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Essential mechanisms of differential activation of eosinophils by IL-3 compared to GM-CSF and IL-5

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Abstract

There is compelling evidence that the eosinophils bring negative biological outcomes in several diseases, including eosinophilic asthma and hypereosinophilic syndromes. Eosinophils produce and store a broad range of toxic proteins and other mediators that enhance the inflammatory response and lead to tissue damage. For instance, in asthma, there is a close relationship between increased lung eosinophilia, asthma exacerbation, and loss of lung function. The use of an anti-IL-5 therapy in severe eosinophilic asthmatic patients is efficient to reduce exacerbations. However, anti-IL-5-treated patients still display a relatively high amount of functional lung tissue eosinophils, indicating that supplemental therapies are required to damper the eosinophil functions. Our recent published works, suggest that compared to IL-5, IL-3 can more strongly and differentially affect eosinophil functions. In this review, we will summarize our and other investigations that have compared the effects of the three β-chain receptor cytokines (IL-5, GM-CSF and IL-3) on eosinophil biology. We will focus on how IL-3 differentially activates eosinophils compared to IL-5 or GM-CSF.

Keywords

IL-3; IL-5; GM-CSF; beta-chain cytokine receptors; eosinophils

INTRODUCTION

Mature eosinophils are non-dividing leukocytes that are recruited to tissues in response to parasites, allergens, and solid tumors, among other causes. Tissue eosinophils are implicated in harmful outcomes in a variety of diseases, including allergy, asthma and hypereosinophilic syndromes. Eosinophils perform their damaging functions through degranulation and the release of intracellularly stored toxic granule proteins and a variety of cytokines.[1] IL-3, IL-5, and GM-CSF are critical cytokines involved in eosinophil development and biology. These three cytokines trigger intracellular signals via a common β-chain (βc) receptor, and yet can differentially affect eosinophil functions. Multiple, potential reasons may explain these differential properties among IL-3, IL-5, and GM-CSF.

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Obvious explanations are the regulation and trafficking of their specific α-chain receptors on the eosinophil surface, and their specific downstream intracellular signaling in a βc-chain-independent manner.[2] We and others have shown that IL-3 is more potent than IL-5 or GM-CSF to increase eosinophil proteins, including CD48, CD13, semaphorin-7A and CD32.[3–6] In addition, we have shown that when combined with TNF-α, IL-3 was more effective than IL-5 or GM-CSF to increase the expression level of a group of genes, including MMP-9, activin-A and the thymic stromal lymphopoietin receptor (TSLPR).[7–9] Therefore, among the βc cytokines, IL-3 possesses unique characteristics to activate eosinophils.

Because IL-5 receptor alpha (IL5Rα) expression is typically limited to eosinophils and basophils, IL-5 is an ideal therapeutic target to reduce eosinophilia. Anti-IL-5 biologics (mepolizumab/Nucala® and reslizumab/Cinqair®) have been recently approved to treat severe eosinophilic asthma.[10, 11] The use of anti-IL-5 therapies reduces asthma exacerbations by ~50% and allows reduction of high dose corticosteroid maintenance therapy in severe asthmatic patients with persistent eosinophilia. Unfortunately, these therapeutics do not completely block all exacerbations and are not effective in all patients with eosinophilic asthma.[12, 13] This may be due, in part, to the limited effect of IL-5-neutralizing antibodies on airway eosinophils.[14] Thus, it is important to study the mechanisms of action of other eosinophil-activating cytokines, such as IL-3. In this article, we will review how the three βc-chain cytokines differentially impact eosinophil biology and how their specific α-chain receptors are regulated on the cell surface. We will discuss critical biological functions for the eosinophil, including cell differentiation, survival, adhesion, degranulation, and migration. Finally, we will review what is known regarding the mechanisms involved in prolonged signaling and specific protein translation in IL-3-activated eosinophils. For the most part, we have limited our discussion to human eosinophils, as there are considerable differences between human and mouse eosinophils.[15] that are beyond the scope of this review. A number of the studies reviewed are from the late 1980's to early 1990's because these earlier works concomitantly studied all three cytokines, thus allowing a better comparison of their differential functions.

I. SOURCE OF IL-3 AND ITS RELEVANCE IN EOSINOPHILIC ASTHMA

In addition to its effects on myeloid progenitors and macrophages, IL-3 activates eosinophils and basophils,[16] which are key players in allergic inflammation. The main cellular sources of IL-3 are T cells and mast cells.[17] In fact, all three βc family cytokines are produced by *ex vivo*-activated blood[18] and airway[19] T cells obtained from allergic asthmatics. Mast cells also secrete IL-3 and GM-CSF after allergen-mediated IgE stimulation.[20] Interestingly, eosinophils and neutrophils can release preformed or newly synthetized IL-3 between 1 and 24 hours after activation with phorbol myristate (PMA).[21]

Along with IL-5 and GM-CSF, IL-3 was detected by immunohistochemistry in nasal polyps; however, levels of IL-3 were comparatively lower in tissue homogenates.[22] In a report by Koller *et al.*, an IL-3 sputum level of >30 ng/ml was measured in patients with cystic fibrosis and bronchial asthma, while IL-3 was undetectable in subjects with pneumonia.[23] In addition, the amounts of sputum IL-3 strongly correlated with levels of eosinophil granule proteins, and with decreased lung function.[23] In asthma, polymorphisms in the IL-3 gene

have been associated with disease development.[22, 24] IL-3 is elevated in serum from poorly controlled asthmatic patients;[25] and, in a population of symptomatic asthmatics, IL-3 positive cells in the bronchoalveolar lavage (BAL) were associated with asthma severity.[26] Of note, in addition to subjects with airway allergy or asthma, subjects with eosinophilic esophagitis also have elevated levels of IL-3 in their blood plasma.[27] Together with IL-4, IL-5 and GM-CSF, IL-3 transcript elevation was observed in skin biopsies during an allergen-induced late-phase cutaneous reaction.[28] Additionally, a local airway allergen challenge in mild asthma leads to increased levels of IL-3 in the BAL fluid.[5, 29] Finally, and importantly, while airway eosinophils display reduced surface α-chain for the IL-5 receptor, they show increased surface α-chain for the IL-3 receptor compared to blood eosinophils.[30, 31]

