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IL-3 Up-regulates and Activates Human Eosinophil CD32 and α MB2 Integrin Causing Degranulation

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Abstract

Background—Eosinophils contribute to the pathogenesis of multiple diseases, including asthma. Treatment with antibodies targeting IL-5 or IL-5 receptor α reduces the frequency of asthma exacerbations. Eosinophil receptors for IL-5 share a common β -chain with IL-3 and GM-CSF receptors. We recently reported that IL-3 is more potent than IL-5 or GM-CSF in maintaining the ERK/p90S6K/RPS6 ribosome-directed signaling pathway, leading to increased protein translation.

Objective—We aimed to determine disease-relevant consequences of prolonged eosinophil stimulation with IL-3.

Methods—Human blood eosinophils were used to establish the impact of activation with IL-3 on IgG-driven eosinophil degranulation, which was then compared to IgG-mediated degranulation of freshly isolated (unstimulated) airway eosinophils activated *in vivo* by segmental allergen challenge.

Results—When compared to IL-5, continuing exposure to IL-3 further induced degranulation of eosinophils on aggregated IgG via increased production and activation of both CD32 (low affinity IgG receptor) and α MB2 integrin. In addition, unlike IL-5 or GM-CSF, IL-3 induced expression of CD32B/C (FCGR1B/C) subtype proteins, without changing CD32A (FCGR1A) protein and CD32B/C mRNA expression levels. Importantly, these *in vitro* IL-3-induced modifications were recapitulated *in vivo* on airway eosinophils.

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Conclusions—We observed for the first time upregulation of CD32B/C on eosinophils, and identified IL-3 as a potent inducer of CD32- and α M β 2-mediated eosinophil degranulation.

Keywords

Allergen challenge; CD32; degranulation; eosinophils; IL-3

Introduction

Asthma exacerbations are often associated with eosinophilic airway inflammation, and therapies targeting eosinophils decrease the frequency of asthma exacerbations [1]. The molecular events that link airway eosinophils with asthma exacerbations are not fully known but probably involve eosinophil release of toxic granule proteins (including eosinophil cationic protein/ECP and eosinophil-derived neurotoxin/EDN) and inflammatory mediators (cytokines and chemokines).

Blood or airway eosinophilia defines a major asthma phenotype. Most of the patients with this phenotype control their disease with inhaled corticosteroids, while a subset of patients with severe eosinophilic asthma have little or no response to high doses of inhaled corticosteroids, and are candidates for eosinophil-targeted anti-IL-5 therapies [2]. IL-5 is a cytokine that is critical for eosinophil development, survival and functions. Treatments with monoclonal antibodies targeting IL-5 dramatically reduce circulating eosinophils and decrease the frequency of asthma exacerbations in severe asthmatic subjects with eosinophilia [3, 4]. However, the effect on lung tissue eosinophils is much less pronounced compared to blood [5]. Because IL-5, GM-CSF and IL-3 signal via a common β -chain receptor and therefore possess many redundant functions [6, 7], blocking one cytokine may not mitigate all their effects on eosinophil functions. Importantly, in addition to the redundancy among these cytokines, each cytokine-specific α -chain receptor can signal via separate signaling pathways. In fact, GM-CSF and IL-5 can impact cell survival in a β -chain-independent manner [8-10]. Also, these cytokines can differentially regulate their receptors, with IL-5 and IL-3 decreasing and increasing, respectively, their specific α -chain receptor [11-13]. We and others have demonstrated that compared to IL-5 or GM-CSF, IL-3 is a more potent inducer of certain eosinophil functions, including cell surface expression of CD48 [14], aminopeptidase N (CD13) [15], ICAM-1 (CD54) [16], activin-A [17], metalloproteinase-9 [18], thymic stromal lymphopoietin receptor [19] and semaphorin-7A [20]. More recently, we found that unlike GM-CSF and IL-5, IL-3 maintains intracellular signaling via the ERK/p90S6K/RPS6 pathway for >24 h, which leads to increased protein translation of semaphorin-7A [12]. Notably, IL-3 maintains signaling in basophils leading to production of proteins otherwise not induced by IL-5 or GM-CSF [21].

