IgE AND IL-33-MEDIATED TRIGGERING OF HUMAN BASOPHILS INHIBITS TLR4-INDUCED MONOCYTE ACTIVATION

Chapter 3

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ABSTRACT

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.

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 (FcγRIIb) on inflammatory macrophages, leading to the suppression of antibody-dependent inflammation. Thus, IL-33 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 alarmin (*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) ($CD123^+$, $BDCA\text{-}2^+$), 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.

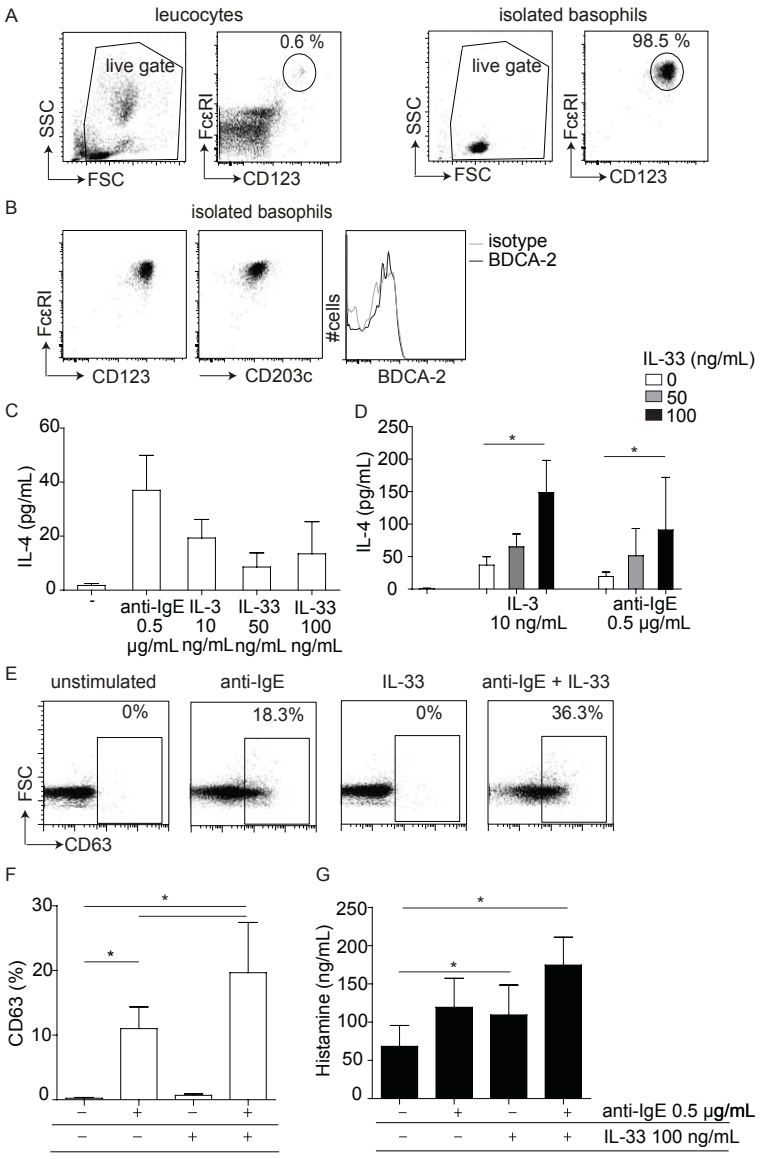


Figure 1. Activation of human basophils. (A) Basophils were isolated from peripheral blood leucocytes of healthy donors by immune-magnetic negative selection. Cells were analyzed by FACS to verify purity. A representative example is shown, with total leucocytes (left), and isolated basophils (right). After gating out debris using FSC/SSC, basophils were identified as $CD123^{+}Fc\varepsilon RI^{++}$ 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.

