Late IL-3–induced phenotypic and functional alterations in human basophils require continuous IL-3 receptor signaling

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ABSTRACT
Cytokines of the GM-CSF family signal via the same receptor subunit (βc) and, thus, have overlapping effects on cells that express all cytokine-specific α-chains (IL-3Rα, IL-5Rα, GM-CSF-Rα), such as human basophils, whose rapid effector functions are similarly enhanced by IL-3, IL-5, and GM-CSF. However, previous work has shown that IL-3, but not IL-5 or GM-CSF, supports and induces allergy-associated functions of human basophils at later time points. This includes induction of Th2 cytokine and chemokine secretion, high-affinity IgE receptor-independent leukotriene C4 (LTC4) formation, expression of enzymes (e.g., RALDH2, granzyme B), and kinases (e.g., Pim1). Here, we address the question of why IL-3, but not IL-5 or GM-CSF, is capable of inducing these late responses in human basophils, and we investigate the mechanism that underlies the unique regulatory capacity of IL-3. We find that IL-3, IL-5, and GM-CSF rapidly activate the same canonical signaling cascades in a qualitatively identical manner with comparable strength, but we identify signaling duration as a major discriminating factor. IL-5 and GM-CSF rapidly down-regulate surface levels of their receptors within minutes, concomitant with a rapid decay in signaling molecule activation and time-dependent loss of ability of these cytokines to prime basophils for functional responses. By contrast, IL-3 hardly down-regulates the α-chain of its receptor without depleting the common β-chain, which enables extraordinarily sustained signaling events, predominantly the activation of Stat5. Of interest, acute IL-3 signaling is not sufficient to induce persistent phenotypical and functional changes in human basophils. Induction of these functional late responses depends on continuous IL-3 receptor activation and signaling.

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Introduction
Basophils are increasingly recognized as key players in allergic diseases, such as atopic asthma. This rare granulocyte type is selectively recruited to sites of allergen challenge [1–3] and contributes to immediate-type hypersensitivity reactions by virtue of IgE-dependent and -independent release of mediators, such as histamine and LTC4 [4–11]. Activated basophils are also a prime source of the archetypal Th2 cytokines IL-4 and IL-13 [12–15], which are crucial to induction of isotype class switching to IgE [16, 17] and to amplify the differentiation of T helper cells toward a Th2 phenotype [18–20]. Both mast cells and basophils are able to secrete IL-13 independent of high-affinity IgE receptor signaling [12, 21]; however, of note, basophils are the only cell type capable of secreting IL-4 in an allergen-independent manner [7] and, hence, are considered as a source of early IL-4 that initiates the Th2 differentiation program [19, 22, 23]. The potential immunomodulatory role of basophils is further supported by our recent finding that basophil-derived retinoic acid provides an additional and novel mechanism for Th2 polarization [24].

Former studies by other groups and ours have shown that basophils are regulated by cytokines of the GM-CSF family (IL-3, IL-5, GM-CSF), which are produced by T cells or mast cells at sites of allergic inflammation [10–12, 25–28]. Receptors for IL-3, IL-5, and GM-CSF consist of a cytokine-specific α-chain [IL-3Rα (CD123), IL-5Rα (CD125), or GM-CSF-Rα (CD116)], and a common βc (CD131). βc is necessary for high-affinity binding and signal transduction [29, 30]. Hence, differential α-chain expression determines the reactivity of a cell type to the corresponding cytokine. Furthermore, a cell that expresses >1 type of α-chain is expected to respond in an identical manner to any of these cytokines as a result of the shared use of the βc signaling subunit.