II. COMMONALITIES AND DIFFERENCES AMONG IL-3, IL-5, AND GM-CSF EFFECTS ON EOSINOPHILS

A. Differentiation

Human eosinophils originate from $CD34^+$ pluripotent hematopoietic stem cells (HSC) located in the red bone marrow. Downstream from the HSC, common myeloid progenitors (CMP) will differentiate into either granulocyte/macrophage progenitors (GMP), megakaryocyte/erythrocyte progenitors (MEP) or $CD34^+IL\text{-}5R\alpha^+$ eosinophil lineage-committed progenitors (EoP).[32] These $CD34^+IL\text{-}5R\alpha^+$ EoP are the exclusive origin of mature human eosinophils and their numbers are increased in the bone marrow, circulation and tissues of patients with eosinophilic diseases.[33] At the transcriptional level, the differentiation of CMP into $CD34^+IL\text{-}5R\alpha^+$ EoP requires downregulation of the GATA-1-binding transcription factor FOG1, allowing increased GATA1 function.[34] PU.1 (SPI1)[35] is then required for the final maturation into eosinophils. In addition, X-box binding protein 1 (XBP1) is a critical requirement for survival and terminal differentiation of murine EoP.[36]

EoP express other key receptors required for its development including IL-3Rα and GM-CSFRα, and the granule proteins eosinophil peroxidase (EPX) and major basic protein 1 (MBP).[37] Therefore, IL-3, IL-5, and GM-CSF can all participate in EoP proliferation and differentiation until the mature non-proliferative stage is reached. IL-5 quickly matures eosinophils from the bone marrow, suggesting that IL-5 activates differentiation from existing eosinophil progenitor cells, rather than from stem cells. It is generally thought that IL-3 and GM-CSF are crucial in the early stage of eosinophil differentiation from $CD34^+$ cells while IL-5 is required for their final maturation.[38, 39] Yet, IL-5 deficient mice[40–42] and anti-IL-5-treated humans[14] retain a limited number of eosinophils. This suggests that the maturation of eosinophils does not require IL-5 and that eosinophilopoiesis can occur in an IL-5-independent manner. Pre-incubation of mouse bone marrow cells with IL-3 (not IL-5, GM-CSF, C-CSF, or CSF-1) significantly increases the numbers of eosinophils produced upon subsequence activation by IL-5.[43] The combination of IL-3 plus IL-1 during the pre-incubation stage further enhances the number of IL-5-responsive eosinophil progenitors.[43] IL-5 activation of human bone marrow induces colonies of highly pure and mature eosinophils, while IL-3 or GM-CSF induces 5 times more colonies that are comprised of less than 20% mature eosinophils and colonies that are contaminated with 5% neutrophils and

basophils.[44] In another study, human bone marrow cultured with IL-5 in liquid medium displayed 38% and 72% of differentiated eosinophils after 14 days and 28 days, respectively.[45] At 21 days, contaminants found were surviving cells already present at the origin of the culture, including macrophages (10%) and lymphocytes/monocytes/neutrophil myelocytes (11%). In this same study, the addition of IL-3 to progenitors in semi-solid medium led to more eosinophil colonies compared to IL-5 or GM-CSF; although, in long-term (3 weeks) liquid medium cultures, IL-5 differentiated more eosinophils than IL-3, and much more than GM-CSF.[45] This suggests that IL-5 activates less precursor types present in the bone marrow compared to IL-3, but it is more potent for the stimulation of EoP proliferation. Unfortunately, beyond recording eosinophil differentiation, these studies did not analyze the biology and functions of eosinophils differentiated in media lacking IL-5. For instance, it is legitimate to ask whether eosinophils matured without exogenous IL-5 are biologically similar to eosinophils matured with IL-5.

B. Survival

The number of eosinophils in the airways is associated with asthma severity.[46] Delayed apoptosis and necrosis are required for eosinophil accumulation in tissues and are thus important factors in eosinophil pathogenesis. All three of the βc family cytokines (IL-3, IL-5, and GM-CSF) attenuate eosinophil death, particularly apoptosis.[47] In a model of mouse bone marrow-derived eosinophils, IL-3 and GM-CSF showed little activity to generate eosinophils compared to IL-5.[48] However, IL-5 and GM-CSF were equivalent in their ability to maintain the viability of differentiated eosinophils, while IL-3 was somewhat less effective.[48] It was also reported that GM-CSF was more potent than IL-3 and IL-5 to prolong *ex vivo* eosinophil survival for 4 days.[49] In another study, increasing concentrations of the three cytokines were used on blood eosinophils for 48 hours with no significant differences among the cytokines on cell survival, although IL-3 seemed a little inferior to IL-5 or GM-CSF for concentrations >100 pg/ml.[50] In agreement with this study, we have recently published data showing human blood eosinophil survival in the presence of either of the three βc family cytokines.[31] The cytokines were used at 2 ng/ml (~130 pM) and survival was determined from 2 to 16 days. IL-5, IL-3 and GM-CSF had a very similar effect on eosinophil survival during the first 6 days in culture. Longer-term cultures (12 days) demonstrated that GM-CSF is significantly superior to IL-5 or IL-3 in promoting survival.[31] However, according to cytokine consumption analyses in this same study, IL-5 consumption by eosinophils is minimal over time. In contrast, as much as 2 ng of IL-3 was consumed by eosinophils in 48 hours, whereas only ~200 pg/ml of the initial 2 ng/ml of GM-CSF was used.[31] In fact, while ~2 ng/ml of IL-5 were still present after 3 days of culture, IL-3 was already totally consumed. Therefore, the rapid consumption of IL-3 should be taken into account when comparing the effect of the three βc family cytokines on eosinophil survival, and studies examining eosinophil survival should compare the ability of the cytokines to prolong eosinophil survival at a constant concentration over time. In accordance with our observations, Ohnishi *et al.* had shown that any of the three βc family cytokines used at a very low dose (20 pg/ml) increased blood eosinophil survival from ~5% to ~80% after 4 days in culture.[51] In a study by Tai *et al.*, blood eosinophils from patients with hypereosinophilic syndrome were incubated for 10 days with IL-5, GM-CSF or IL-3.[52] The authors concluded that all three cytokines significantly prolong eosinophil survival with

