established in the mouse, including in vivo, the present in vitro study on human blood basophils is interesting and potentially relevant. Our enthusiasm is dampened by the observation that isolated basophils spontaneously release histamine. These data undermine the concept that activation of basophils by IL-33 and anti-IgE is required to exert an inhibitory function on activated monocytes via histamine.

Comments

- Fig 1B: The purity of the basophil preparation should be demonstrated and possible contamination by dendritic cells should be excluded more convincingly. Are basophil preparations stimulating allogeneic naïve T cell proliferation or type I IFN production upon appropriate TLR stimulation? Furthermore, the introduction does not relate adequately to the work of others. For example, APC function of human basophils has not been confirmed in ref (11, 12).
- Fig 1D: IL-33 is used at at very high concentrations (100 ng/ml and at 50 ng/ml). No significant effect is observed at 10 ng/ml alone or in combination with IL-3 or anti-IgE.
- Fig 1G: The data do not support the conclusion included in the abstract and the results. The authors stated (Abstract page 2 line 16-18 and page 6 line 14) that IL-33 stimulates histamine release. In fact, IL-33 synergizes with anti-IgE to increase histamine release whereas by, itself, it has no effect. This can be misleading.
- The effects of basophil-derived histamine are blocked by ranitidine used at molar concentration 10 times higher than H1R or H3-4 inhibitors. It cannot be concluded that the effects are H2R-mediated. Dose-response together with signaling studies (cAMP, etc..) would be necessary to support this conclusion.
- The antagonistic activity of IL-4 and histamine should be integrated in the context of the current literature and the significance of these observations should be highlighted.

Reviewer: 2

Comments to the Author

Aim of Rivelese's et al paper is to study the role of IgE and IL33 on the human basophil activation and the inhibition of TLR-4 activation of monocytes by basophil released products. The investigation of regulatory function of basophil on innate and adaptive immune cells is a hot topic in the field of regulation of immune system. However, even if the paper presents some novelty in the interaction between basophil and monocyte, the data presented are present largely in literature. It is already known that histamine is inhibitory on monocyte activation, TNF- α release via H2R. It is also well known the molecular pathway that acts on inhibition of TNF- α gene expression.

- It is interesting the study between the basophil and monocyte; it could be of interest to investigate the role of cell-cell interaction on the monocyte activation.
- The reciprocal influence of the signal transduction pathways activated by H2R and TLR4.
- It should be tested the presence of LPS in the used FCS.
- It should be added to the panel of D of figure 2 the CD80 expression on IL33 stimulated cells.
- Fig. 6: It is of interest to perform intracellular staining of TNF- α to evaluate the contribution of basophil or monocyte to the cytokine production

First Revision – authors' response – 17 June 2014

We thank the Reviewers for their valuable comments, as these have encouraged us to perform additional experiments. Moreover, we have modified the introduction and discussion sections in order to better integrate our data within the current knowledge. All changes to the manuscript are indicated by the track change mode of the word editor and a clear (non highlighted) version of the manuscript is also enclosed to facilitate the review process.

Reviewer: 1

Comments to the Author

The authors report that human blood basophils (isolated by negative selection using magnetic beads) stimulated with IL-33 together with anti-IgE inhibit dose-dependently TNF α production and CD80 expression by LPS-stimulated monocytes. These effects are largely mediated by histamine through H2R. Basophil-derived IL-4 and histamine have antagonistic effects on the production of IL-10 and the expression of Fc γ RIIb by LPS-stimulated monocytes.

Although the concept that basophils may inhibit the pro-inflammatory activity of macrophages has been previously established in the mouse, including in vivo, the present in vitro study on human blood basophils is interesting and potentially relevant. Our enthusiasm is dampened by the observation that isolated basophils spontaneously release histamine. These data undermine the concept that activation of basophils by IL-33 and anti-IgE is required to exert an inhibitory function on activated monocytes via histamine.