Eosinophils express all 3 receptor α-chains [25, 31], and IL-3, IL-5, and GM-CSF regulate cellular functions, such as survival and expression of activation markers, in a qualitatively similar manner [25, 32]. Blood basophils also express the α-chains for all 3 cytokines [25, 27]. Accordingly, earlier studies showed that a short incubation with IL-3, IL-5, and GM-CSF prime these cells with identical efficacy for LTC4 formation and enhanced mediator release in response to diverse agonists, such as the complement fragment C5a [10, 11, 28]; however, more recent

Abbreviations: βc = β-chain, AhR = aryl hydrocarbon receptor, sIL-3Rα = anti–IL-3 receptor α-chain antibody, LTC4 = leukotriene C4, PKB = protein kinase B, RALDH2 = retinaldehyde dehydrogenase II

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studies from our and other laboratories showed that IL-3 is the only family member capable of inducing a variety of long-lasting functional and phenotypical alterations that are crucial to the effector and immunoregulatory functions of these cells. For example, culture with IL-3, but not IL-5 or GM-CSF, for ≥18 h primes basophils for a rapid burst of LTC4 formation in response to a short stimulation with C5a, a phenomenon termed late priming [6, 7]. Prolonged stimulation with a combination of C5a plus IL-3 primes basophils for a rapid burst of LTC4 formation in response to cross-linking, C5a, and IL-33 [7, 12, 14, 15, 27, 33, 34]. Furthermore, only IL-3 induces de novo expression of the retinoic acid–generating enzyme RALDH2 [24], up-regulates receptor activator of NF-κB ligand [35], and mediates expression of granzyme B [36], a cytotoxic serine protease that was believed to be restricted to lymphoid cells. More recently, we reported that IL-3, acid

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RESULTS

GM-CSF family cytokines promote qualitatively identical early signaling events in basophils and eosinophils

Basophils and eosinophils both express α-chains of the receptors of all GM-CSF cytokine family members (IL-3, IL-5, GM-CSF), albeit at different densities (IL-3Rα > IL-5α > GM-CSFRα in basophils and GM-CSFRα ≥ IL-5Rα > IL-3Rα in eosinophils) [25, 27]. Because both cell types express approximately the same number of IL-5Rα but differ in receptor density of IL-3Rα, we compared the activation of signaling events at different time points after IL-3 or IL-5 addition in both cell types. To achieve maximal efficacies, we used receptor-saturating concentrations of IL-3 and IL-5 in these experiments. Figure 1A shows that the Jak-Stat, PI3K-PKB, and Ras-MAPK signaling pathways that are known to be activated by IL-3 in factor-dependent cell lines are rapidly activated in human blood basophils in response to both IL-3 and IL-5, as evidenced by the phosphorylation of Stat5, Stat3, PKB, and Erk1/2. The same was true for eosinophils (Fig. 1B), except that we could hardly detect cytokine-induced phosphorylation of PKB over baseline (Figs. 1B and 2B).

Figures 2A and B illustrate the phosphorylation events induced by all GM-CSF family members 10 min after stimulation of basophils or eosinophils. Because we demonstrated in a previous study a weak activation of p38 MAPK by IL-3 [34], the effect of IL-5 and GM-CSF on p38 phosphorylation was also analyzed. Data show that, within the same cell type, all 3 cytokines activate the same early signaling pathways in a qualitatively identical fashion; however, data in Figs. 1 and 2 also show that strength and duration of the early signals induced by the different growth factors are diverse and also differ between basophils and eosinophils. Particularly prominent were the differences in Stat 3 phosphorylation, possibly because at high signaling strength, activation of the Jak-Stat pathway becomes more promiscuous. The order of efficacies of the early signaling events induced by these cytokines was IL-3 > IL-5 > GM-CSF in basophils and GM-CSF ≥ IL-5 > IL-3 in eosinophils, which reflects the order of expression levels of the corresponding cytokine receptor α-chains at the mRNA and protein levels [25, 27]. Differences in signaling strength elicited by IL-3, IL-5, and GM-CSF are also in line with their potency in inducing early cellular responses, such as rapid priming for C5a-induced LTC4 formation and enhancement of degranulation in basophils, as well as the rapid up-regulation of CD11b in both basophils and eosinophils [25].