IL-3 is relevant in allergy since it is released by activated Th-2 lymphocytes and by mast cells and basophils following IgE cross-linking [22]. In asthma, polymorphisms in the IL-3 gene have been associated with disease development [23, 24]. In addition, IL-3 is elevated in serum in poorly controlled asthmatic patients, and airway IL-3-positive cells are increased with asthma severity [25, 26]. Furthermore, segmental allergen challenge in mild asthma leads to elevated levels of IL-3 in the BAL fluid [20, 27], and while airway eosinophils

display reduced surface α chain for the IL-5 receptor, they show increased IL-3 receptor compared to blood eosinophils [12, 28].

Eosinophil-driven pathology involves the release (degranulation) of preformed toxic granule proteins and mediators of inflammation. A prolonged effect of IL-3 versus IL-5 or GM-CSF on eosinophil degranulation has not been reported. High amounts of IgG are present in human BAL fluid [29], and allergen-specific IgG correlates with EDN in sputum of asthmatic patients [30]. Therefore, antigen-bound or aggregated IgG has been used as a relevant model to investigate eosinophil degranulation [31, 32]. In these studies, the low affinity IgG receptor II, CD32 (FCGR2) [32], and α M β 2 integrin (CD11b/CD18) [31] were reported to be responsible for eosinophil degranulation on IgG.

Therefore, in the present study, we analyzed the effect of prolonged IL-3 activation on CD32 and the α M β 2 integrin level and activation states and their function on IgG-induced eosinophil degranulation.

Materials and Methods

Reagents

Recombinant human (Rh) GM-CSF was purchased from R&D Systems Inc (Minneapolis, MN, USA), while rhIL-3 and rhIL-5 were purchased from BD Biosciences (San Jose, CA, USA). Human serum IgG was from Sigma-Aldrich (St. Louis, MO), LY294002 from Cell Signaling (Danvers, MA), and BI-D1870 and PF-4708671 from Selleckchem (Houston, TX). For the neutralization of IL-3-activated eosinophil degranulation on aggregated IgG, polyclonal goat anti-FCGR2B/C and anti-FCGR2A antibodies and control goat IgG were from R&D systems (Minneapolis, MN). Neutralizing anti- α M/CD11b (mouse, clone 2LPM19c) was from Biomeda (Foster City, CA), anti- β 1 integrin (rat, clone mAb13) was from Sigma, and the anti- β 7 (rat, clone Fib504) was from BD Biosciences (San Jose, CA). The control mouse IgG₁ and the control rat IgG2a were from Dako (Carpinteria, CA) and Santa Cruz Biotechnologies (Dallas, Texas), respectively. All the neutralizing anti-integrin antibodies have been described previously [33].

Subjects, cell preparations and cultures

The study protocol was approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board. Informed written consent was obtained from subjects prior to participation. For studies of *in vitro* eosinophil activation, peripheral blood eosinophils were obtained from allergic subjects with and without mild asthma. Subjects with prescriptions for low doses of inhaled corticosteroids did not use their corticosteroids the day of the blood draw. Eosinophils were purified by negative selection as previously described [20]. Briefly, heparinized blood was diluted 1:1 in HBSS and was overlaid above Percoll (1.090 g/ml). After centrifugation at $700 \times g$ for 20 min at room temperature, the mononuclear cells were removed from the plasma/percol interface and erythrocytes were eliminated from the cell pellet by hypotonic lysis. The remaining pellet was resuspended in 2% NCS in HBSS. Cells were then incubated with anti-CD16, anti-CD3, anti-CD14 and anti-Glycophorin-A beads from Miltenyi (San Diego, CA), and run through an AutoMACS (Miltenyi). Eosinophil

preparations with purity > 99% and viability ~98% were used the same day, ~5 h after the blood draw.

For studies of *in vivo* eosinophil activation, bronchoscopy and bronchoalveolar lavage (BAL) were performed 48 h after segmental bronchoprovocation with an allergen (SBP-Ag) in subjects with mild asthma who were allergic to ragweed, dust mite, or cat dander allergens [20]. Eosinophils were purified, as previously described [12], from the BAL cell preparation (BAL EOS) and from peripheral blood (BBL EOS) of the same allergen-challenged subject. On the same day, eosinophils were also purified from peripheral blood of a control unchallenged subject (control EOS (Ctrl)).

