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Regulation of the interleukin-5 receptor α-subunit on peripheral blood eosinophils from healthy subjects

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SUMMARY

The aim was to study *in vitro* regulation of the IL-5 receptor α (IL-5Rα) on purified peripheral blood eosinophils from healthy subjects. The IL-5Rα was down-regulated, in a dose-dependent manner, by recombinant IL-5 and GM-CSF, with IL-5 being most potent. This down-regulation was not induced by autocrine release of GM-CSF or IL-5, respectively. Incubation of eosinophils with cell-free peritoneal dialysis fluid (PF) collected from a patient with peritoneal fluid eosinophilia (PFE), induced up-regulation of the proportion of CD69 positive eosinophils, in parallel with down-regulation of the proportion of IL-5Rα positive eosinophils. Experiments with neutralizing antibodies against IL-5 and GM-CSF, revealed that IL-5 was the principal cytokine responsible for the down-regulation of the IL-5Rα. When eosinophils were incubated with PF collected from the same patient in remission or with PF collected from a newly started patient or a patient with bacterial peritonitis, less down-regulation of the IL-5Rα was observed.

In conclusion our data indicate that IL-5, as opposed to its proposed action on eosinophil progenitors, down-regulates the IL-5Rα chain on mature eosinophils. We therefore suggest that an IL-5 driven inflammation generates an eosinophil tissue phenotype that is characterized by a low IL-5Rα expression. These aspects of IL-5 action on IL-5Rα expression could gain new insights into the mechanisms of specific immuno-modulatory therapies, such as anti-IL-5.

Keywords CD69 eosinophils GM-CSF IL-5 IL-5 receptor alpha

INTRODUCTION

The eosinophil is regarded as an important effector cell in allergic inflammatory responses such as asthma [1], and an increased number of activated eosinophils has been observed in peripheral blood [2–5] and airways [1,6–9] during allergic responses. Moreover, the eosinophil has been implicated to be involved in other eosinophilic disorders like parasitic infection [10], hypereosinophilic syndrome [11], peritoneal fluid eosinophilia [12] and nasal polyposis [13]. In addition, data indicate that eosinophils might exert cytotoxic action on cancer cells [14].

Eosinophils exert their effect through the release of cytotoxic basic granule proteins like eosinophil cationic protein (ECP), major basic protein (MBP) and other eosinophil-derived products [15]. These inflammatory products are believed to contribute to the pathophysiology of asthma [6,16].

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Interleukin (IL)-5 and granulocyte/macrophage-colony stimulating factor (GM-CSF) are haematopoietic growth factors essential for eosinophil development, activation and survival. Interleukin-5 acts specifically on eosinophils, while GM-CSF acts on many different cell types [17]. Increased levels of both cytokines have been detected in sera, bronchoalveolar lavage (BAL) fluid and lung biopsies from asthmatic individuals [18–21]. In guinea pigs, an intravenous injection of recombinant IL-5 resulted in rapid blood eosinophilia [22] and the level of IL-5 have been shown to significantly correlate to the number of eosinophils detected in the airways of asthmatics [20]. Furthermore, experiments with gene deletion and blocking antibodies have demonstrated IL-5's role to attenuate eosinophilic responses in different models of allergic inflammation [23,24]. Together these results suggest that IL-5 is a major determinant of eosinophilic inflammation.

The biological signal from IL-5 is mediated through a receptor consisting of a specific IL-5-binding α-chain, and a signal-transducing common β-chain, which is shared with the receptors for IL-3 and GM-CSF [25,26]. When the β-chain associates with the specific IL-5-binding α-chain, a high affinity complex is formed

[27]. The IL-5 receptor α-subunit (IL-5Rα) can appear in either a membrane-bound active form or a soluble antagonistic form. The various expressions of these forms are regulated at the transcriptional level by alternative splicing [28].

Previous studies have demonstrated that IL-5 specifically up-regulates the expression of the transmembrane IL-5Rα on CD34+ cord blood cells [29]. Moreover, there is an increase in the number of CD34+/IL-5Rα+ cells in the airways of asthmatics as well as in the bone marrow after allergen challenge of asthmatic subjects [30,31]. These results indicate that IL-5 could act early in eosinophil differentiation by up-regulating its own receptor on progenitors. However, the regulation of the transmembrane IL-5Rα on mature peripheral blood eosinophils is poorly studied. We have previously demonstrated that the transmembrane IL-5Rα is down-regulated on eosinophils in peritoneal dialysis fluid, as compared to blood eosinophils, from patients with peritoneal fluid eosinophilia [32], which indicates that the receptor on mature eosinophils can be dynamically regulated at the extravascular site.