IL-3 selectively induces continuous signaling in basophils with prominent and persistent Stat5 phosphorylation

Figure 2A provides a direct comparison of the phosphorylation status of key components of the different signaling pathways in basophils induced by the 3 related growth factors early (10 min) and late (18 h) after addition of stimuli. The most striking finding is a strong persistent activation of Stat5 observed upon culture with IL-3, but not with IL-5 or GM-CSF. The strong activation of Stat3, PKB, and Erk1/2 induced early after stimulation seemed to be only transient (see also Fig. 1A); however, prolonged exposure of Western blots revealed a weak residual IL-3-induced activity of Stat3, Erk1/2, and PKB (Ser743) in cells from most donors, whereas IL-5-dependent phosphorylation of these proteins was identical to the medium control (Fig. 2C). An approximately 2- to 3-fold increase in p38 phosphorylation was observed in basophils that were exposed for 18 h to IL-3 compared with cells that were cultured in medium alone or with IL-5 or GM-CSF (Fig. 2A). This phenomenon may be the result of the IL-3-specific induction of the serine/threonine kinase Pim1, as proposed earlier [32].

Figure 1. Kinetic of rapid signaling events in basophils and eosinophils. (A and B) Representative Western blots (n = 3) show a comparison of signaling strength and duration in response to IL-3 and IL-5 in basophils (A) and eosinophils (B) for the activation of Stat5, Stat3, PKB, and Erk1/2. Cells were stimulated with IL-5 or IL-5 (50 ng/ml each) for indicated time periods. The 0 time point represents extracts from unstimulated cells. β-Actin and total Stat3 served as loading controls.
IL-3, but not the other cytokine family members, also enhanced phosphorylation of the ribosomal protein S6 in a persistent manner (Fig. 2C and Supplemental Fig. 5).

All GM-CSF family members promote late Stat5 phosphorylation in eosinophils

A direct comparison of phosphorylation events in eosinophils exposed to IL-3, IL-5, or GM-CSF for 10 min and 18 h showed that, in contrast to basophils, all 3 cytokines were able to promote a sustained phosphorylation of Stat5, albeit to different degrees (Fig. 2B, D, and E). Of interest, the cytokine with the weakest effect on rapid signaling (IL-3) was the most effective in inducing late and sustained Stat5 activation. The order of efficacy of the cytokines to promote late Stat5 phosphorylation in eosinophils (IL-3 > GM-CSF > IL-5) contrasts with the order of efficacy of early signaling (GM-CSF ≥ IL-5 > IL-3) and was a consistent finding. These findings reflect the efficacy of cytokines to promote other late responses in eosinophils, such as protection from apoptosis [32] and up-regulation of CD69, and has been suggested to be the result of the up-regulation of the IL-3Rα chain by the GM-CSF receptor cytokine family, including IL-3 itself, and the down-regulation of IL-5Rα [25].

Figure 2D provides a comparison of sustained Stat5 activation induced by IL-3, IL-5, and GM-CSF in eosinophils after 18 h of culture. Bars represent mean ± SEM of n = 3. Cells cultured in medium only (nil) served as control sample for Dunnett’s multiple comparison post test. **P < 0.001.
Effects of blocking IL-3 receptors on basophils before or after IL-3 addition