Flow cytometry

Blood eosinophils were activated with IL-3 (2ng/ml), IL-5 (4ng/ml) or GM-CSF (2ng/ml), or cultured without cytokine (Resting) for 20 h and then stained for flow cytometric analysis. PE-conjugated anti-CD32 (FCGR2) (clone FUN-2) was from Biolegend (San Diego, CA), FITC-conjugated anti-CD64 (FCGR1-clone 10.1), FITC-conjugated anti-CD16 (FCGR3-clone 3G8), PE-conjugated anti CD11b (αM-clone D12), FITC-conjugated anti-CD18 (β2-clone L130) and corresponding isotype controls, PE- and FITC-conjugated mouse IgG₁ were all from BD Biosciences. The activation state of CD32 was measured using the previously described monoclonal phage antibody A17 [34, 35]. Activation-sensitive anti-αM CBRM1/5 was from BioLegend and anti-β₁ N29, from Chemicon/Millipore/Sigma-Aldrich [36]. PE-conjugated goat anti-mouse Ab was used as the secondary Ab. Five to 10 thousand viable (no propidium iodide uptake) cells were acquired on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo (TreeStar Inc., Ashland, OR).

Real-time PCR

Total RNA was extracted from eosinophils using RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY). mRNA expression was determined by real-time quantitative PCR (RT-qPCR) using SYBR Green Master Mix (SABiosciences, Frederick, MD) and Applied Biosystems 7500 Sequence detector (ABI/Invitrogen, Carlsbad, CA) as previously described [37]. Specific primers shown in the supplementary Table 1 were designed using Primer Express 3.0 (Applied Biosystems) and blasted against the human genome to determine specificity using <http://www.ncbi.nlm.nih.gov/tools/primer-blast>. The housekeeping gene, β-glucuronidase (GUSB), forward: CAGGACCTGCGCACAAGAG, reverse: TCGCACAGCTGGGGTAAG, was used to normalize the samples. Standard curves were performed and efficiencies were determined for each set of primers. Efficiencies ranged between 94 and 99%. Data are expressed as fold change using the comparative cycle threshold ($2^{-\Delta\Delta C_t}$) where $\Delta C_t = C_t \text{ target gene (FCGR1A or FCGR1B or FCGR1C)} - C_t \text{ of the housekeeping gene (GUSB)}$; $\Delta\Delta C_t = \Delta C_t \text{ after cytokine treatment} - \Delta C_t \text{ of eosinophils at 0 h}$; and fold change = $2^{-\Delta\Delta C_t}$ [38].

Western blot

Cells were lysed either in RIPA buffer (Cell Signaling, Danvers, MA) plus 0.2 % SDS and protease inhibitors, or directly in Laemmli buffer (10% SDS), before boiling and loading

onto 10-12% SDS-polyacrylamide gels. Immunoblot analysis was performed as previously described [12] using rabbit polyclonal anti-FCGRIIA antibody (GeneTex, Irvine, CA), rabbit monoclonal anti-FCGRIIB/C (Sino Biological Inc. North Wales, PA), mouse monoclonal anti-FCGRIII antibody (MEM-154; Invitrogen) and mouse monoclonal anti- β -actin (Sigma-Aldrich). Secondary HRP-conjugated anti-rabbit IgG antibody and anti-mouse IgG were from Pierce/Thermo Fisher Scientific (Rockford, IL). Immunoreactive bands were visualized with Super Signal West Femto chemiluminescent substrate (Pierce/Thermo Fisher Scientific). Bands were quantified using the FluorChem® Q Imaging System (Alpha Innotech/ProteinSimple, Santa Clara, CA) and data are expressed as a ratio of target to β -actin.

Total cellular EDN measurement and degranulation on heat aggregated IgG (HA-IgG)

Peripheral blood eosinophils were cultured at 1×10^6 /ml in medium (RPMI 1640 plus 10% fetal bovine serum), without cytokine (resting), or with IL-3 (2ng/ml), or with IL-5 (4ng/ml) for 20h. Eosinophils were then lysed with 50 mmol/L HCl and 0.5% Triton X-100 to release total granule contents [39] or added to plates treated with heat-aggregated IgG to assess degranulation. Heat aggregation of human IgG (HA-IgG) was performed in PBS for 30 min at 63°C as previously described [40]. After 20 h cultures with or without cytokines, cells were washed and suspended at 1×10^6 /ml in fresh medium (no cytokine), and 100 μ l was added to a 96-well plate, which had been coated overnight with or without HA-IgG or monomeric IgG (both at 10 μ g/ml; 50 μ l/well) and saturated with 0.1 % gelatin for 30 min at 37°C. After a 6 h incubation, photomicroscopy was performed (digital camera and microscope, both from Olympus) and supernatant fluids were analyzed by ELISA for EDN release. Human EDN ELISA (MBL, Woburn, MA) has a minimum detection limit of 0.62 ng/ml. For the neutralization experiments, IL-3-activated peripheral blood eosinophils were pretreated with the neutralizing antibodies 15 min before their incubation on HA-IgG. Degranulation of BAL EOS on HA-IgG was performed using freshly prepared (no culture) cells.