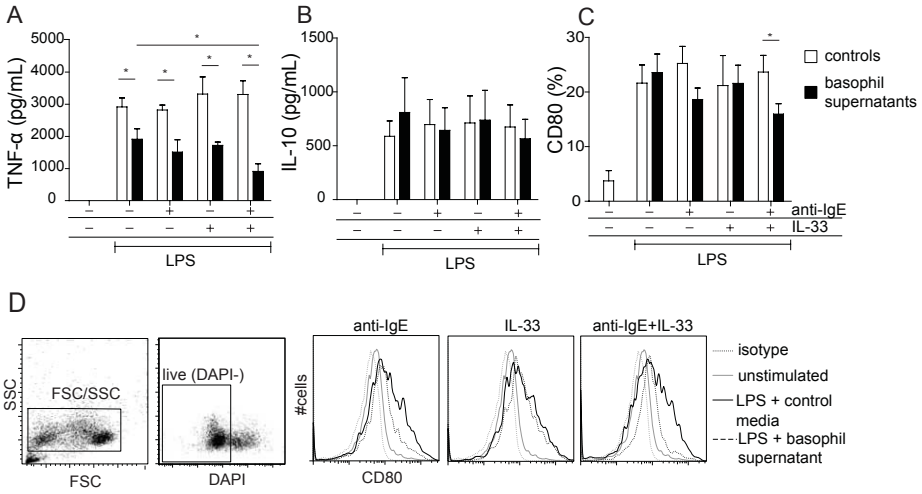


Figure 2. Effects of basophil supernatants on LPS-induced activation of monocytes. (A–C) Monocytes were isolated by positive selection using CD14 immunomagnetic beads, incubated with the supernatants of basophils (black bars) or with control media (white bars) and activated with LPS (5 ng/mL). The stimuli 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-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.

To further study monocytes activation, we analyzed surface markers by flow cytometry. While the expression of several markers, including activating and inhibitory Fc γ receptors (CD14, HLA-DR, CD16, CD64, CD32a, CD32b), were not significantly influenced (data not

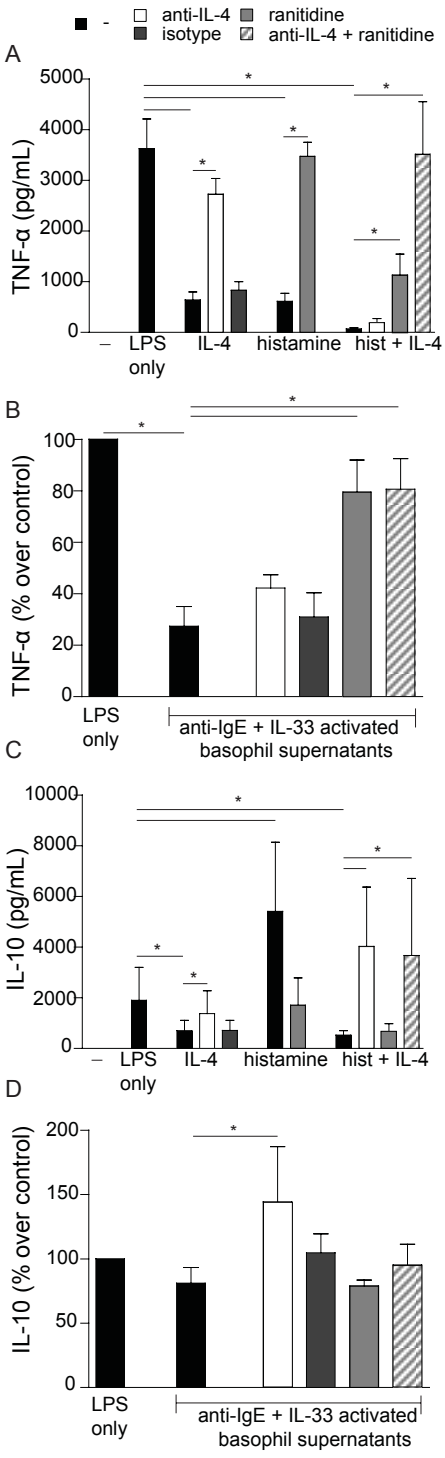
shown), basophils supernatants reduced the percentage of monocytes expressing the costimulatory molecule CD80 upon LPS stimulation (Fig. 2C and D). Although this effect was statistically significant only for the combined activation of basophils with IgE and IL-33, these data further confirm the ability 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 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 (Supplementary 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.

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-IL4 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.

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 basophils 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 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.