We next investigated to what extent IL-3 receptor activation at early or later time points contributes to the different late phenotypic and functional changes in human blood basophils. To manipulate the cells as little as possible, we used an aIL-3Rα blocking antibody that was added either 30 min before or 3 h after culture with IL-3. Readouts were obtained after culture for either 24 h (Supplemental Fig. 3) or 48 h (Fig. 3). In the case of 48 h cultures, the antibody was also added after 24 h of culture with IL-3. In some experiments, basophils were treated with up to 10 µg/ml aIL-3Rα only, which did not promote cell activation or release of LTC4, IL-8, and IL-13 itself (data not shown). The most obvious finding was that the degree of inhibition was virtually identical regardless of whether aIL-3Rα was added 30 min before or 3 h after stimulation with IL-3; however, the degree of inhibition was variable depending on the readout. After 48 h of culturing, inhibition was complete for Stat5 and Erk1/2 phosphorylation, RALDH2 and granzyme B induction, IL-8 and IL-13 release, and late priming for C5a-induced LTC4 formation, but was only partial for Pim1 and AhR induction as well as CD44, CD54, and CD69 up-regulation. The findings obtained by adding aIL-3Rα after 24 h are more difficult to interpret as most late effects are already maximally induced after 24 h. Furthermore, the amount of the measured product also depends on the balance between production and degradation. However, inhibition of Erk1/2 and Stat5 phosphorylation and expression of Pim1, which has a rapid turnover and is transcriptionally regulated by Stat5 [40, 41], as well as the blocking of late priming indicate a requirement for continuous IL-3 signaling at later time points (see also Activation of basophils by IL-3 during 3 h does not promote any late effects).

To better understand the reasons for the differences in inhibitory efficacy illustrated in Fig. 3, we studied the effects of variable aIL-3Rα concentrations on late responses and phenotypical changes induced by a near maximally effective concentration of IL-3 (10 ng/ml). Of interest, aIL-3Rα inhibited the distinct responses with similar and high potency, despite some differences in efficacy (Fig. 4). Furthermore, examination of the capacity of a maximally effective dose of aIL-3Rα to inhibit the effects of different concentrations of IL-3 showed marked differences in the shift of the dose-response curve between the various late responses (Supplemental Fig. 4). Taken together, these data indicate that the partial inhibition only of some of the responses is not simply the result of insufficient affinity of aIL-3Rα; rather, it seems that the capacity of aIL-3Rα to reverse IL-3-mediated effects is inversely proportional to the efficacy of IL-3 to induce late responses.

Activation of basophils by IL-3 during 3 h does not promote any late effects

Rapid priming and early signaling in basophils, which is already optimally induced at low IL-3 concentrations, may not be efficiently inhibited by aIL-3Rα at the IL-3 concentration needed to induce late effects. Indeed, we found that aIL-3Rα did not affect acute Stat5 activation and only partially inhibited PKB and Erk1/2 phosphorylation upon stimulation with 10 ng/ml IL-3 (Supplemental Fig. 5). To exclude that the partial inhibition of some late responses is the result of insufficient suppression of early activation events, we cultured basophils for 3 h with and without IL-3, followed by washing and addition of buffer or IL-3 and/or aIL-3Rα, as depicted in Fig. 5. Data show that the combination of depleting IL-3 by washing and receptor blockade completely blocked all late responses if IL-3 was not re-added. Thus, our data demonstrate that all signaling events and gene induction programs that are triggered by IL-3 during the first 3 h are not sufficient to promote late phenotypic and functional changes in basophils.

Continuous IL-3 receptor signaling is required for inducing and maintaining functional and phenotypic properties

Data in Fig. 3 indicate that IL-3 signaling is ongoing, even after 24 h. To further examine the importance of these sustained signaling events, we analyzed IL-3–mediated up-regulation of the α-chain of the high-affinity IL-2 receptor, CD25, whose expression is increased over a longer time period [35]. By using this activation marker, we found that expression of CD25 steadily increased for up to 5 d and that this IL-3–induced CD25 up-regulation could be blocked by aIL-3Rα, even when added 2 d after initiation of the culture (Fig. 6A).

Late IL-3 priming for C5a-induced lipid mediator formation requires gene induction and protein expression, but the underlying mechanisms are only poorly understood [6, 26]. Late priming becomes optimal after overnight culture with IL-3 [6, 7]. Figure 6B illustrates that this functional change in basophils can be reversed and that the decay of late priming after removal of IL-3 was quite rapid. C5a-induced LTC4 formation was reduced by only 1 h after removal of IL-3, and basophils returned to an unprimed state within 24 h; thus, continuous signaling is required for inducing and maintaining the late effects of IL-3. Data also indicate that, whatever mechanism may be responsible for late priming, at least one of the necessary components must be short lived.