Statistical analyses

Statistical analyses were performed using the SigmaPlot 11.0 software package. Differences between two groups were analyzed using the paired Student's *t*-test. One Way ANOVA was used to compare more than 2 groups, and $p < 0.05$ was considered statistically significant.

Results

Flow cytometric analysis of eosinophils cultured without or with IL-3 (2ng/ml), IL-5 (4ng/ml) or GM-CSF (2ng/ml) for 20 h (Figure 1A) indicated that eosinophils do not express surface CD16 or CD64. However, CD32 was present on resting eosinophils' surface and was increased by cytokine activation (Figure 1A). Figure 1B shows that IL-3 enhanced surface CD32 more potently than did IL-5.

CD32 comprises 3 different genes: FCGRIIA, FCGRIIB and FCGRIIC. To learn if any of these 3 genes is (are) differentially regulated by IL-3, IL-5 and GM-CSF, we analyzed their expression level over time following activation by the 3 cytokines (Figure E1). All 3 gene

messengers (FCGRIIA, FCGRIIB and FCGRIIC) were readily detectable in freshly isolated eosinophils by real-time PCR (Figure E1) with cycle threshold (Ct) values of 23 ± 1 , 28 ± 2 and 25 ± 1 , respectively. Expression levels of FCGRIIA and FCGRIIC mRNA did not change over the 9-hour culture under any condition, while there was a trend toward a rise in FCGRIIB in response to any of three cytokines compared to resting cells (Figure E1), which then partially decreased at 20 h (not shown).

Eosinophil lysates were immuno-blotted with antibodies that specifically recognize FCGRIIA and FCGRIIB/C (Figure 2). At the protein level, cellular FCGRIIA was detected in resting eosinophils and there was a slight apparent increase of FCGRIIA by IL-3, IL-5, or GM-CSF treated eosinophils compared to cells cultured without cytokine, although statistical significant differences were not reached (Figure 2A). In contrast, FCGRIIB/C proteins were up-regulated more than 15-fold by IL-3 compared to no cytokine and more than 4-fold compared to IL-5 or GM-CSF (Figure 2B). IL-3-induced FCGRIIB/C proteins was observed between 20h and 44h, while IL-5 or GM-CSF failed to significantly induce FCGRIIB/C at any time-points between 1h and 44h (Figures E2 and 2). The strong increase of FCGRIIB/C proteins and the lack of change at the mRNA levels implicate control at the level of translation. We recently reported that semaphorin-7A protein, but not mRNA, is IL-3- up-regulated in eosinophils by a pathway that requires 90-KDa ribosomal S6 kinase (p90S6K) [12]. Similarly, Figure 2B shows that the p90S6K inhibitor (BI-D1870) completely blocked IL-3-induced FCGRIIB/C production, whereas the inhibitors of the p70 ribosomal S6 kinase (PF-4708671) and PI3K (LY294002) had no effect. These data suggest that FCGRIIB/C is up-regulated at the translational level by IL-3 via the p90S6K, similarly to semaphorin-7A. Of note, FCGRIII (CD16) protein was not detected by Western-blot in eosinophils under any of our culture conditions (not shown).

CD32 binds complexed IgG, therefore, eosinophil degranulation on heat-aggregated (HA)-IgG was analyzed. Figure 3A shows total EDN present in eosinophils cultured with IL-3, IL-5 or without cytokine for 20 h. Figure 3B indicates that eosinophils pre-activated with IL-3 for 20 h and then seeded on HA-IgG for 6 h released EDN to a concentration of ~ 800 ng/ml, which was > 2.5 -fold higher than EDN release by IL-5-activated eosinophils (Figure 3B). Of note, 800 ng/ml corresponds to $\sim 25\%$ of total cellular EDN (Figure 3A) which is in line with EDN release to the frequently reported eosinophil degranulation activator, fMLP that typically induces release of around 20% of total EDN [39]. Unlike HA-IgG, and in agreement with Kaneko et al [31], the monomeric IgG coated at the concentration of 10 μ g/ml, did not significantly induce EDN degranulation (Figure 3B). The slight increase of EDN release from cells not exposed to cytokine or IgG was possibly due to cell death after the 20 h priming and during the incubation on IgG or no IgG, as we have previously shown that resting eosinophils started dying when cultured for more than 24 h, reaching $\sim 50\%$ death at 48 h [12] and (Figure E3). Figure 3C illustrates adhesion and spreading of IL-3-activated eosinophils on HA-IgG after 6 h.