IL-5>GM-CSF>IL-3. However, IL-5 was used at 0.5 ng/ml, whereas GM-CSF and IL-3 were used at a very high concentration (~150 ng/ml). It is our experience that high concentrations of cytokines (≥10ng/ml), can trigger eosinophil cytolysis. Therefore, under these conditions, it is uncertain whether IL-5 was more potent than IL-3 and GM-CSF to maintain eosinophil survival, or whether eosinophil lysis was induced by the high concentration of IL-3 and GM-CSF. In conclusion, it is safe to say that as long as they remain present in culture at a moderate level (≤2 ng/ml), all three βc family cytokines have a very similar effect on eosinophil survival.

C. Adhesion/Chemotactism/Migration

Adhesion is an important event in eosinophil biology. By means of eosinophil interaction with endothelial cells, adhesion directs the early step for eosinophil recruitment into the tissue, preceding extravasation.[53] It is generally accepted that *in vivo*, expression of the α4β1 integrin on eosinophils interacting with the adhesion molecule VCAM-1 present on endothelial cells is critical for eosinophil recruitment into tissues.[53] Ip *et al.* demonstrated that adhesion of eosinophils to epithelial cells was increased by IL-3 and IL-5, but not by GM-CSF.[54] It may be important to note that, in this study, IL-3 and IL-5 were used at 20 ng/ml while GM-CSF was used at an even higher concentration (50 ng/ml). In another model, the effect of βc family cytokines on human blood eosinophil adhesion to serum-treated zymosan (STZ) coated with complement fragment iC3b was measured.[55] All three cytokines strongly and rapidly (within 5 minutes) increased eosinophil adhesion to STZ via enhanced surface presence of the complement receptor type 3 (CR3).[55] Reimert *et al.* showed that eosinophil adhesion to human serum albumin-coated wells was induced to a similar extent by all three cytokines.[56] Of note, adhesion was CD18 (integrin β2)-dependent. Fattah *et al.* reported that all three cytokines increased eosinophil binding to IgG, ICAM-1 or VCAM-1, with IL-3 being about 10-fold less potent than either IL-5 or GM-CSF.[57] This last study seems to be in disagreement with the work by Ip *et al.*, although Ip used 20 ng/ml of cytokines, and at this high concentration, Fattah also observed no difference among the three cytokines to stimulate eosinophil adhesion on ICAM-1 and VCAM-1.

Following eosinophil differentiation in the bone marrow, mature eosinophils must enter the blood stream and migrate to the assaulted tissue. After extravasation through the vascular endothelium, the recruitment of the eosinophils to the inflamed site is directed by chemotactism toward a chemical gradient. Complement C5a, platelet activating factor (PAF), the eicosanoids (leukotriene B4 (LTB_4) and prostaglandin D2), and the ligands for CC-chemokine receptor 3 (CCR3) (RANTES, MCP-4, and eotaxin 1–3) are the major chemoattractants for eosinophils.[58] Warringa *et al.* compared chemotactism of eosinophils for 2.5 hours toward PAF, LTB_4, C5a and IL-8 after a short pre-activation (30 min) with increasing concentrations of either GM-CSF or IL-3.[59] Both GM-CSF and IL-3 increased eosinophil chemotactism toward LTB_4 and IL-8, although at different cytokine concentrations. While the optimal concentration of GM-CSF for both LTB_4- and IL-8-mediated chemotactism was as low as 15 pg/ml, 10 to 100 fold more IL-3 was required. Interestingly, only IL-3, not GM-CSF, strongly increased eosinophil chemotactism toward PAF, and the effective concentration of IL-3 was as low as 15 pg/ml.[59] Of note, GM-CSF and IL-3 themselves were chemotactic for eosinophils during the 2.5 hour assay, although

10-fold more IL-3 (1.5 ng/ml) was required to observed a significant effect. This was confirmed by another paper that showed chemoattractant activity for the three βc family cytokines, with IL-5>GM-CSF>IL-3, on blood eosinophils from subjects having eosinophilia and asthma.[60]