Receptor α-chains for IL-5 and GM-CSF, but not IL-3, are rapidly down-regulated by their ligands

One possible reason for the selectivity of IL-3 to promote late effects in basophils could be a result of differences in receptor down-regulation. To study IL-3, IL-5, and GM-CSF receptor expression, we incubated human basophils with 10 ng/ml IL-3, 3 ng/ml IL-5, or 1 ng/ml GM-CSF that did not interfere with the capacity of anti–CD123-PE, anti–CD125-PE, and anti–CD116-PE, respectively, to bind the corresponding receptors (Supplemental Fig. 6A). We found a rapid ligand-induced down-regulation of IL-3Rα and GM-CSFRα without recovery after 18 h. By contrast, IL-3Rα remained highly expressed after exposure to IL-3 (Fig. 7A). Absolute quantification of CD123, CD125, and CD116 confirmed the high and stable expression of IL-3Rα over 18 h and the down-regulation of IL-3Rα and GM-CSFRα within 1 h (Supplemental Fig. 6B). Absence of IL-3Rα down-regulation was not a result of nonsaturating IL-3 concentrations, as CD125 expression remained stable in cells, which were treated with a 3-fold higher concentration of IL-3 (300 ng/ml) than the receptor-saturating concentration (Supplemental Fig. 6C). The rapid receptor down-regulation correlated with a rapid decay of early priming for C5a-induced LTC4 formation promoted by IL-5.
Figure 3. Effects of IL-3 receptor blockade on basophils before or after IL-3 addition. Purified basophils were cultured for 48 h in absence (nil) or presence of IL-3 (10 ng/ml) or IL-3Ra (10 μg/ml) was administered to cells 30 min before (-0.5 h) or 3 or 24 h after addition of IL-3.  

Phosphorylation of Stat5 and Erk1/2 and expression of Pim1, RALDH2, granzyme B, and AhR was analyzed by Western blotting; Stat3 and β-actin were used as loading controls. Depicted are a representative blot (upper left) and the quantified results (n = 3–5) expressed as means ± SEM. (continued on next page)
and GM-CSF, whereas, in the case of IL-3, decay of early priming overlapped with the induction of the late priming phenomenon (Fig. 7E). Furthermore, the temporal pattern of decay of the acute signaling events induced by the diverse cytokines (Fig. 1) closely matched the time-dependent loss of early priming ability of the different GM-CSF family members (Fig. 7E).

**βc is down-regulated, but not depleted, by GM-CSF cytokine family members**

Measurements of absolute numbers of receptors showed that surface expression of βc is approximately one tenth of all-3Rα and at least 5 times higher than IL-5Rα and GM-CSFRα surface expression (Supplemental Fig. 6). Consistent with an internalization of α-β chain complexes, βc was more strongly down-regulated by IL-3 and more weakly by IL-5 and GM-CSF (Fig. 7B and Supplemental Fig. 6B); however, in contrast to IL-5Rα and GM-CSFRα, βc surface expression was always above staining of the isotype-matched control antibody. Of interest, 18 h after IL-3 addition, surface expression of βc slightly recovered and became similar to cells that were treated with GM-CSF and IL-5. Total βc expression as revealed by Western blotting was even higher in IL-3-treated cells (Fig. 7C and D), which indicated that IL-3 induces replenishment of βc. Curiously, after culture for 18 h, βc was strongly down-regulated, even without cytokine addition. The reason for this βc down-regulation in untreated cells is unknown and not a result of loss of cell viability and is also not found for other basophil surface proteins (e.g., CD25, CD44, CD122, AITR, BDCA-2) ([35] and unpublished observations).