Figure 4 shows the inhibition of IL-3-activated eosinophil degranulation with anti-FCGRIIB/C (10 μ g/ml) or anti- α M integrin (1 μ g/ml) antibodies. The inhibition of degranulation with anti-FCGRIIA (10 μ g/ml; Figure 4) was somewhat unexpected since IL-3 did not increase FCGRIIA production (Figure 2). The inhibition suggests that IL-3 possibly

enhanced the activation state of FCGR1A rather than its production. Of note, according to the neutralizing antibody's provider (R&D Systems), the cross-reactivity of the anti-FCGR1A antibody with FCGR1B was less than 5%, indicating it is unlikely that the anti-FCGR1A cross-reacted with FCGR1B/C under our conditions. Thus, we analyzed the activation state of CD32 by flow cytometry using the A17 phage antibody [34, 35]. Figure 5A shows that, unlike IL-5, long term (20 h) activation with IL-3 increased CD32 activation on eosinophils.

The amount of surface α M and β 2 on eosinophils was also augmented following activation with IL-3 for 20 h (Figure 5B), and there was increased expression of the highly active conformation of α M β 2 [41] on IL-3-activated, compared to resting or IL-5-activated eosinophils (Figure 5C). Conversely, staining with N29, which recognizes the intermediate- and high-activity state of β 1 [41], tended to decrease on cytokine-activated eosinophils, although differences did not reach statistical significance (Figure 5C). Collectively, these data indicate that IL-3 induces prolonged surface expression and activation of both CD32 and α M β 2 integrin.

To evaluate the *in vivo* relevance of these *in vitro* data, we analyzed airway eosinophils (BAL EOS) obtained by bronchoscopy 48 h after a segmental allergen challenge. Because we have reported that BAL EOS display more activated CD32 [34] and α M β 2 [36] integrin compared to blood eosinophils, we analyzed whether BAL EOS display increases of both degranulation on HA-IgG and expression of FCGR1B/C, compared to blood eosinophils. Freshly isolated BAL EOS were seeded on HA-IgG for 4 h and EDN released in the cell supernatant fluids was measured by ELISA. As shown on Figure 6A, BAL EOS demonstrated greater degranulation compared to blood eosinophils in the presence of coated HA-IgG. In addition, BAL EOS produced a higher amount of FCGR1B/C compared to both control blood eosinophils and blood eosinophils from the allergen-challenged subjects (BBL EOS) (Figure 6B). Altogether, these data indicate that after allergen challenge, airway eosinophils have a similar phenotype to blood eosinophils activated *ex vivo* with IL-3.

Discussion

In light of our recent findings that IL-3 is unique among the three β -chain cytokines in its ability to increase translation of semaphorin-7A by maintaining p90S6K/RPS6 intracellular signaling [12], the current report demonstrates that while the β -chain cytokines have many overlapping functions, IL-3 exhibits a clear distinction when it comes to CD32-mediated degranulation. In here, we demonstrate that unlike IL-5 or GM-CSF, activation of eosinophils with IL-3 induced prolonged CD32 and α M β 2 surface expression and activation, leading to eosinophil degranulation on immobilized and aggregated IgG. Moreover, while FCGR1A protein amount remained largely unaffected by eosinophil activation, we showed that FCGR1B/C on eosinophils was upregulated as a result of sustained IL-3-mediated activation and prolonged activity of the p90S6K.

We have recently reported that unlike IL-5 and GM-CSF, IL-3 maintains p90S6K-dependent RPS6 phosphorylation in eosinophils, leading to semaphorin-7A protein production [12]. We now show that IL-3-driven FCGR1B/C protein production is also dependent on p90S6K.