Cell migration is closely related to chemoattraction since chemoattraction leads to cell movement. However, different types of chemoattractants and environmental conditions can induce different types of cell migration. Cells in a liquid phase moving directionally toward an increasing gradient of a chemoattractant, such as PAF or $LTB_{4,}$ define “chemotaxis” while “chemokinesis” is defined by random and non-directional movement. Otherwise, “haptotaxis” is used to describe movement of cells along an insoluble extracellular matrix protein (ECM). We have previously reported that IL-3-activated eosinophils adhere to plexin-C1.[5] Plexin-C1 is expressed by stromal cells and is known to only bind to semaphorin-7A.[61] We wanted to know whether eosinophils could migrate on plexin-C1, and whether compared to IL-5, IL-3-activated eosinophil migration on plexin-C1 was enhanced. We had previously shown that compared to IL-5 or GM-CSF, IL-3 activation for 20 hours further enhanced semaphorin-7A levels on the eosinophil surface.[5] Therefore, we anticipate that compared to IL-5, eosinophil pre-activation with IL-3 for 20 hours would induce greater migration on plexin-C1. As shown on Figure 1A, we found that eosinophil migration on plexin-C1 was increased by IL-3 activation compared to both non-activated cells and IL-5-activated cells. Eosinophil migration was further enhanced by the presence of the chemoattractant, eotaxin, which tended to limit the differential effect of IL-3 compared to IL-5. Migration on plexin-C1 without the requirement of a chemotactic compound (no eotaxin) indicates that eosinophils use a haptotaxic process on plexin-C1, similarly to their migration on an ECM protein such as periostin.[62] Although, the only known ligand for plexin-C1 is sempahorin-7A,[63] IL-3-activated eosinophil migration on plexin-C1 was significantly, yet only slightly, inhibited by a peptide inhibitor analog to the semaphorin-7A binding site for plexin-C1 (Figure 1B). In contrast, neutralizing antibodies to the αMβ2 integrin almost totally blocked eosinophil migration on plexin-C1 (Figure 1C). It is unknown whether the peptide inhibitor was not efficient in blocking plexin-C1/semphorin-7A interaction, or whether plexin-C1 possesses another ligand besides semaphorin-7A.

Moser *et al.* have reported that eosinophils migrate almost equally across a non-activated and IL-1-activated endothelial cell line monolayer after a 24 hour activation of eosinophils, with only 150 pg/ml of IL-5, IL-3 or GM-CSF.[64] Yet, IL-3 activation tends to be less potent than IL-5 or GM-CSF. However, as described above, 150 pg/ml of IL-3 is rapidly consumed by eosinophils.[31] It would be interesting to compare the effect of cytokine concentration on eosinophil migration. In another study, higher concentrations of the βc family cytokines (5 ng/ml) were added on blood eosinophils during transmigration through a layer of epithelial cells.[65] In this condition, all three cytokines equally enhanced eosinophil migration compared to non-activated eosinophils, and increased migration was blocked by an antibody to the β_2 integrin.[65] Likewise, it has been reported that an addition of either of the three βc family cytokines at the beginning of an eosinophil migration assay through Matrigel® (a gelatinous mixture of ECM proteins such as laminin and collagen), significantly increased eosinophil migration; although, at a lower concentration, IL-3 seemed less efficient than IL-5 or GM-CSF.[66]

In summary, altogether these studies indicate that all three βc family cytokines increase eosinophil adhesion, chemotactism and migration. Although, at lower doses (~100 pg/ml), IL-3 was less efficient than IL-5 or GM-CSF. The decreased potency of IL-3 was probably partially due to the high consumption of IL-3 by eosinophils and as described later, the requirement of a longer activation time for IL-3 compared to IL-5 or GM-CSF. Yet, the three cytokines display differential effects on adhesion, chemoattraction, and migration depending on the substrate (IgG, VCAM-1, ICAM-1, iC3b or plexin-C1), and the chemoattractant (LTB4, IL-8 or PAF).

D. Degranulation

Degranulation remains the main event by which eosinophils can affect tissue integrity and inflammation. The release of stored mediators including toxic granule proteins, cytokines, chemokines, and growth factors from eosinophil granules may happen when 1) intact granules are discharged due to cell lysis, 2) granule contents are secreted through the cytoplasmic membrane (exocytosis), or 3) specific granule proteins are released by a process known as piecemeal degranulation (reviewed[67]). The release, or not, of toxic or pro-inflammatory intracellular materials *in vivo* is closely linked to the mechanism associated with eosinophil death (either apoptosis or necrosis or necroptosis). Apoptotic eosinophils are phagocytosed by macrophages and epithelial cells that digest eosinophil contents, while necrotic and necroptotic[68] eosinophils lose membrane integrity and consequently release granules and mediators leading to tissue damage and inflammation. Importantly, eosinophil adhesion via interaction of its integrins (particularly αMβ2) with appropriate ligands is a required step for eosinophil degranulation.[69, 70] In Reimert's study mentioned above, GM-CSF-induced adhesion of eosinophil on albumin- or IgG-coated tissue culture plates preceded degranulation and the release of granule proteins by about 1 hour.[56] One of the first *in vitro* models to study eosinophil degranulation was the binding of eosinophils on aggregated immunoglobulins (IgG or IgA).[71] Notably, degranulation on immunoglobulins typically leads to eosinophil lysis and does not involve piecemeal release of cytokines.[72] Therefore, the studies using IgG or IgA are exclusively measuring release of toxic proteins, such as EDN. Eosinophils activated with either IL-5, GM-CSF, or IL-3 for 1 hour and then cultured in the presence of secretory IgA for 4 hours release a very similar amount of the granule protein, EDN.[73] In another study, eosinophils activated for 4 hours with GM-CSF, IL-5 or IL-3 (10 ng/ml) in human serum albumin-coated tissue culture plates released 12, 8, and 6% of their total EDN content, respectively.[74] We have, however, recently shown that blood eosinophils activated for 20 hours with IL-3 and seeded on aggregated human IgG-coated on tissue culture plates, highly degranulate (25 % of total cellular EDN); whereas, activation with IL-5 results in release of only 10% of total cellular EDN.[6] As described by us and others, eosinophil degranulation on aggregated IgG is αMβ2 integrin and CD32 (low affinity receptor for IgG; FCγRII)-dependent.[6, 75, 76] The more potent ability for IL-3, compared to IL-5 or GM-CSF, to prime eosinophils to degranulate on IgG is probably due to increased production and a prolonged activation state of both the αMβ2 and CD32.[6] The notion of a delayed and prolonged effect of IL-3 compared to IL-5 or GM-CSF, and the mechanisms involved in production and prolonged activation of αMβ2 and CD32 in IL-3-activated eosinophils, will be further developed below in the section on "Differential protein translation of semaphorin-7A and CD32 by IL-3". Therefore, for longer periods of

activation, IL-3 seems to be superior to IL-5 or GM-CSF to trigger eosinophil degranulation on immunoglobulins.