**DISCUSSION**

An increasing number of reports in recent years have shown that prolonged exposure of mature human blood basophils to IL-3 can dramatically alter the phenotype and function of this granulocyte type [6, 7, 12, 24, 27, 32, 35, 36, 42, 43]. This bioactivity of IL-3 on basophils is not shared by any other cytokine, including other members of the GM-CSF family, which affect cellular functions only transiently [10, 11, 25-28]. Thus, the major aim of the present study was to better understand the unique capacity of IL-3 to regulate the functional late responses of blood basophils. Although this study clearly focuses on basophils, we have also included some comparative experiments with eosinophils, as they respond to all 3 GM-CSF family members in a similar fashion [25, 44, 45] and because basophil and eosinophil granulocytes are developmentally, phenotypically, and functionally closely related cell types, at least in the human system [46-48].

Most of our knowledge about the signaling pathways that are involved in mediating the functions of GM-CSF family cytokines comes from studies with cell lines and is limited to the examination of events that occur immediately after addition of a bolus of stimuli. Here, we show that IL-3, IL-5, and GM-CSF rapidly and transiently activate several canonical signaling pathways in both basophils and eosinophils in a similar manner that is consistent with the capacity of these cytokines to promote some early cellular functions in both cell types. However, the signaling strength elicited by the different family members was found to be different, closely reflecting the density of surface expression of the corresponding receptor α-chains on basophils and eosinophils [25, 27]. The diverse late phenotypic and functional changes that are induced by IL-3 in basophils in a unique and restricted manner require gene induction and de novo protein synthesis and proceed gradually over time.

Therefore, we also studied the degree of activation of critical components of several signaling pathways at later time points in cells that were cultured with IL-3, IL-5, or GM-CSF. The most obvious and striking finding was a strong and persistent activation of Stat5 selectively induced by IL-3 in a dose-dependent manner. The degree of Stat5 phosphorylation closely parallels the induction of the diverse late phenotypic and functional changes [24, 27, 32-34, 36] and this study). We thus propose that continuous Stat5 activation is critical in driving these cellular responses. This interpretation is supported by our comparative experiments with eosinophils, in which persistent Stat5 phosphorylation is induced by all 3 GM-CSF family cytokine members, albeit to different degrees that closely reflect the relative efficacies of the growth factors to promote CD69 expression [25, 45], induce the kinase Pim1 ([32] and this study), and inhibit apoptosis [25, 32]. In this regard, it is interesting to note that aberrant Stat5 activation has been implicated in the induction of certain leukemias and cancers [49, 50] and that Stat5 also regulates the proliferation and differentiation of diverse lymphocyte subsets [51, 52]. The present study suggests that Stat5 may also be an important regulator of the functions of nondividing, terminally differentiated granulocytes.

Another consistent finding was sustained phosphorylation of the ribosomal protein S6 in basophils that were cultured with IL-3 for prolonged periods of time. It is conceivable that Pim1, which is known to partially overlap with PKB in substrate specificity [53], is involved in the prolonged activation of S6 and other IL-3-induced late effects, but a lack of specific inhibitors makes it difficult to dissect the precise role of this kinase. Thus, the responsible signaling pathway and the functional role of the IL-3-induced enhancement of S6 phosphorylation in basophils are still unclear.