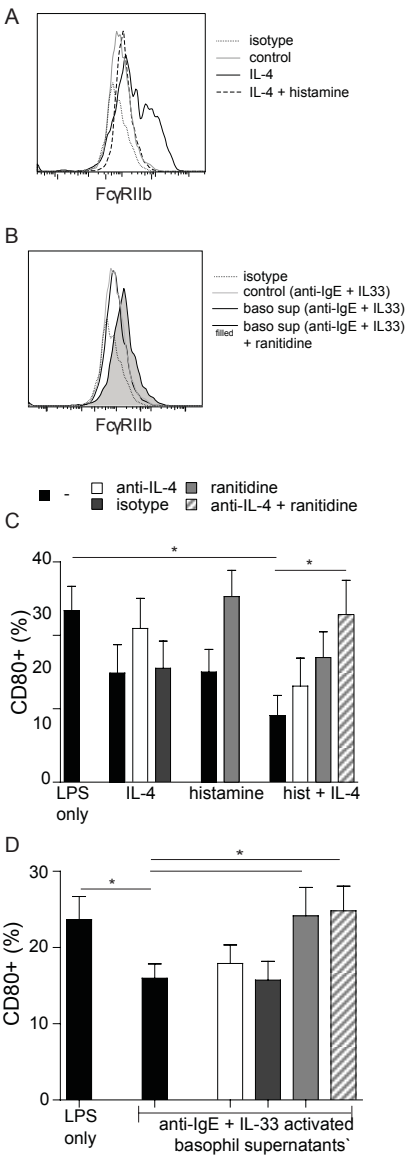


Figure 4. FcγRIIb and CD80 modulation by basophil supernatants. After 18 h of incubation with LPS (5 ng/mL) monocytes were analyzed by flow cytometry for the expression of surface markers. (A) FcγRIIb expressed by monocytes stimulated with rIL-4 (10 ng/mL), histamine (10−5 M), or both. (B) FcγRIIb expressed 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 as shown in Figure 2D. (C, D) Data are shown as 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.

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).

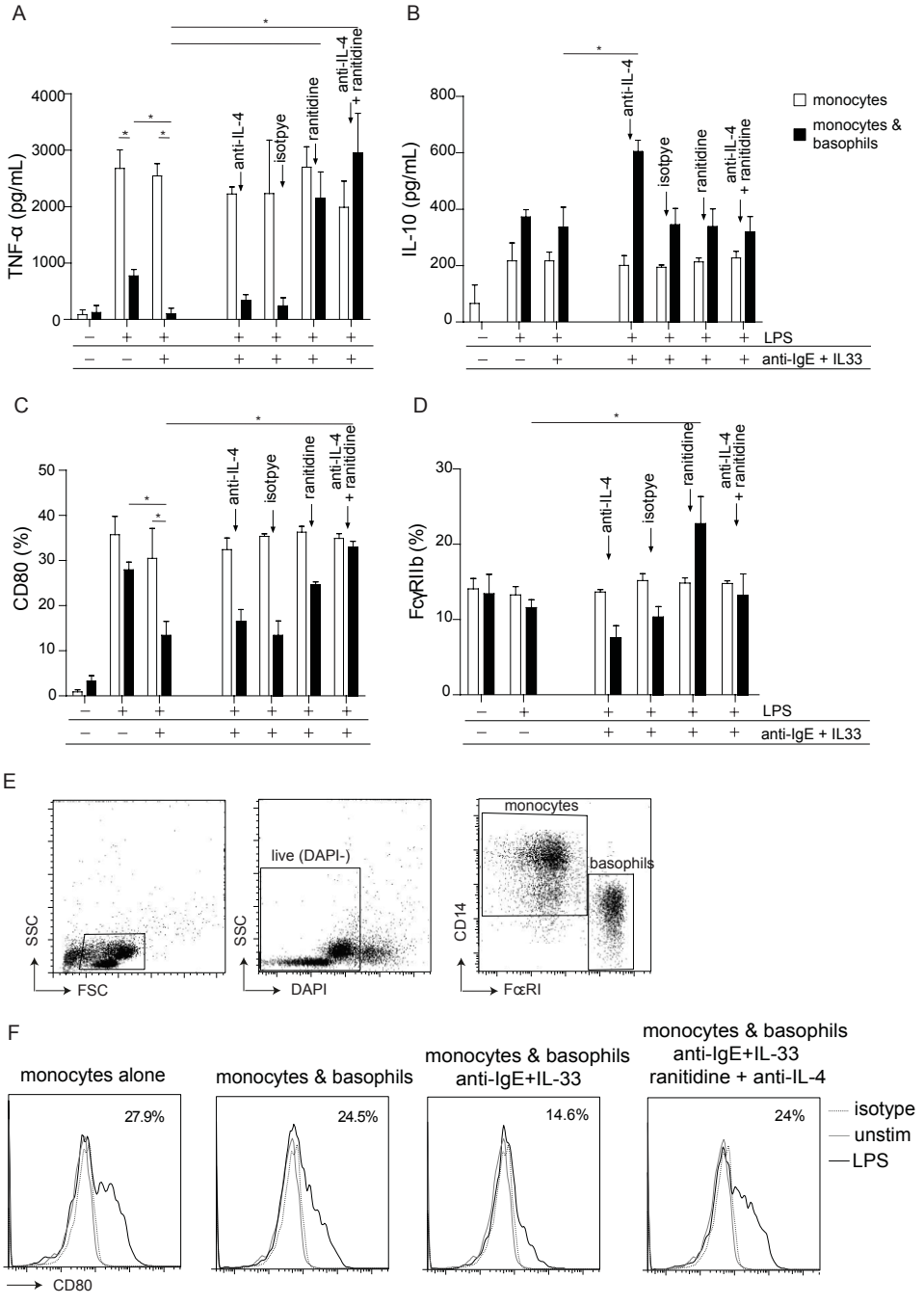
Furthermore, activated basophils in coculture with monocytes significantly inhibited LPS-induced CD80 expression with a contribution of both histamine and IL-4 (Fig. 5C and F). On the other hand, the expression of FcγRIIb was not influenced by the presence and activation of basophils, unless histamine 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 FcεRI 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 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*).