Interestingly, sustained intracellular signaling and RPS6 phosphorylation exclusively downstream of IL-3 has been recently shown in basophils as well [21]. Although, the function of RPS6 in its phosphorylated state remains uncertain, Roux *et al* have demonstrated that the activation of the Ras/ERK/p90S6K/RPS6 pathway stimulates cap-dependent translation [42]. It is possible that this pathway also controls translation of FCGR1B/C in IL-3-activated eosinophils. Taken together, IL-3-induced prolonged signaling, and consequently production of important specific proteins in basophils and eosinophils, suggest that IL-3 may support some of the disease-associated functions of these granulocytes.

The modest degranulation of IL-3-activated eosinophils on monomeric compared to aggregated IgG under our conditions suggested the involvement of a low affinity receptor for IgG rather than a high affinity receptor like CD64. In accordance with precedent quantitative proteomic and RNAseq analyses [43, 44], we did not detect FCGR1 (CD64) nor FCGR3 (CD16) on eosinophils. Therefore, CD32 was most likely the candidate that could trigger the interaction with aggregated IgG. The requirement of CD32 for eosinophil degranulation on IgG is in agreement with several studies. Kaneko *et al* reported eosinophil degranulation in a CD32-dependent manner when in contact with antigen-IgG1 or -IgG3 complexes [31]. This was later confirmed in another model where immune complexes of the four IgG subclasses (IgG 1-4) could trigger eosinophil degranulation [32].

The low affinity receptor for IgG, CD32 is a type I trans-membrane glycoprotein expressed by granulocytes, macrophages/monocytes and platelets. The constitutive presence of CD32 on the eosinophil surface is well-known [45, 46]; however, in humans, three different genes code for CD32: CD32A (FCGR2A), CD32B (FCGR2B) and CD32C (FCGR2C). Although FCGR2A is a well-known membrane protein present on eosinophils and neutrophils, we show for the first time upregulation of FCGR2B/C protein on human eosinophils, exclusively after long-term (20 h) activation with IL-3. Munitz *et al* had previously shown the presence of a high level of surface FCGR2B on blood eosinophils; although no data on the used antibody was described in this study [47]. Therefore, it is uncertain whether the antibody used in this previous study was specific for FCGR2B, particularly since the extracellular and membrane parts of the 3 proteins are highly homologous (>95%) and these portions of FCGR2B and FCGR2C are 100% identical. Interestingly, our results implicate both FCGR2A and FCGR2B/C in IL-3-induced eosinophil degranulation on IgG. The intracellular tails are much less similar (~25%) than the extracellular portions, with FCGR2B containing an immunoreceptor tyrosine-based inhibitory motif (ITIM), while FCGR2A and FCGR2C contain identical tyrosine-based activating motifs (ITAM) [48]. Therefore, while FCGR2A and FCGR2C can trigger signal transduction through their ITAM, FCGR2B and its ITIM triggers an inhibitory intracellular signal. Consequently, it is very likely that the inhibition of IL-3-induced degranulation using the neutralizing anti-FCGR2B/C antibody occurs via FCGR2C rather than FCGR2B. We did not investigate whether IL-3 induces solely FCGR2B or FCGR2C or both. However, due to the high level of both FCGR2B and FCGR2C mRNAs in resting eosinophils, and the similarity of their 5' untranslated region, it is likely that IL-3-activated eosinophils produce both proteins.

Another novel observation is the ability of IL-3 to maintain the activation state of CD32 on eosinophils *in vitro*. CD32 activation state was measured using the phage A17, an anti-active CD32 antibody, which was previously utilized to demonstrate both eosinophil priming *in vivo* in asthmatic subjects [35], and the activation state of airway eosinophils [34]. The association between CD32 activation state and IL-3-induced eosinophils degranulation suggests that activated CD32 plays a role in eosinophil degranulation. Of note, the presence of A17 staining on a small population of *in vitro* non-activated eosinophils from our allergic population may indicate that some circulating eosinophils from these subjects display a CD32 active state *in vivo*, which persists during the 20 h culture. This is in agreement with a previous study that reported higher CD32 activation state on eosinophils from asthmatic versus healthy subjects [35]. Notably, the A17 antibody recognizes activated FCGR1IA [34, 35] but its interaction with activated FCGR1IB/C is unknown.