→ Answer to the general comments of Reviewer 1

We understand the feeling that the spontaneous histamine release by basophils, together with the partial effect of unstimulated basophils on monocyte activation, might undermine the relevance of our observations. Nonetheless, the spontaneous release of histamine by basophils is well described in the literature, especially, but not exclusively, in atopic patients (1-2). Additionally, most of the publications exploring the activation of basophils use indirect ways of measuring degranulation (e.g. beta-hexosaminidase or membrane markers as CD63). When histamine is directly measured, the spontaneous release is usually subtracted and/or set as 0%. In Fig. 1G we didn't subtract the spontaneous release because the presence of histamine in the supernatants of unstimulated basophils helps to understand their partial effect on monocytes activation. Nonetheless, we believe this observation does not undermine the concept that activation of basophils by IL-33 and anti-IgE is required to exert an inhibitory function on activated monocytes via histamine. Indeed, as shown in figure 2A, supernatants of unstimulated basophil exert only a partial effect, with a mean of 31 % inhibition of LPS-induced TNF- α production, while the inhibition obtained with supernatants of anti-IgE

and IL-33-triggered basophils rises to 73% ($p=0.002$ compared to unstimulated basophils). Therefore, activation of basophils by IL-33 and anti-IgE contributes in a significant manner to the immunomodulatory effects of human basophils.

References

1. Sampson HA, Broadbent KR, Bernhisel-Broadbent J. Spontaneous release of histamine from basophils and histamine-releasing factor in patients with atopic dermatitis and food hypersensitivity. *N Engl J Med.* 1989 Jul 27;321(4):228-32
2. Akagi K and Townley R G. Spontaneous histamine release and histamine content in normal subjects and subjects with asthma. *J Allergy Clin Immunol* 1989. 83: 742-749.

Comments

Comment#1 Reviewer1

Fig 1B: The purity of the basophil preparation should be demonstrated and possible contamination by dendritic cells should be excluded more convincingly. Are basophil preparations stimulating allogeneic naïve T cell proliferation or type I IFN production upon appropriate TLR stimulation? Furthermore, the introduction does not relate adequately to the work of others. For example, APC function of human basophils has not been confirmed in ref (11, 12).

→Answer #1 Reviewer 1 :

We agree with the Reviewer that contamination with pDCs could influence the outcome of our study. Therefore, we have been very cautious in excluding this possibility by several measures and controls.

Several arguments point against the presence of pDC in our isolations:

- the antibody mix used for depletion of non-basophils during the isolation procedure includes antibodies against CD36 and CD45RA, which are expressed by pDCs (1), allowing for depletion of this cell type;
- as for the evaluation of purity by flow cytometry, it is indeed known that pDCs express CD123 and low levels of Fc ϵ RI. Nonetheless, gating basophils as CD123++Fc ϵ RI++CD203c+ (2-3) allows exclusion of pDCs, as confirmed by the gating strategy further explained in Figure R1. As an additional control, with an example already shown in figure 1B of the manuscript, we verified that isolated basophils, positive for CD203c, were negative for the pDC marker BDCA-2. This can also be seen again in the bottom panel of figure R1. We have now added the analysis of CD203c, which was routinely performed for each isolation, to Fig 1B to better show that the gating strategy we used included only CD203c+ basophils and no pDCs;
- as also noticed by the Reviewer, an additional evidence to rule out the contamination by pDCs would be to measure typical DC products in the supernatants of isolated basophils. We have recently demonstrated, in a previous publication (4) (Suurmond et al. *Eur J Immunol.* 2014

Feb;44(2):386-96), that typical pDCs products such as type I interferon and IL-12 were not detected or were only present at very low concentrations in the supernatants of isolated basophils and, most importantly, were not upregulated upon stimulation with TLR ligands. These data are also shown below for the Reviewers. Please note that the basophils used in this publication were isolated with the same technique and experiments were performed in parallel to the current manuscript.

Regarding the introduction, we modified the text and added the appropriate references (15-17 of the revised manuscript), to state more clearly that the APC function of human basophils has not been confirmed by several groups.