E. Gene and protein expression

Compared to IL-5 and GM-CSF, IL-3 is more potent in activating eosinophil's release of certain proteins. We have shown that, compared to IL-5 or GM-CSF, the combination of IL-3 with TNF-α leads to a high mRNA expression level of several genes, including MMP-9 and activin-A.[7, 8] MMP-9 and activin-A mRNA expression levels were dependent on the mRNA stabilization downstream of mitogen-activated protein (MAP) kinase signaling. More than IL-5, but similarly to GM-CSF, IL-3 potently induced newly synthetized CR3 when activation was prolonged for 24 hours.[77] Calcium ionophore-induced leukotriene C_4 ($LTC_{4)}$ production by blood eosinophils obtained from subjects with moderate-to-severe asthma is potentiated by all three βc family cytokines after 30 min, with IL-5>GM-CSF>IL-3.[78] However, while the effect of GM-CSF and IL-5 declined after 1 hour, the effect of IL-3 was delayed but gradually augmented LTC_4 release from 1 to 6 hours.[78] Interestingly, the ionophore-induced release of LTC_4 after incubation with IL-3 for 6 h was observed in eosinophils obtained from subjects with severe asthma, but not eosinophils from normal or mild asthmatic subjects.[78]

IL-3 has a strong effect on eosinophil surface proteins. Surprisingly, eosinophil activation for 24 hours with IL-3, but not IL-5 or GM-CSF, results in down-regulation of cell surface CCR3, via activation of the phosphatidylinositol-3 kinase (PI3K) pathway and receptor internalization.[79] On a longer term (24 h), IL-3 reduced CCR3 mRNA expression levels. In contrast to the down-regulation of CCR3, a short activation (30 min) of blood eosinophils with ≥ 10 ng/ml of cytokines indicates that IL-3 was less effective than IL-5 or GM-CSF to induce eosinophil expression of CD11b (αM)[50] However, we found that after 20 hours of activation, IL-3 augments both αM and β2 on the eosinophil surface.[6] Similarly, Hartnel *et al.,*[80] showed that compared to IL-5 or GM-CSF, long-term activation (24 h) led to a stronger effect of IL-3 on expression of the activation marker CD69 for cytokine concentrations ranging from 0.5 ng/ml to 10 ng/ml.[50] CD13 (ANPEP) is another surface protein strongly increased by a 24 hour activation of eosinophils with IL-3 (1 ng/ml) compared to IL-5 or GM-CSF (both at 1 ng/ml), while CD13 mRNA was not changed.[4] Furthermore, at a concentration >2ng/ml, IL-3 was the only of the three βc family cytokines to increase the production of surface CD48 on eosinophils at 18 h.[3] Kinetic studies indicate that IL-3-induced CD48 production is significant only after 10 hours,[3] suggesting that IL-3-induced IL-3 receptor augmentation may be required. Therefore, unlike IL-5 and GM-CSF, long-term activation with IL-3 enhances the production of a group of proteins that can dramatically impact the function of eosinophils.

In summary, compared to IL-5 and GM-CSF, long-term activation with IL-3 is better suited to induce expression and production of a subset of genes. Potential mechanisms include the regulation and the dynamics of the surface α-chain receptors and differential intracellular signaling, which we discuss in Sections III and IV.

III. THE COMMON BETA-CHAIN CYTOKINE RECEPTORS

IL-3, IL-5, and GM-CSF receptors are heterodimeric with a cytokine-specific α-chain and a common β-chain that initiates intracellular signaling. The cytokines bind to their respective α-chain with low affinity (nanomolar); however, subsequent recruitment of the β-chain results in a conformational change to a high affinity (picomolar) binding complex. βc is unique in that it is displayed as a preformed interlocked homodimer.[81] At the point of insertion into the cell membrane the C-terminal tails are separated by over 120 Å, preventing interaction of βc subunits.[81] Thus, a dynamic change in the receptor is required for signaling. The crystal structure of the human GM-CSF/GM-CSFRα/βc ternary complex revealed a hexamer structure composed of 2 binary complexes of GM-CSF/GM-CSFRα bound together via a single βc homodimer. Two hexamer complexes are packed head-to-head to form a dodecamer complex, which is required for signaling.[82] The dodecamer formation provides a conformational structure that moves the central intracellular βc domains into close proximity (~10 Å versus ~120 Å in the hexamer formation) to enable JAK2 transphosphorylation and initiation of the JAK2/STAT5 signaling pathway.[82, 83]