A
TNF-α (pg/mL)
anti-IL-4
isotpye
ranitidine
anti-IL-4 + ranitidine
B
IL-10 (pg/mL)
monocytes
monocytes & basophils
LPS
anti-IgE + IL33
C
CD80 (%)
D
FcγRIIb (%)
E
SSC
FSC
live (DAPI-)
DAPI
monocytes
basophils
CD14
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monocytes alone
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monocytes & basophils anti-IgE+IL-33
monocytes & basophils anti-IgE+IL-33 ranitidine + anti-IL-4
27.9%
24.5%
14.6%
24%
isotype
unstim
LPS
CD80

Figure 5 *(left)*. 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 µg/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) FcγRIIb 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 experi-ments. (A–D) Data are shown as mean + 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 comparisons of coculture conditions, ANOVA was used with Bonferroni's post-test.

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 (Supplementary 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-kB 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-kB 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 × 106 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 × 106 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 4ºC 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-PerCpCy5.5 (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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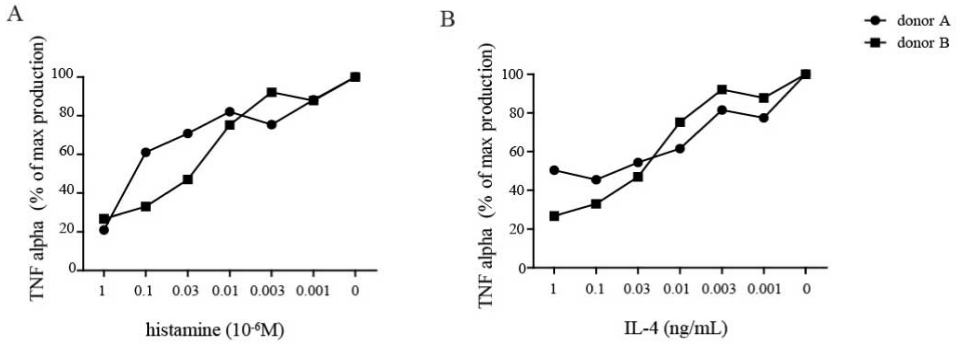
SUPPLEMENTARY FIGURE



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. Results from 2 independent experiments (2 donors) are shown.