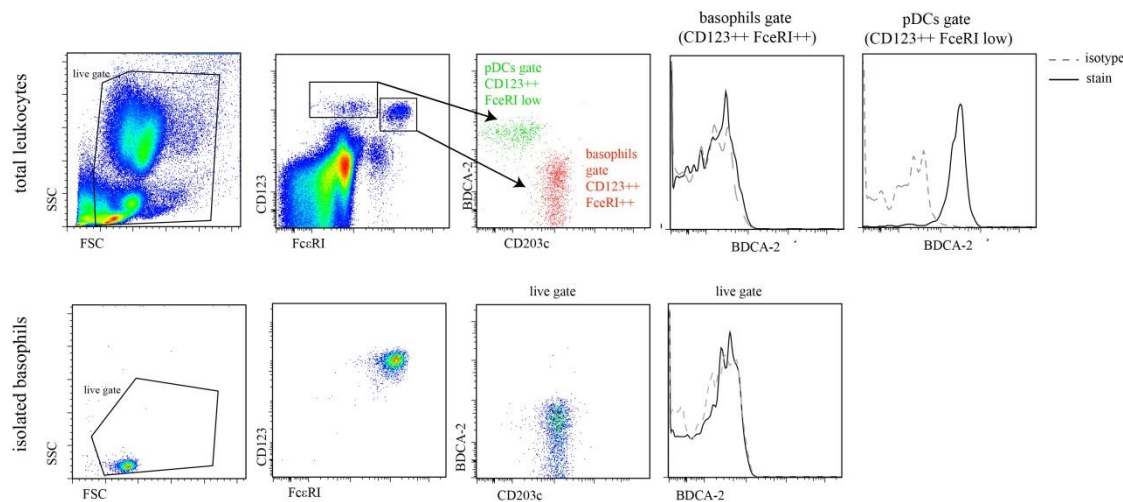


Figure R1 (for Reviewers):

Staining of total leukocytes and isolated basophils for CD123, FcεRI, CD203c, and BDCA-2.

Top panels: Total leukocytes. pDCs are gated as CD123+ FcεRI low (green) and basophils as CD123+FcεRI++ (red). On the right, histograms show expression of BDCA-2 vs isotype matched control by gated basophils and pDCs. **Low panels:** Isolated basophils. In the third and fourth panel cells from the live gate are shown (not gated on CD123++ FcεRI++) to confirm that among all cells present in the basophil preparation there are no cells positive for BDCA-2.

	ctr	TLR-1/2	TLR-2/2	TLR-3	TLR-4	TLR-5	TLR-6/2	TLR-7	TLR-8	TLR-9	Anti-IgE
IFN-alpha2	1.3 ± 0.8 pg/mL	3.4 ± 3.4 pg/mL	4.7 ± 2.4 pg/mL	0.9 ± 0.9 pg/mL	4.6 ± 3.9 pg/mL	2.5 ± 2.1 pg/mL	1.7 ± 1.0 pg/mL	1.4 ± 1.2 pg/mL	1.1 ± 0.9 pg/mL	2.1 ± 1.3 pg/mL	2.7 ± 3.9 pg/mL
IL-12p70	0.7 ± 1.1 pg/mL	1.2 ± 1.2 pg/mL	2.4 ± 0.6 pg/mL	0.8 ± 1.2 pg/mL	1.4 ± 1.4 pg/mL	1.3 ± 1.1 pg/mL	0.8 ± 1.2 pg/mL	0.8 ± 1.2 pg/mL	0.9 ± 1.1 pg/mL	0.8 ± 1.2 pg/mL	1.1 ± 1.2 pg/mL

Table R1 (for Reviewers): This table shows that typical DC cytokines are not upregulated in response to TLR ligands. Mean (± StDev) of the production in pg/mL of the particular cytokine in response to the TLR

ligand, anti-IgE or control medium is shown. None of these cytokines were significantly increased in a paired samples T test, compared to unstimulated (ctr). Adapted from (4).