The receptors for IL-3, GM-CSF and IL-5 can be controlled by their respective ligand and by other members of the βc family cytokines. IL-3Rα mRNA and protein levels are up-regulated by all three cytokines with GM-CSF>IL-5 or IL-3.[31, 84–87] Increased eosinophil IL-3Rα occurs at the transcription level[87] and is dependent on activation of the PI3K pathway.[86] Of note, whereas IL- 5 or GM-CSF induces a rapid (within 3 h) and transient increase in IL-3Rα mRNA accumulation, the effect of IL-3 is delayed such that accumulation of mRNA does not peak until 24 h.[85] This delayed effect of IL-3 is reminiscent of what we have observed for IL-3-mediated up-regulation of genes for activin,[7] MMP-9[8], and IL-1β (unpublished observation). In contrast to their positive effect on IL-3Rα, the three βc cytokines induce a strong and prolonged down-regulation of IL-5Rα on human peripheral blood eosinophils, whereas surface GM-CSFRα remains generally constant.[31, 84–87] Down-regulation of IL-5Rα occurs very rapidly (within 30 min) and is cytokine dose-dependent. Corresponding with the nearly undetectable level of cell surface IL-5Rα, eosinophils are no longer able to bind IL-5[87] or release EDN[84] or superoxide[86] upon subsequent IL-5 exposure. They do however, remain functionally intact in response to IL-3 or GM-CSF. We demonstrated that attenuation of membrane IL-5Rα (mIL-5Rα) corresponds to eosinophil release of a soluble form of the receptor (sIL-5Rα).[84] Inhibition of matrix metalloproteinases (MMPs) reversed both the down-modulation of mIL-5Rα and the increase in sIL-5Rα,[84] indicating that proteolytic release of mIL-5Rα may be a mechanism for accumulation of sIL-5Rα. Due to high affinity binding to IL-5, sIL-5Rα is a potent mIL-5Rα antagonist.[88] In addition to surface cleavage, sIL-5Rα can also be generated by alternative splicing of the mIL-5Rα gene.[88] Transcripts for sIL-5Rα are predominant in early eosinophil progenitors. IL-5 (but not IL-3 or GMCSF) induces a switch to expression of mIL-5Rα.[89] Conversely, in mature human eosinophils, IL-5, IL-3, or GM-CSF down-regulate both receptor isoforms.[86, 87]

Importantly, much of the described *ex vivo* data have been recapitulated *in vivo.* We have demonstrated that eosinophils recruited to the airway following an allergen challenge of atopic subjects, have elevated surface levels of GM-CSFRα and IL-3Rα, and significantly

less mIL-5Rα than their circulating counterparts.[30, 31] The observed decrease in mIL-5Rα on allergen-induced BAL eosinophils has been confirmed by Julius, *et al.*[90] We have also established the presence of sIL-5Rα in BAL fluid of allergic subjects.[30] Concentrations were increased after an airway allergen challenge and were associated with increased levels of IL-5. We did not observe up-regulation of sIL-5Rα gene expression on BAL eosinophils compared to circulating eosinophils, suggesting that cell surface shedding of mIL-5Rα in response to IL-5 may contribute to the increase in sIL-5Rα. Gevaert *et al.* showed that sIL-5 is present in nasal polyp homogenates and that eosinophils from nasal polyps have less cell surface mIL-5Rα than blood eosinophils.[91] Another study demonstrated sIL-5Rα in serum of patients with highly elevated circulating eosinophils.[92] Concentrations of sIL-5Rα were associated with increased numbers of blood eosinophils and concentrations of serum IL-5.[92]

Taken together, these *in vitro* and *in vivo* studies indicate that IL-5Rα expression and function are regulated by multiple mechanisms including alternative IL-5Rα gene splicing and cell surface shedding of IL-5Rα. Conversely, IL-3Rα expression is increased in activated-eosinophils explaining the differential consequences of IL-3 and IL-5 stimulations.

IV. DIFFERENTIAL PROTEIN TRANSLATION OF SEMAPHORIN-7A AND CD32 BY IL-3

A. The ERK/RSK/RPS6 signaling

The βc family cytokines trigger an intracellular kinase cascade leading to the phosphorylation of a number of intracellular proteins. IL-3, IL-5, and GM-CSF activate (PI3K), the MAPK (ERK and p38) and the signal transducer and activator of transcriptions (STAT) pathways. Just downstream of the ligand-induced β-chain receptor activation, members of the proto-oncogene tyrosine kinase SRC (c-SRC), such as LYN (LCK/YES novel tyrosine kinase), and SYK, can tyrosine phosphorylate a number of intracellular proteins in eosinophils, including the β-chain receptor itself.[93] Van der Bruggen *et al.*[94] have analyzed the effect of the three βc family cytokines on general tyrosine phosphorylation in eosinophils. The optimal cytokine concentrations to induce tyrosine phosphorylation after 30 min were 150 pg/ml, 1.5 ng/ml and 15 ng/ml for GM-CSF, IL-5 and IL-3, respectively. At these optimal concentrations, high protein tyrosine phosphorylation was observed by activation with any of the cytokines between 5 and 60 min, but phosphorylation was dampened at the next 120 min time point.[94] The weakest effect from IL-3 compared to the other two cytokines on early intracellular signaling was expected for the reason that fresh blood eosinophils possess less IL-3Rα than IL-5Rα and GM-CSFRα.[85] Interestingly though, after 1 hour of activation, IL-3 and IL-5 have a very comparable strength to prime eosinophils in order to respond to a second activation with a ligand to a G protein-coupled receptor.[95] Unlike for a short-term activation, long-term cytokine activation of eosinophils substantially changes the potential of the three βc family cytokines on eosinophil signaling. We have shown that IL-3 activation leads to a prolonged (> 48 h) phosphorylation of the ribosomal protein S6 (RPS6), downstream of p90S6K (RSK) and ERK phosphorylations, while IL-5 and GM-CSF could not maintain this intracellular signaling for more than a few hours.[5] Intriguingly, prolonged signaling by IL-3 compared to IL-5 or GM-CSF has also been reported in basophils.[96] Yet, we do not know whether the strong and rapid stimulation

of IL-5 and GM-CSF quickly turns on a receptor containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) that would damper the RSK/RPS6 signaling. It has been shown, for instance, that activation of the ITIM-containing receptor, CD300a suppresses some of the IL-5 and GM-CSF effects, including ERK phosphorylation, on eosinophils.[97] Another explanation for the prolonged effect of IL-3 on eosinophils, is the enhanced presence of its specific receptor (IL-3Rα) at the cell surface between 4 and 24 hours after the beginning of activation.[31, 85] Conversely, cell surface IL-5Rα and GM-CFRα levels are reduced or maintained, respectively, during eosinophil activation.[31]