Taken this observation into account, we sought to study the regulation of IL-5Rα on peripheral blood eosinophils from healthy individuals upon *in vitro* stimulation with relevant cytokines. We analysed the impact of the haematopoietic growth factors IL-5 and GM-CSF as well as peritoneal fluid from the site of eosinophilic inflammation, representing an eosinophilic promoting milieu.

MATERIALS AND METHODS

Preparation of peripheral blood eosinophils

Peripheral blood from healthy blood donors (age 18–64 years) was collected in tubes containing citrate (Vacutainer, 5 ml, with 0·5 ml 0·129 M 9NC, Becton Dickinson, San Jose, CA, USA). Eosinophils were purified by the magnetic cell separation system MidiMacs (Miltenyi Biotec, Bergisch Gladbach, Germany) [33]. Briefly, citrate blood was layered onto Percoll solution (Pharmacia-Upjohn, Uppsala, Sweden) and centrifuged (30 min, 1000 × g, 20°C). The mononuclear cells layer was removed and the remaining cell suspension was haemolysed in isotonic lysing solution (154 mM NH_4Cl, 10 mM $KHCO_3$, 0·1 mM EDTA, pH 7·2). Neutrophils and eosinophils were washed in PBS and anti-CD16 magnetic beads were added for 20 min at +4°C. The eosinophils were obtained by negative selection by using a separation column in a magnetic field where magnetically labelled cells (CD16+ neutrophils) were trapped and unlabelled cells (eosinophils) were collected.

This study was approved by the local ethics committee.

Preparation of peritoneal dialysis fluids (PF)

Newly drained effluents from night bags (Standard double bag system) were collected from three patients (Patient A-C, Table 1) treated with continuous ambulatory peritoneal dialysis (CAPD). Patient A had peritoneal fluid eosinophilia (PFE) and was referred with cloudy effluent and a negative bacterial culture but without abdominal pain. Effluents were collected both during disease and after remission of the disease. Patient B was a newly started CAPD-patient with clear PF. Patient C had bacterial peritonitis and appeared with cloudy dialysate and a positive bacterial culture but without abdominal pain. The fluids were centrifugated and supernatants were stored at −70°C. The leukocyte counts were analysed by flow cytometry. The IL-5, IL-8, GM-CSF and eotaxin levels were measured with Quantikine human immunoassays (R & D Systems Inc, Minneapolis, MN, USA). Levels of ECP were measured with Pharmacia ECP CAP FEIA System (Pharmacia & Upjohn, Uppsala, Sweden). Leukocyte counts and soluble factors in PF are presented in Table 2 and have partly been reported previously [32].

In vitro *incubation of purified eosinophils with recombinant IL-5, GM-CSF and peritoneal dialysis fluids*

Purified eosinophils ($0{\cdot}4 \times 10^6$/ml) were incubated in recombinant human (rh) IL-5 (0·1–1000 ng/ml) (Immunokontact, Frankfurt,

Table 1. Patient characteristics

Patient	Age	Sex	Time on CAPD	Disease	Bag/manufacturer*
A	52	Female	10 days	Diabetes mellitus	Dianeal,Physioneal/Baxter
B	70	Male	<3 days	Nephrosclerosis	GambrosolBio/Gambro
C	56	Male	10 months	Renal obstruction	Lockolys/Fresenius

*Baxter, Deerfield, IL, USA; Gambro, Lund, Sweden; Fresenius, Bad Homburg, Germany.

Table 2. Cell counts and soluble factors in dialysis fluids from CAPD-patients

	Counts					Soluble factors				
Patients	Leukocytes (count $\times 10^3$/ml)	Eosinophils % (count $\times 10^3$/ml)	Lymphocytes % (count $\times 10^3$/ml)	Monocytes % (count $\times 10^3$/ml)	Neutrophils % (count $\times 10^3$/ml)	ECP (μg/l)	IL-5 (pg/ml)	Eotaxin (pg/ml)	IL-8 (pg/ml)	GM-CSF (pg/ml)
A	4500	44·8 (2016)	13·0 (585)	12·9 (581)	28·4 (1278)	314	454	996	52	49
A*	30	17·7 (5)	28·8 (9)	57·9 (17)	0·0 (0)	2·2	27	260	<10	<2·8
B	31	2·9 (1)	11·8 (4)	82·5 (26)	2·8 (1)	<2·0	<3·0	37	<10	4·3
C	1080	2·2 (24)	19·0 (205)	14·2 (153)	63·9 (690)	2·1	<3·0	501	47·1	13·9

ECP, Eosinophil Cationic Protein; *Patient A after remission of disease.