References:

1. Dzionic A et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol.* 2000. 165(11):6037-46
2. Dijkstra D et al. Basophils from humans with systemic lupus erythematosus do not express MHC-II. *Nat Med.* 2012. 18(4):488-9.
3. Charles N et al. Reply to: Basophils from humans with systemic lupus erythematosus do not express MHC-II. *Nat Med.* 2012. 18(4):489-90.
4. Suurmond et al. Activation of human basophils by combined toll-like receptor- and FcεRI-triggering can promote Th2 skewing of naive T helper cells. *Eur J Immunol.* 2014 Feb;44(2):386-96

Comment #2 Reviewer 1

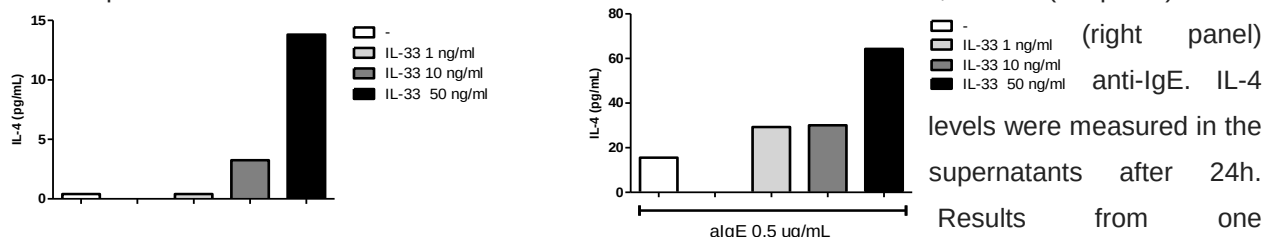
Fig 1D: IL-33 is used at very high concentrations (100 ng/ml and at 50 ng/ml). No significant effect is observed at 10 ng/ml alone or in combination with IL-3 or anti-IgE.

→ Answer #2 Reviewer 1

Although the concentrations of IL-33 used seem relatively high, the results shown in Fig 1C and 1D are in line with previously published papers (1-3), in which the same concentrations were used (50 or 100 ng/mL). Likewise, when IL-33 was titrated, as in ref 3, it showed dose-dependent effects, but reached statistical significance, for most of the parameters analyzed, only at high concentrations (100 ng/mL). To further address this comment, we performed a titration experiment (shown as in figure R2) which gave comparable results: low doses of IL-33 (1 and 10 ng/mL) induced a minor activation of basophils (e.g. IL-4 production) and partially enhanced their IgE-mediated activation, whereas higher doses (50 ng/mL) gave strong responses. This confirms that doses higher than 50 ng/mL are needed in order to induce a proper activation of basophils. Although such high concentrations can appear non-physiological, it is important to underline that IL-33, as an alarmin, can be released upon cell necrosis and reach very high levels locally during inflammatory processes. Following this comment, we modified the results section of fig 1D, to specify that only high concentrations of IL-33 had a statistically significant effect.

Figure R2 (for Reviewers)

Basophils were incubated for 24h with the indicated concentrations of IL-33, without (left panel) or with



experiment are shown.

References

1. Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. and Dahinden, C. A., Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009. 113: 1526-1534.
2. Smithgall, M. D., Comeau, M. R., Yoon, B. R., Kaufman, D., Armitage, R. and Smith, D. E., IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* 2008. 20: 1019-1030.
3. Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S., Matsushima, K., Ohta, K., Yamamoto, K. and Yamaguchi, M., An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J Immunol* 2008. 181: 5981-5989.