Along with CD32, IL-3-activated eosinophils also showed increased expression and activation of α M β 2 integrin, and blockade of this integrin with a neutralizing antibody greatly inhibited IL-3-induced eosinophil degranulation on IgG. This indicates that CD32-driven degranulation on IgG is α M β 2-dependent, and it suggests that CD32 and the α M β 2 are partners to trigger eosinophil degranulation. Eosinophils possess a unique repertoire of seven integrins, $\alpha_4\beta_1$, $\alpha_6\beta_1$, $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$ and $\alpha_4\beta_7$ [41], and the crucial role of the β_2 integrin for eosinophil adhesion/degranulation on IgG has been previously described [31]. In a report by Kaneko *et al*, the interplay between CD32 and the α M β 2 integrin was discussed and close proximity or even direct interaction between these 2 membrane proteins was suggested [31]. The physical and functional interactions between CD32 and α M β 2 have been well-recognized and described in details in a review by Petty *et al*. [49]. The ligand(s) for the α M β 2 integrin in our present study remain(s) unknown, yet as previously suggested, the extracellular matrix and other proteins present in the serum of the cell culture medium are potential candidates [31, 50] Such proteins include vitronectin and fibrinogen, which support α M β 2-mediated adhesion of freshly isolated BAL eosinophils and *ex vivo* cytokine-activated blood eosinophils [51, 52].

A limitation of the present study is that the exact mechanism of degranulation was not studied. It probably involves adhesion, and possibly cell spreading and membrane rupture; however, it is not known whether FCGR1IB/C and/or FCGR1IA increase eosinophil degranulation on IgG by triggering cell death. In this regard, previous studies have demonstrated that ligation of CD32 on eosinophils can promote either survival or apoptosis [40, 53].

While anti-IL-5 therapies reduce the occurrences of asthma exacerbations in severe eosinophilic asthma[3, 4], airway eosinophil precursors remain mostly unaffected by these therapies [54-56]. We and others previously showed that IL-5 receptor α is downregulated on IL-5-activated blood eosinophils as well as airway eosinophils obtained after airway allergen challenge [11-13]. Corresponding to the low level of IL-5 receptor, airway eosinophils do not degranulate in response to IL-5 [28]. This is consistent with our recent finding that administration of anti-IL-5 in mild asthmatic subjects did not decrease the level and activation state of α M β 2 integrin on airway eosinophils [36], implying a role for other mediator(s), such as IL-3, in achieving and maintaining airway eosinophil activation. In

addition, since IL-5 and GM-CSF were unable to induce the production of FCGR1B/C on blood eosinophils *in vitro*, it is doubtful that these cytokines alone could induce FCGR1B/C in BAL eosinophils. Altogether, the increases of IL-3 and its receptor on eosinophils after an allergen challenge, and the phenotypic similarities between IL-3-activated blood eosinophils with the airway eosinophils, suggest that a therapy targeting an essential and specific piece of the IL-3 pathway in eosinophils could reduce important airway eosinophil functions, including degranulation. Yet, we cannot rule out that *in vivo*, effectors other than IL-3 may also change airway eosinophils into a similar phenotype as IL-3-activated blood eosinophils.

In conclusion, our data demonstrate increased production of FCGR1B/C on eosinophils when activated with IL-3. In contrast to IL-5 or GM-CSF, prolonged IL-3 activation induced increased production and functional activity of CD32 and the α MB2 integrin on eosinophils enhancing their degranulation. Moreover, the phenotype of IL-3-activated eosinophils *in vitro* mirrored the phenotype of airway eosinophils *in vivo*, suggesting that IL-3 has an important impact on airway eosinophil biology independent of that related to IL-5 or GM-CSF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAL bronchoalveolar lavage

EDN	eosinophil-derived neurotoxin
EOS	eosinophils
FCGR2	receptor for Fc fragment of IgG, low affinity II
gMCF	geometric mean channel fluorescence
HA-IgG	heat aggregated IgG
p90S6K	90-KDa ribosomal S6 kinase
SBP-Ag	segmental bronchoprovocation with an allergen

Figure 1. Surface expression of CD32 (FCGR2B), CD64 (FCGR1), or CD16 (FCGR3B) in response to IL-3, IL-5, or GM-CSF

Peripheral blood eosinophils were cultured under the indicated conditions for 20 h and then examined by flow cytometry. A. Representative histograms are shown (n=3 donors). B. *Box plots* depicting medians and interquartile ranges of surface CD32 (specific geometric mean channel fluorescence, gMCF) for n=7 donors. # indicates the difference between IL-5-activated cells and resting (Rest) cells, and * indicates that IL-3 activation increases CD32 compared to resting and IL-5 (p<0.0001 for both comparisons, mixed-effect ANOVA).