Germany) and/or rhGM-CSF (0·2–2000 ng/ml) diluted in HEPES (10 mM)-buffered RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (RPMI). In another assay eosinophils were resuspended in 500 μl undiluted PF from CAPD-patients (see above). As control, eosinophils were incubated in RPMI alone. The suspensions were incubated in 24-well plates for 120 min at +37°C in 5% CO_2, supernatants were removed and the cells were washed twice in PBS (300 × g for 5 min at +4°C). In a time-related study eosinophils were incubated in rhIL-5 (10 ng/ml) or RPMI alone for 0–120 min at +37°C in 5% CO_2, and then washed twice in PBS.

Inhibition of cytokines with neutralizing antibodies

Peritoneal dialysis fluid, rhIL-5 (10 ng/ml), rhGM-CSF (2 ng/ml) or RPMI (500 μl) were preincubated with the neutralizing antibodies anti-IL-5 (5 or 10 μg/ml) (R & D Systems), anti-GM-CSF (5 or 10 μg/ml) (R & D Systems) or the IgG1 isotypic control (5 or 10 μg/ml) (DAKO A/S, Glostrup, Denmark), for 60 min at +37°C, whereafter the eosinophils were added ($0{\cdot}4 \times 10^6$/ml). When both antibodies were used in PF (5 μg/ml each), the isotypic control antibody was used at 10 μg/ml. Eosinophils were subsequently cultured for 120 min at +37°C, resuspended and washed in PBS.

Immunofluorescence staining of eosinophils and flow cytometry

Surface antigens were incubated with nonconjugated monoclonal antibodies (mAb) to IL-5Rα (10 μg/ml, Clone: α16, non-neutralizing) [29] (Kind gift from Prof Jan Tavernier, University of Ghent, Belgium), fluoroscein isothiocyanate (FITC)-conjugated mAb to CD69 (5 μg/ml, Clone: L78) (Becton Dickinson, Meylan-Cedex, France) for 30 min at +4°C, and then washed in PBS. Secondary immunostaining was performed with FITC-conjugated rabbit-anti-mouse immunoglobulin, $F(ab')_2$ (50 μg/ml, Code: F313) (DAKO A/S), for 30 min at +4°C and washed with PBS. The nonspecific binding was determined with isotype-matched control antibodies in corresponding concentrations. Cells were finally diluted in 0·5 ml PBS and a minimum of 2000 eosinophils was analysed in an EPICS XL-MCL (Beckman Coulter Inc., Fullerton, CA, USA) flow cytometer. The flow cytometer was calibrated daily with Flow Check and Flow Set (Beckman Coulter).

Statistics

Non-parametric methods were used; data were analysed by one-way ANOVA, followed by Wilcoxon paired within groups. $P < 0{\cdot}05$ were considered significant. Results are presented as medians (interquartile ranges) and mean ± SD.

RESULTS

Dose-response curves of IL-5Rα expression

The proportion of IL-5Rα positive eosinophils was down-regulated in a dose-dependent manner by rhIL-5 (Fig. 1a) and GM-CSF (Fig. 1b) when incubated for 120 min at +37°C in 5% CO_2 ($n = 3$). For the following experiments 10 ng/ml of rhIL-5 and 2 ng/ml of rhGM-CSF were selected.

Time-related expression of IL-5Rα after incubation with rhIL-5

The kinetics of IL-5-induced down-regulation of IL-5Rα positive eosinophils was analysed after incubation of eosinophils with rhIL-5 or RPMI alone for 0, 5, 15, 30, 60 and 120 min ($n = 3$). RhIL-5 induced a down-regulation of the IL-5Rα at 30 min, which was further pronounced at 60 and 120 min (Fig. 2). Incubation of eosinophils with RPMI for different time points did not effect the IL-5Rα expression.