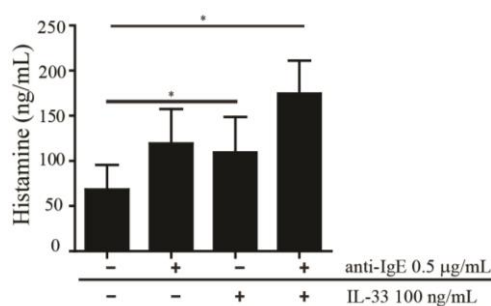
Comment #3 Reviewer 1

Fig 1G: The data do not support the conclusion included in the abstract and the results. The authors stated (Abstract page 2 line 16-18 and page 6 line 14) that IL-33 stimulates histamine release. In fact, IL-33 synergizes with anti-IgE to increase histamine release whereas by, itself, it has no effect. This can be misleading.

→Answer #3 Reviewer 1

We agree with the Reviewer that that the results, as presented in Fig. 1G, were not sufficiently clear to support the conclusions made, as we did not include a statistical comparison between unstimulated and IL-33-triggered basophils (i.e. column 1 and 3 of the graph). Therefore, we have now compared these two conditions and the results of this test ($p=0.014$) show a significant effect of IL-33. Therefore, we have now modified Fig. 1G, as shown below, to show the difference of IL-33 vs unstimulated basophils, which is supporting the conclusions that IL-33 induces the release of histamine.

Figure 1G of the manuscript (modified)



Comment #4 Reviewer 1

The effects of basophil-derived histamine are blocked by ranitidine used at molar concentration 10 times higher than H1R or H3-4 inhibitors. It cannot be concluded that the effects are H2R-mediated.

Dose-response together with signaling studies (cAMP, etc..) would be necessary to support this conclusion

→Answer #4 Reviewer 1

We now performed additional experiments to confirm that the described effects of histamine on monocytes are H2R dependent. The histamine receptor inhibitors were titrated at doses ranging from 10^{-6} to 10^{-4} M with different concentrations of exogenously added histamine. As shown in Fig. R3 below, only ranitidine (H2R blocker) is able to block the inhibitory effects of histamine on LPS induced TNF- α , while cetirizine (H1R blocker) and thioperamide (H3/4R blocker) are ineffective at all doses tested, up to 10^{-4} . These findings are in line with previously published data (1-2) in which more detailed dose-response studies and signaling studies (including cAMP) have already been performed, confirming that the histamine effects we observe on monocytes are H2R dependent. We now describe these data in the revised manuscript.

Given the limited the availability of basophils, dose-response experiments with different blockers are not feasible with basophil supernatants. Nonetheless, we believe that the evidence that ranitidine blocks the effects of basophil supernatants, together with the evidence that ranitidine specifically blocks the effects of exogenous histamine (as shown below and in line with the literature), are adequate to conclude that the suppression of LPS induced TNF- α production is mediated by basophil-derived histamine acting via the H2R.