Erk1/2 is believed to play a central role in the generation of C5a-mediated LTC4 by delivering one of the 2 signals needed for cytosolic phospholipase A2 activation [54]. In contrast, as only a transient IL-3-mediated Erk1/2 phosphorylation was observed,
**Figure 4. aIL-3Rα shows a high potency but variable efficacy in suppressing IL-3–induced late responses.** Basophils were cultured for 24 h without (nil) or with IL-3 (10 ng/ml) in the absence or presence of titrated concentrations of aIL-3Rα. (A–C) Stat5 activation and expression of Pim1, RALDH2, granzyme B, and AhR was analyzed by Western blotting (A and B), and IL-8 and IL-13 were measured in culture supernatants (C). Representative Western blot (A) and quantification of band intensity (B) are shown. (C) Cytokine release expressed as percentage of IL-3–induced response in the absence of antibody that ranged from 2216 to 2344 and from 355 to 387 pg/10^6 basophils for IL-8 and IL-13, respectively. Bars in panels B and C represent means ± SEM of n = 3. Cells cultured in medium only (nil) served as control sample for Dunnett’s multiple comparison post test. ns = not significant. *P < 0.05; **P < 0.01; ***P < 0.001.
Vilarion et al. [55] proposed an alternative pathway for how IL-3 primes human basophils for anti–IgE-dependent LTC4 formation; however, by prolonged exposure of Western blot membranes, we observed in almost all experiments a weak sustained activation of the PI3K-PKB and, especially, Ras-Erk1/2 pathways. In light of our data, it is thus conceivable that the preconditioning of this enzyme by weak persistent Erk1/2 activity is involved in the poorly understood phenomenon of late IL-3 priming for C5a-induced LTC4 formation.

Of interest, experiments that were aimed at arresting the bioactivity of IL-3 at different time points after initiation of the culture by addition of an IL-3 receptor blocking antibody
and/or by removal of IL-3 by washing showed that all signaling events and gene induction programs that occur within the first 3 hours are sufficient to promote any of the late effects that we have studied. This was a rather surprising finding, because in light of the conventional view of how acute signaling is transformed into long-lasting and permanent phenotypic alterations, one might expect that the cascade of events that likely follows IL-3 addition irreversibly commits the cells to manifest at least some of the late IL-3–mediated responses. Our results, however, indicate that continuous signaling via the IL-3 receptor is necessary at any time to promote and maintain the different phenotypic and functional changes in basophils. A number of observations are consistent with this interpretation, such as the inhibition of Stat5 phosphorylation, Pim1

Figure 6. Continuous activation of the IL-3 receptor is required to sustain CD25 up-regulation and maintain late priming in human basophils. (A) IL-3–induced up-regulation of CD25 requires persistent receptor activation. Basophils were cultured for 1, 2, or 5 d with IL-3 (30 ng/ml). At the indicated day of culture, IL-3 receptor was blocked by administering all-3Rα (20 μg/ml) for the remaining culture period. After 1, 2, or 5 d, surface expression of CD25 was analyzed by flow cytometry. Data are given as percentage of CD25 expression of basophils cultured with IL-3 for 1 d (mean ± SEM, n = 3). Cells that were cultured with IL-3 for 1 d served as a control sample for Dunnett’s multiple comparison post test. (B) Decay of IL-3–induced late priming for C5a-mediated LTC4 formation. Basophils were cultured for 18 h in the presence of IL-3 (10 ng/ml), then washed to remove IL-3. Cells were further cultured in medium that contained either no (black columns) or 10 ng/ml IL-3 (gray columns) and stimulated with C5a (10 nM) for 20 min at indicated times (after washing). Percentage of LTC4 (mean ± SEM, n = 3) relative to LTC4 formed by IL-3–primed cells upon triggering with C5a added after 10 min is shown. Maximal response of LTC4 formation was 1114–18,234 pg/10⁶ basophils. Cells that were exposed to medium, IL-3, or C5a only did not produce LTC4 (data not shown). ns = not significant. ***P < 0.001.