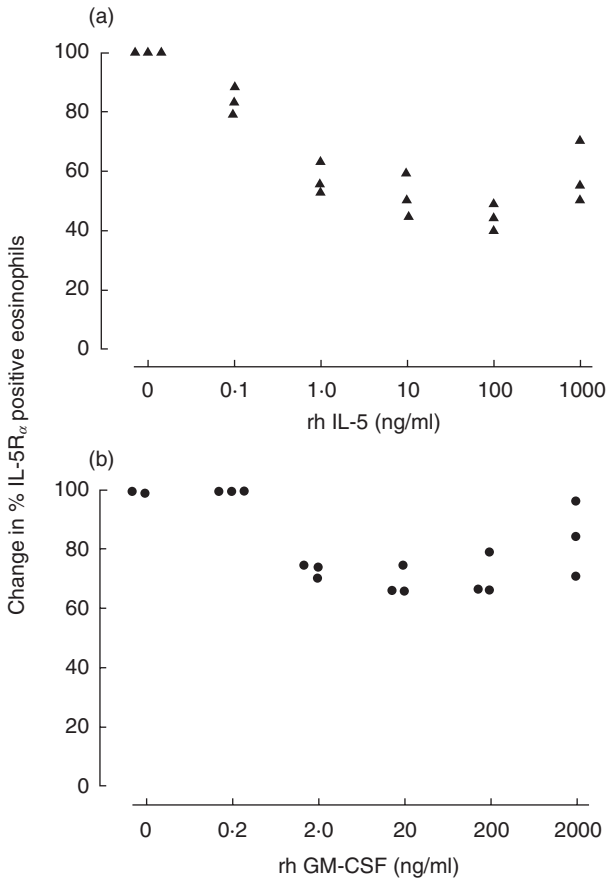

Fig. 1. (a) Dose–response curve ($n = 3$). Purified peripheral blood eosinophils ($0{\cdot}4 \times 10^6$/ml) from healthy subjects were incubated with rhIL-5 (0–1000 ng/ml) for 120 min at +37°C. Results are presented as change in the proportion of IL-5Rα positive eosinophils as compared to the RPMI control. (b) Dose–response curve ($n = 3$). Purified peripheral blood eosinophils ($0{\cdot}4 \times 10^6$/ml) from healthy subjects were incubated with rhGM-CSF (0–2000 ng/ml) for 120 min at +37°C. Results are presented as change in the proportion of IL-5Rα positive eosinophils as compared to the RPMI control.

IL-5Rα and CD69 expression after incubation with rhIL-5 and GM-CSF

When purified eosinophils from seven healthy subjects were incubated with rhIL-5, rhGM-CSF or the combination of both for 120 min, the proportion of IL-5Rα positive eosinophils was significantly ($P = 0{\cdot}018$ for all) down-regulated (14·2%, range 5·2–24·8; 46·7%, 36·3–66·6; 28·8%, 15·8–39·8; respectively) as compared to the RPMI control (74·8%, range 65·3–82·9) (Fig. 3). The proportion of CD69 positive eosinophils was significantly ($P = 0{\cdot}018$ for all) up-regulated (72·4%, range 47·7–78·5; 70·4%, 44·0–76·9; 74·2%, 48·3–80·7; respectively) as compared to the RPMI control (0%, range 0–0·2) (Fig. 3). The proportion of IL-5Rα and CD69 positive eosinophils obtained at +4°C, (79·2%, 69·6–87·8; 0·4%, 0–0·9; respectively) did not significantly differ from the values obtained after incubation with RPMI (see above).

Effect of addition of neutralizing antibodies to rhIL-5 and rhGM-CSF suspensions

To analyse whether the results from the incubation with rhIL-5 could be an effect of an autocrine production of GM-CSF and vice versa, neutralizing antibodies were added to the cell suspensions. However, anti-GM-CSF antibodies did not effect the IL-5-induced down-regulation of the IL-5Rα, 22·2% ± 1·1 *versus* 22·1% ± 1·2, and anti-IL-5 did not restore the GM-CSF-induced down-regulation of the IL-5Rα, 75·9% ± 4·3 *versus* 69·8% ± 6·7, as compared to RPMI, 87·3% ± 3·8 ($n = 3$).

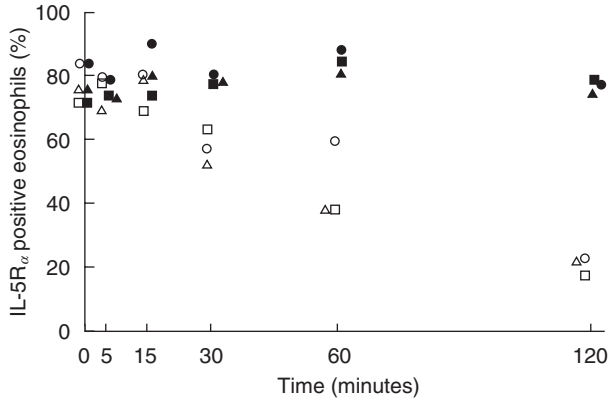

Fig. 2. Purified peripheral blood eosinophils ($0{\cdot}4 \times 10^6$/ml) from healthy subjects ($n = 3$) were incubated with rhIL-5 (10 ng/ml) (○,□,△) or RPMI (●,■,▲) for 0–120 min at +37°C. Results are presented as the proportion of IL-5Rα positive eosinophils.