B. IL-3 induces translation of a subset of mRNAs in eosinophils

For some genes, including MMP-9, TSLPR and activin-A, a differential effect of IL-3 compared to IL-5 or GM-CSF is not seen unless eosinophils are stimulated with the cytokines plus TNF-α.[7–9] In TNF-α plus IL-3-activated eosinophils, the MMP-9 and activin-A mRNA expression levels were highly increased, and mRNA increased levels were controlled at least partially at the post-transcriptional level through increased mRNA stabilization. Conversely, the first protein that we found differentially increased by IL-3 used alone (no TNF-α) was semaphorin-7A, which was not altered at the mRNA expression level, but was enriched in the polyribosome cell compartment indicating increased semaphorin-7A mRNA translatability.[5] Downstream of ERK, IL-3-induced increased semaphorin-7A translation was p90S6K-dependent. P90S6K is a well-known substrate for ERK, and is a RPS6-phosphorylating kinase.[98] Although it was not evaluated in our study, semaphorin-7A increased translation was also likely RPS6-phosphorylation-dependent. This is because it has been suggested previously, in proliferating cells, that phosphorylation of RPS6 enhances protein synthesis from a subset of mRNAs by stimulating their binding to the 40S ribosomal subunit.[99] It is thought that this mechanism involving RPS6 phosphorylation allows rapid translation of pre-existing transcripts. Semaphorin-7A mRNA is abundant in resting eosinophils; however, semaphorin-7A increased translation does not happen during the first 4 hours of activation with IL-3, raising the possibility that in addition to RPS6 phosphorylation, there is another factor requiring long-term cytokine stimulation. Importantly, while IL-3 induced the translation of semaphorin-7A by >10-fold, it increased global translation by less than 1.5-fold compared to GM-CSF.[5] This demonstrates that IL-3 strongly targets a subset of mRNAs in eosinophils. Another identified IL-3-mRNA target is the low affinity IgG receptor, CD32.[6] In humans, three different genes code for CD32: CD32A (FCGRIIA), CD32B (FCGRIIB) and CD32C (FCGRIIC). Though, FCGRIIA protein amount was not changed by IL-3, we were able to show the up-regulation of FCGRIIB/C protein in eosinophils, exclusively after long-term (20 h) activation with IL-3 (not with IL-5 or GM-CSF).[6] In addition, as for semaphorin-7A, CD32B/C was also regulated at the translational level by the RSK pathway. The direct implication of RPS6-phosphorylation on semaphorin-7A and CD32B/C protein translation remains to be demonstrated. Importantly, all of the differential characteristics of *in vitro* IL-3-activated eosinophils were found *in vivo* in eosinophils recovered from the airways of allergen challenged patients, including increased surface expression of semaphorin-7A and CD32B/C, and activation of the p90S6K/RPS6 pathway.[5, 6, 31] These *in vivo* data do not prove, yet they do suggest that IL-3 could be involved in these mechanisms *in vivo* as well.

CONCLUSION

Via their βc-chain receptor subunit, IL-3, IL-5 and GM-CSF have many common functions on eosinophils. The differential effect of IL-3, among the three βc cytokines, seems to be partially directed by the dynamic of their specific α-chain receptor on the cell surface. Freshly prepared blood eosinophils have low levels of surface IL-3Rα, which increases overtime when eosinophils are activated. Due to low level of IL-3Rα on fresh mature eosinophils, functions needing only low doses of cytokine, such as survival, are very similarly affected by either of the three cytokines. However, for functions requiring higher doses of cytokine and higher IL-3/IL-3Rα signaling, IL-3 displays typically weaker effects on short-term incubation. Conversely when eosinophil activation is prolonged (>10 hours), surface IL-3Rα is increased and IL-3 displays more potent effects on most of the eosinophil functions, compared to IL-5 or GM-CSF, which specific receptor decreases or remains at a constant level on the cell surface overtime. Among the critical enhanced functions due to prolonged IL-3-induced eosinophil activation and its consequent prolonged intracellular signaling (MAPK/p90S6K/RPS6), is the translatability of specific transcripts including semaphorin-7A and CD32B/C. Consequently for instance, IL-3-driven CD32 and integrin surface augmentation and activation lead eosinophils to strongly degranulate on IgG. To date, it remains important to identify the exhaustive list of proteins specifically augmented by IL-3 to further acknowledge the dramatic impact of IL-3 on the eosinophil biology. Because IL-3Rα is active on other critical cells besides the eosinophils, it remains important to identify specific mechanisms implicated in IL-3-induced eosinophil functions in order to propose new therapeutic targets to treat eosinophilia.

A summary of the differential functions of IL-3 compared to IL-5 and GM-CSF is shown in Table. 1.

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Abbreviations

BAL	bronchoalveolar lavage
βc	β chain
CCR3	CC-chemokine receptor 3
EDN	eosinophil-derived neurotoxin
EOS	eosinophils
EoP	eosinophil lineage-committed progenitors

FCGRII	receptor for Fc fragment of IgG, low affinity II
IL5Rα	IL-5 receptor-α
HA-IgG	heat aggregated IgG
LTB4	leukotriene B4
p90S6K	90-KDa ribosomal S6 kinase
PAF	platelet activating factor
SBP-Ag	segmental bronchoprovocation with an allergen