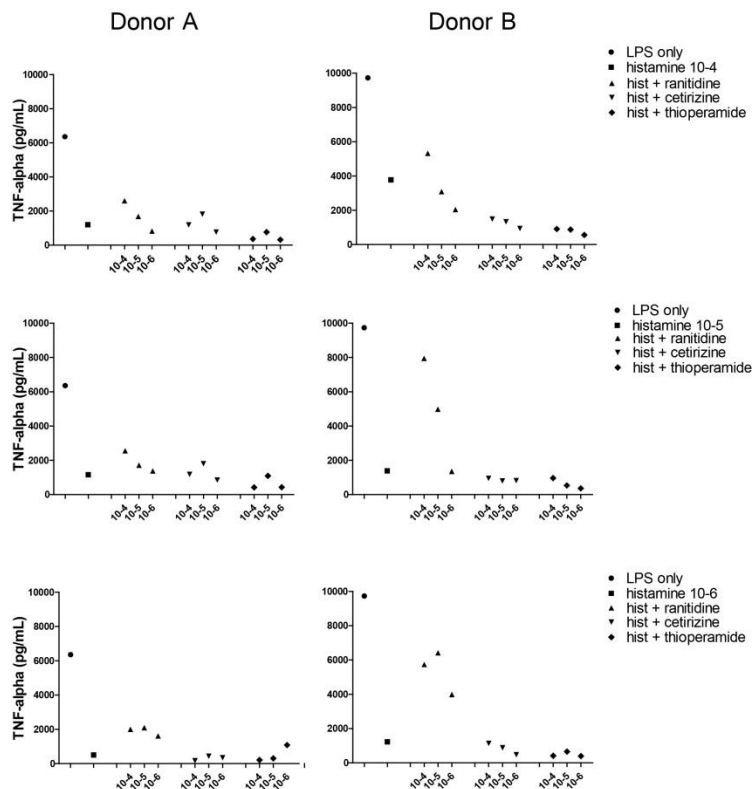


Figure R3 (for Reviewers)

Monocytes were pre-incubated with the doses of histamine receptors antagonist indicated on the x axis and stimulated with LPS (5ng/mL) in the presence of the indicated amount of histamine (from 10^{-4} , upper panels, to 10^{-6} , lower panels). After 18h incubation supernatants were harvested and TNF- α was measured by ELISA. Results from two donors are shown.

References

1. Elenkov, I. J., Webster, E., Papanicolaou, D. A., Fleisher, T. A., Chrousos, G. P. and Wilder, R. L., Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J Immunol* 1998. 161: 2586-2593.
2. Morichika T1, Takahashi HK, Iwagaki H, Yoshino T, Tamura R, Yokoyama M, Mori S, Akagi T, Nishibori M, Tanaka N. Histamine inhibits lipopolysaccharide-induced tumor necrosis factor- α production in an intercellular adhesion molecule-1- and B7.1 dependent manner. *J Pharmacol Exp Ther*. 2003 Feb;304(2):624-33.

Comment #5 Reviewer 1

The antagonistic activity of IL-4 and histamine should be integrated in the context of the current literature and the significance of these observations should be highlighted.

→Answer #5 Reviewer 1

We thank the Reviewer for this suggestion. We have indeed expanded the discussion to integrate our findings in the context of current literature, including a newly published paper (Ref n 30 of the revised manuscript) and giving more insights into the interactions between histamine and IL-4 at intracellular levels to further explain and expand our findings.

Reviewer: 2

Comments to the Author

Aim of Rivelese's et al paper is to study the role of IgE and IL33 on the human basophil activation and the inhibition of TLR-4 activation of monocytes by basophil released products. The investigation of regulatory function of basophil on innate and adaptive immune cells is a hot topic in the field of regulation of immune system. However, even if the paper presents some novelty in the interaction between basophil and monocyte, the data presented are present largely in literature. It is already known that histamine is inhibitory on monocyte activation, TNF- α release via H2R. It is also well known the molecular pathway that acts on inhibition of TNF- α gene expression.

→Answer to the general comments of Reviewer 2:

We agree with the Reviewer that the mechanisms of histamine-mediated inhibition of monocyte activation have been described, including the intracellular signaling. Nonetheless, the novelty of our manuscript relies on the analysis of the interaction of basophils with monocytes, which, to the best of our knowledge, has never been performed in a human setting. Although histamine effects are known, we show for the first time strong immunomodulatory effects of basophils on human monocytes. These effects of basophils are, at least in part, mediated by histamine, as we describe here for the first time. Likewise, we show that the histamine-mediated effects can overcome, in our in-vitro settings, some of the effects of IL-4, such as the up-regulation of the inhibitory receptor Fc γ RIIb.

Comment #1 Reviewer 2

-It is interesting the study between the basophil and monocyte; it could be of interest to investigate the role of cell-cell interaction on the monocyte activation.

→Answer #1 Reviewer 2

We agree with the Reviewer that the investigation of cell-cell interaction is of interest. Therefore, we have included such experiments (Fig. 5). However, the results obtained with the coculture are equivalent to the supernatants, suggesting that soluble mediators are most likely involved in the interaction between basophils and monocytes. Indeed, when both IL-4 and histamine receptor 2 were

blocked, TNF- α production by monocytes was fully restored (Fig. 5A of the manuscript), confirming that soluble mediators, and in particular histamine, are responsible for the suppressive effects of basophils. These observations are now better highlighted and discussed in the revised manuscript.