Figure 2. IL-3 up-regulates the production of FCGRIB/C protein via a p90S6 kinase-dependent pathway

Blood eosinophils were cultured with the indicated cytokines or without cytokine (Rest) for 20 h. Additional cultures were treated with 10 μ M of the inhibitors to 90-KDa ribosomal S6 kinase (BI-D1870, BI), p70 ribosomal S6 kinase (PF-4708671, PF) or PI3K (LY294002, LY) for 15 min before addition of IL-3. Cell lysates were analyzed by Western-blot. Graphs represent the mean $\pm$ SEM of the ratio FCGRIB/C/ β -actin (n=3 donors). Data were normalized to resting eosinophils fixed at 1 in panel A and B. A. None of the cytokine treatments resulted in statistically significant changes in FCGR1IA. B. * indicates that IL-3 treatment significantly increased FCGRIB/C compared to IL-5 and GM-CSF, and to IL-3 plus BI pretreatment (p<0.001 for each comparison, ANOVA).

Figure 3. Degranulation of IL-3- or IL-5-treated eosinophils on heat aggregated (HA)-IgG
Peripheral blood eosinophils were cultured for 20 h with IL-3 (2 ng/ml) or IL-5 (4 ng/ml) or without cytokine (R). A. Total cellular EDN was measured by ELISA (mean $\pm$ SEM for 4 donors). B. and C. Cells were added (1×10^5 per 100 μ l) on coated HA-IgG or monomeric (mono-) IgG (10 μ g/ml) for 6 h. B. EDN in the supernatant fluids was measured by ELISA. Bar graphs represent mean $\pm$ SEM (n=4 donors), * indicates statistically significant difference compared to resting cells (R) and IL-5, for eosinophils on HA-IgG (p=0.01 for both comparisons, ANOVA). C. Images of resting (R), IL-3 or IL-5-activated eosinophils on HA-IgG, and IL-3-activated eosinophils on monomeric (Mono)-IgG.

Figure 4. Neutralizing antibodies anti-FCGRIB/C, anti-FCGRIIA and anti-αM integrin strongly reduce IL-3-activated eosinophil degranulation on HA-IgG

Blood eosinophils were activated with IL-3 (2 ng/ml) for 20 h, and were treated with the indicated antibodies for 15 min before the incubation on HA-IgG (10 μg/ml) for 6 h. The anti-FCGRII antibodies (anti-FCGRIIA or anti-FCGRIB/C) and goat IgG were used at 10 μg/ml, and the other antibodies were used at 1 μg/ml. EDN release was measured by ELISA.

* and # indicate a significant decrease compared to goat and mouse IgG, respectively (p<0.04 for all paired comparisons, paired *t* test; n=4 experiments with 4 different donors).

Figure 5. IL-3 activates surface CD32 and α M integrin

Blood eosinophils were cultured with IL-3 (2 ng/ml) or IL-5 (4 ng/ml), or without cytokine (Resting or rest) for 20h. Cells were stained with the phage monoclonal antibody A17 (anti-active CD32), CBRM1/5 (anti-active α M integrin), N29 (anti-active β 1 integrins), anti-CD11b (α M) or anti-CD18 (β 2) antibodies and analyzed by flow cytometry. A. A representative histogram is shown. A.B.C. Graphs show *box plots* depicting medians and interquartile ranges between the 25th and 75th percentiles of surface proteins for 3 or 4 experiments with different donors, and y-axes are specific geometric mean channel fluorescence (gMCF). * indicates significant difference between IL-3 and IL-5 ($p < 0.001$ for A17, $p = 0.009$ for CD11b, $p = 0.021$ for CD18, $p = 0.036$ for CBRM1/5; paired t test).

Figure 6. Airway eosinophils degranulate on HA-IgG and express FCGR11B/C

Freshly isolated BAL and blood eosinophils (BBL EOS) from the same Ag-challenged donor and control blood eosinophils from another donor (Control (Ctrl) EOS) were A. cultured on HA-IgG for 4 h and then analyzed for EDN release (ELISA) and B. analyzed for FCGR11B/C production (Western-blot). A. and B., graphs are mean $\pm$ SEM for 3 donors. A. * indicates statistical difference from control EOS on HA-IgG and BAL EOS on no coating ($p < 0.002$ for both comparison, ANOVA). B. * indicates difference with Ctrl blood EOS and BBL EOS ($p < 0.001$ for both comparison, ANOVA).