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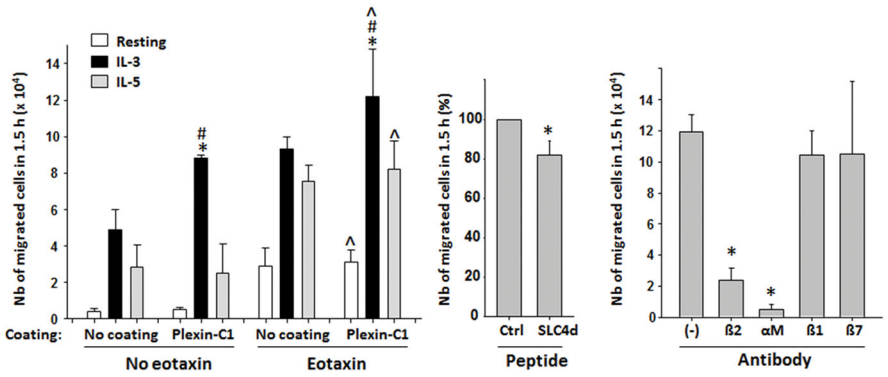

Figure 1. IL-3 increases eosinophil migration on plexin-C1

Peripheral blood eosinophils were activated with IL-3 (2ng/ml), IL-5 (4ng/ml) or were left without cytokine (resting) for 20 h. Cells (3×10^5) were placed into Transwell inserts (polycarbonate membrane, 3μm pores, 6.5 mm, Corning, Lowee, MA) coated with plexin-C1 (R&D Systems, 10μg/ml, overnight at 4°C) or into uncoated inserts (no coating). Then the inserts were saturated with fetal bovine serum (FBS) for 1 hour at 37°C. Cell culture medium with or without eotaxin (10 nM) was added into the bottom chamber. One hundred fifty μl of 2×10^6 eosinophils were added to each insert, and the migrated cells in the bottom chamber were counted at 90 min. A/Graph represent an average ±SEM of 3 (no coating, no eotaxin) or 5 experiments. * indicates that IL-3-activated cells migrated more than IL-5-activated cells. # indicates that IL-3-activated eosinophils migrated more on plexin-C1 than on an uncoated membrane. ^ indicates that migration on plexin-C1was increased by eotaxin versus no eotaxin. B/Cells were activated with IL-3 for 20 hours and were added on plexin-C1-coated inserts pre-incubated with either SLC4d (CRGDQGGESSLSVSKWNTF; ProteGenix, France) or the control (Ctrl) peptide (KLGFTYVTIRVTYQIRVAG) (100 nM). The number of migrated cells in 90 min in the presence of SLC4d is a % of the migrated cells with control peptide. Graph is an average ±SEM of 3 experiments. * indicates difference is statistically significant. C/Cells were activated with IL-3 for 20 h, were incubated with the indicated neutralizing antibodies (10μg/ml) for 15 min and were seeded on plexin-C1-coated inserts. Migrated cells in the bottom chamber after 90 min were counted and the graph is an average ±SEM of 3 experiments. * indicates statistical differences from no antibody.

Table 1

Differential IL-3 functions on eosinophils, compared to IL-5 or GM-CSF

Functions	Take-home messages
Differentiation	IL-3 and GM-CSF are crucial in the early stages, while IL-5 is important for final maturation during eosinophil differentiation. However, maturation can happen without IL-5. Human bone marrow activated with IL-5 forms colonies of highly pure and mature eosinophils, while IL-3 or GM-CSF forms more colonies, but with less mature eosinophils.
Survival	All three β-chain cytokines have a very similar effect on eosinophil survival, which requires only a low dose of cytokines. On long–term eosinophil cultures, the high consumption of IL-3 (1 ng of IL-3 per million eosinophils per day) must be taken into account when comparing cytokines.
Adhesion	IL-3, IL-5, and GM-CSF have relatively similar effects on eosinophil adhesion. Although, after a short-term activation a low dose of IL-3 may be less potent than IL-5 or GM-CSF to promote adhesion via the integrins.
Chemotactism/Migration	Activation with either of the cytokines may have differential effects on eosinophil chemoattraction depending on the chemoattractant. Long-term pre-activation with the cytokines indicates that IL-3 is more potent than IL-5 to induce eosinophil migration on plexin-C1 via semaphorin-7A and the αMβ2 integrin. At ng doses of cytokine for 24 h, IL-3, IL-5 and GM-CSF have similar efficacy to induce eosinophil transmigration through a layer of epithelial cells via the β2 integrin.
Degranulation	After a short-term (4 h) activation, eosinophil degranulation is slightly higher for GM-CSF and IL-5, compared to IL-3. However, a longer-term (20 h) activation places IL-3 ahead of the three cytokines via higher production and activation state of αMβ2 integrin and CD32.
Protein production	After a long-term activation (~24 h) activation, more potently than IL-5 or GM-CSF, IL-3 induces production of CD11b (αM), CD13, CD32B/C and CD48, CD69, CR3 and ICAM-1.
Signaling and Protein Translation	Whereas IL-5 and GM-CSF activate the ERK/p90S6K/RPS6 signaling for a few hours (1 to 6 h), IL-3 prolongs this signaling for >48 h. Subsequently, a subset of transcripts, including CD32B/C and semaphorin-7A are translated into proteins.
β-chain Receptor surface expression	Fresh mature blood eosinophils display a lower level of IL-3Rα compared to IL-5Rα and GM-CSFRα on their surface. However, eosinophil activation with either of the three β-chain cytokines leads to a strong increase of the IL-3Rα, maintenance of the GM-CSFRα, and reduction of the IL-5Rα.
	Summary: the impact of each cytokine on eosinophil biology depends on the function that is analyzed. Although globally, it is safe to say that fresh blood and mature eosinophils possess a low level of IL-3Rα and require a higher dose of IL-3 compared to IL-5 and GM-CSF. Conversely, after >4 h of activation, unlike IL-5Rα and GM-CSFRα, surface IL-3Rα is upregulated, and IL-3 becomes more effective than IL-5 or GM-CSF to enhance some eosinophil functions, such as migration and degranulation.