Comment #2 Reviewer 2

The reciprocal influence of the signal transduction pathways activated by H2R and TLR4.

→Answer #2 Reviewer 2

As correctly pointed out by the Reviewer in the general comments, the signal transduction pathways of H2R, including its effects on TLR4 signaling, have been previously studied (1-2). In addition, cAMP, which is produced upon triggering of H2R, is known to inhibit NF- κ B (3-4) and, consequently, LPS-induced TNF- α production. Similarly, cAMP has been described to induce IL-10 production (5). At the same time, IL-4 intracellular signaling has been already studied (6-7). We believe that investigating the intracellular pathways activated in monocytes in response to the combination of histamine, IL-4 and TLR-4 triggering, although interesting to further understand the exact interaction of these factors, would be a topic for a different study focusing on monocyte signaling. Nonetheless, to address this comment, we now extended the discussion about the interaction of IL-4 and histamine in regards of TLR-4 signaling in monocytes.

References

1. Elenkov, I. J., Webster, E., Papanicolaou, D. A., Fleisher, T. A., Chrousos, G. P. and Wilder, R. L., *Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. J Immunol* 1998. 161: 2586-2593.
2. Morichika T1, Takahashi HK, Iwagaki H, Yoshino T, Tamura R, Yokoyama M, Mori S, Akagi T, Nishibori M, Tanaka N. *Histamine inhibits lipopolysaccharide-induced tumor necrosis factor- α production in an intercellular adhesion molecule-1- and B7.1 dependent manner. J Pharmacol Exp Ther.* 2003 Feb;304(2):624-33.
3. Minguet S1, Huber M, Rosenkranz L, Schamel WW, Reth M, Brummer T. *Adenosine and cAMP are potent inhibitors of the NF- κ B pathway downstream of immunoreceptors. Eur J Immunol.* 2005 Jan;35(1):31-41.
4. Parry GC, Mackman N. *Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF- κ B-mediated transcription. J Immunol.* 1997 Dec 1;159(11):5450-6.
5. Brenner, S. et al. *cAMP-induced interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. J. Biol. Chem.* 278, 5597–5604 (2003).
6. Ohmori Y1, Hamilton TA. *Interleukin-4/STAT6 represses STAT1 and NF- κ B-dependent transcription through distinct mechanisms. J Biol Chem.* 2000 Dec 1;275(48):38095-103.
7. Yao Y1, Li W, Kaplan MH, Chang CH. *Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells. J Exp Med.* 2005 Jun 20;201(12):1899-903.

Comment #3 Reviewer 2

It should be tested the presence of LPS in the used FCS.

→Answer #3 Reviewer 2

As requested by the Reviewer, we tested the FCS for the presence of endotoxins, using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza) and endotoxin levels in FCS were below 0.5 EU*/mL (0.45 EU/mL). Considering that FCS has been used in medium with a dilution factor of 10, this would correspond to an endotoxin contamination of <0.05 EU (<5 pg/mL), which is very low endotoxin levels.
*1 EU (endotoxin units) roughly corresponds to 0.1 ng/mL.

References

Gorbet MB1, Sefton MV Endotoxin: the uninvited guest. *Biomaterials*. 2005 Dec;26(34):6811-7.

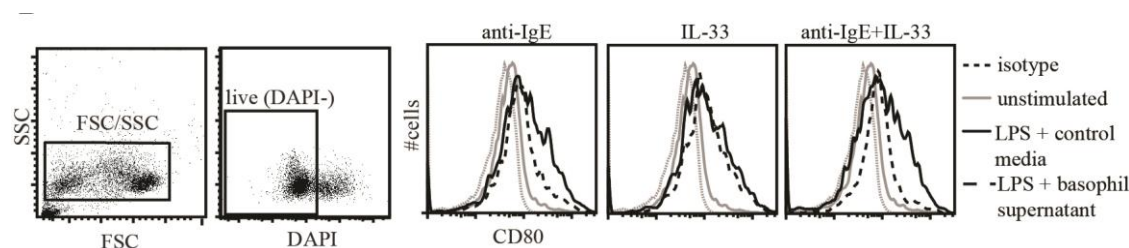
Comment #4 Reviewer 2

It should be added to the panel of D of figure 2 the CD80 expression on IL33 stimulated cells.