Figure 7. Ligand-induced down-regulation of receptors and decay of rapid priming after stimulation of basophils with GM-CSF family cytokines. (A–D) Basophils were exposed to medium only (nil) or to IL-3 (10 ng/ml), IL-5 (3 ng/ml), or GM-CSF (1 ng/ml) for the indicated time periods. (A and B) IL-5 and GM-CSF, but not IL-3, rapidly down-regulate their corresponding receptor α-chains. After incubation with IL-3, IL-5, or GM-CSF, surface expression of IL-3Rα, IL-5Rα, and GM-CSFRα (A) and βc (B) was measured by flow cytometry. Shown are the means ± SEM mean fluorescence intensity (MFI) values, n = 3. Dotted line represents MFI of isotype control. (C and D) Expression of βc in freshly isolated (FR1) and cultured basophils was analyzed by Western blotting. Representative Western blot (C) and quantification of band intensities (mean ± SEM, n = 3) (D) are shown. (E) Time course of the decay of rapid priming and of the induction of late priming. Basophils were primed for the indicated time periods in medium that contained IL-3, IL-5, or GM-CSF (10 ng/ml each), then stimulated with C5a (10 nM) for 20 min. LTC4 release into the supernatant is shown (mean ± SEM, n = 3). Cells that were cultured in medium only (nil) served as control sample for Dunnett’s multiple comparison post test. ns = not significant. ***P < 0.001.
expression, and the late priming for C5a-induced LTC4 formation at 48 h that is observed when the receptor is blocked 24 h after the initiation of the culture with IL-3. The study also shows that IL-3 signaling with concomitant sustained Stat5 activation is ongoing as long as IL-3 is present and that this continuous IL-3 receptor activity is needed for long-lasting up-regulation of CD25 and the permanence of late priming for LTC4 formation. The search for the component(s) that is (are) responsible for late priming can thus be limited to genes that are up-regulated by IL-3 in a persistent manner and that, in addition, encode for a protein with a rapid turnover. As an example, the constitutively active kinase Pim1 qualifies as such a candidate.

The lack of sustained signaling in basophils that were cultured with IL-5 or GM-CSF can be explained by a rapid ligand-induced down-regulation of IL-5Rα and GM-CSFRα that does not recover over time. By contrast, IL-3Rα surface expression remains high for prolonged time periods, even in the presence of high IL-3 concentrations. The kinetics of relative and absolute surface expression of the α- and β-chains indicate which the α-β receptor complex is internalized, which results in rapid depletion of α-chains for IL-5 and GM-CSF. The hardly detectable down-regulation of IL-3Rα chain can be explained by the large excess of α-chain compared with βc. Although IL-3 leads to a rapid and strong down-regulation of βc, which is reflected by a particularly high strength of early signaling, the remaining βc seems to be sufficient to allow sustained signaling. Whether and to what extent IL-3-induced de novo expression of βc is necessary for sustained signaling remains uncertain, as inhibitors of mRNA and protein synthesis are not comfortable with survival of basophils and because knockdown is not feasible in this terminally differentiated cell type. The importance for replenishing receptors in promoting persistent Stat5 activation is also supported by the data on eosinophils presented here, together with earlier investigations that show that stimulation of eosinophils with IL-5 promotes ligand-induced down-regulation of surface receptors and IL-5Rα mRNA expression [31, 45]. By contrast, IL-3Rα mRNA is up-regulated by all GM-CSF family cytokines, including IL-3 itself [31, 45]. This may explain why, in human eosinophils, sustained Stat5 phosphorylation is highest in response to IL-3 but not IL-5. Taken together, these data indicate that persistent receptor expression, either as a result of very high levels at start and/or replenishment of receptors during activation, strongly influences the cellular response to a ligand. The findings reported here might also be applicable to other receptors and ligands. We thus propose that the receptor expression pattern of a cell mainly determines which ligand is able to promote rapid cellular functions, whereas persistent receptor expression and signaling is required to promote more profound and long-lasting cellular responses.

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DISCLOSURES

The authors declare no conflicts of interest.

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AUTHORSHIP

S.S.K. acquired, analyzed, and interpreted data, wrote the draft of the manuscript, and prepared the figures. A.O. acquired, analyzed, and interpreted data and revised the manuscript. C.A.D. and M.F. initiated and supervised the project and approved the final version of the manuscript.

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Late IL-3–induced phenotypic and functional alterations in human basophils require continuous IL-3 receptor signaling

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