→Answer #4 Reviewer 2

In panel D of figure 2 the black line (control medium) represents monocytes incubated with medium containing IL-33+anti-IgE, to control for a possible direct effect of these mediators on monocytes.
If the Reviewer's comment referred to the CD80 expression by monocytes conditioned with IL-33-triggered basophil supernatants (single stimulation with IL-33), we inserted additional panels with anti-IgE- and IL-33-triggered basophil supernatants to figure 2D, to give a more complete overview of the experiment.

Fig. 2D of the manuscript (modified)



Comment #5 Reviewer 2

Fig. 6: It is of interest to perform intracellular staining of TNF- α to evaluate the contribution of basophil or monocyte to the cytokine production

→Answer #5 Reviewer 2

We thank the Reviewer for this suggestion. Although intracellular staining would allow to discriminate the contribution of basophil or monocytes to the cytokine production in the co-culture, we already have control conditions with basophils alone, in which TNF- α was not detectable after stimulation with LPS +

anti-IgE and IL-33. These conditions are not shown in the figure, as stated in the text. We now highlighted this information, also in the figure legend, to improve the readability of the manuscript. Furthermore, we have previously published, using the same methods for basophil isolation, that triggering of human basophils with several TLR ligands (including TLR-4) or anti-IgE do not induce significant amounts of TNF- α (Suurmond et al. Eur J Immunol. 2014 Feb;44(2):386-96, supporting information table 1). In fact, the maximum amount of TNF- α , produced by basophils upon triggering with TLR-2 ligands, was minimal (23 ± 3 pg/ml), especially when compared to monocytes. These data are reported below as table R2 for the Reviewers.

	TLR-1/2	TLR-2	TLR-3	TLR-4	TLR-5	TLR-6/2	TLR-7	TLR-8	TLR-9	Anti-IgE
TNF- α	3.5 (ns)	28.9 (*) 23 ± 3 pg/ml	2.4 (ns)	5.3 (ns)	4.2 (ns)	5.8 (*) 3 ± 1 pg/ml	0.6 (ns)	3.7 (ns)	1.1 (ns)	4.1 (ns)

Table R2 (for Reviewers)

Production of TNF- α by basophils upon triggering with the indicated TLR ligands. Top of each cell shows the mean stimulation index (production of cytokine with TLR ligands or anti-IgE divided by the production by unstimulated cells). Bottom of cells in which significant differences were found shows mean $\pm$ standard error of the mean (SEM) of the absolute production of the particular cytokine in response to the TLR ligand. Cells with bold text showed a significantly increased cytokine production when corrected for multiple testing; n= 10 stimulations; * indicates p value <0.05 using One-Sample T test for stimulation index higher than 1. Adapted from Suurmond et al Eur J Immunol. 2014 Feb;44(2):386-96.

References:

Suurmond et al. Activation of human basophils by combined toll-like receptor- and Fc ϵ RI-triggering can promote Th2 skewing of naive T helper cells. Eur J Immunol. 2014 Feb;44(2):386-96

Second Editorial Decision – 15 July 2014

Dear Dr. Rivellesse,

Please accept my sincere apologies for the prolonged delay in processing the re-review of your revised manuscript. It is a pleasure to provisionally accept your manuscript entitled "IgE and IL-33-mediated triggering of human basophils inhibits TLR4-induced pro-inflammatory activation of monocytes" for publication in the European Journal of Immunology.

For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Laura Soto Vazquez

on behalf of Dr. Silvano Sozzani

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