IL-5Rα and CD69 expression after incubation with peritoneal dialysis fluids

Eosinophils from eight healthy subjects were incubated with PF from CAPD-patients (Tables 1 and 2) for 120 min. The proportion of IL-5Rα positive eosinophils was significantly ($P = 0{\cdot}01$) down-regulated by PF from the patient with PFE (patient A) both during disease, 24·5% (range 19·7–26·9), and after remission of the disease (patient A), 78·7% (66·8–84·1), when compared to RPMI, 86·6% (74·5–89·7) (Fig. 4). The PF collected during disease did however, induce a significantly ($P = 0{\cdot}01$) lower IL-5Rα expression as compared to PF collected after remission. A small but significant ($P = 0{\cdot}01$) down-regulation of the IL-5Rα was found when eosinophils were incubated with PF from the patient with bacterial peritonitis (patient C), 67·8% (47·9–87·3) as well as from the patient newly started on CAPD (patient B), 69·1% (52·0–80·4), as compared to RPMI (see above) (Fig. 4). Recombinant IL-5 was used as a positive control and down-regulated the receptor significantly ($P = 0{\cdot}01$) to 16·1% (8·4–21·2).

In three experiments, the proportion of CD69 positive eosinophils was up-regulated by PF from the patient with PFE (patient A) during disease, 70·5% ± 12·6, and after incubation with recombinant IL-5, 70·3% ± 10·7, when compared to RPMI where no CD69 positive eosinophils could be detected. In addition, no CD69 positive eosinophils was observed after incubation with PF from the patient with PFE after remission of the disease,

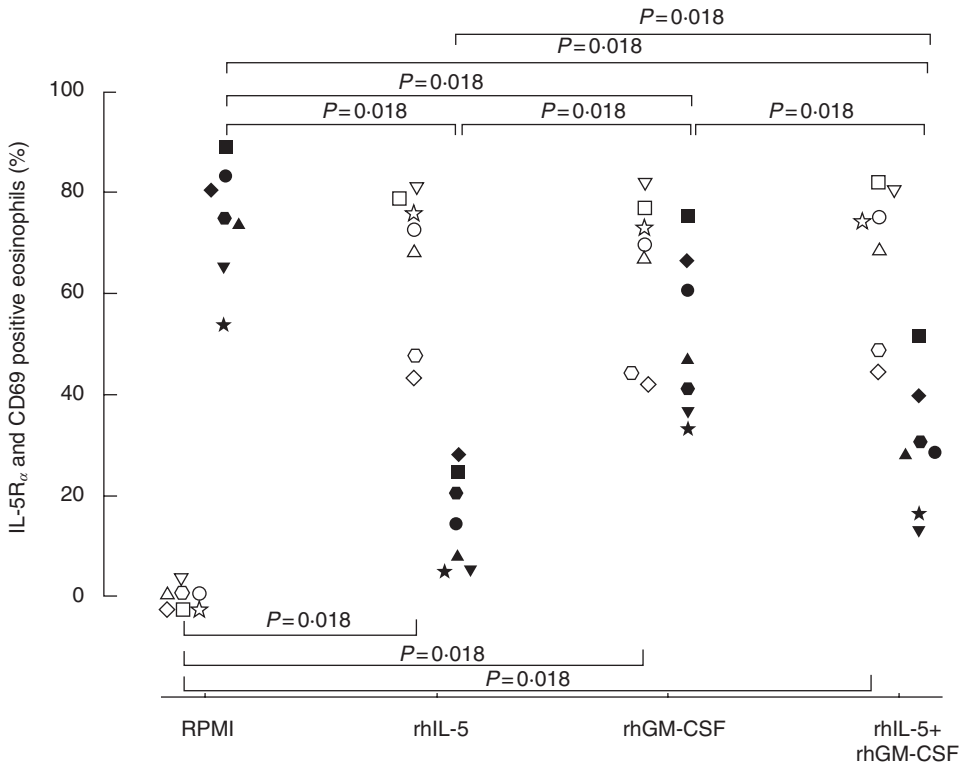

Fig. 3. Purified peripheral blood eosinophils ($0{\cdot}4 \times 10^6$/ml) from healthy subjects ($n = 7$) were incubated with RPMI, rhIL-5 (10 ng/ml), rhGM-CSF (2 ng/ml) or the combination of both cytokines for 120 min at +37°C. Results are presented as the proportion of IL-5Rα (filled symbols) and CD69 (open symbols) positive eosinophils.

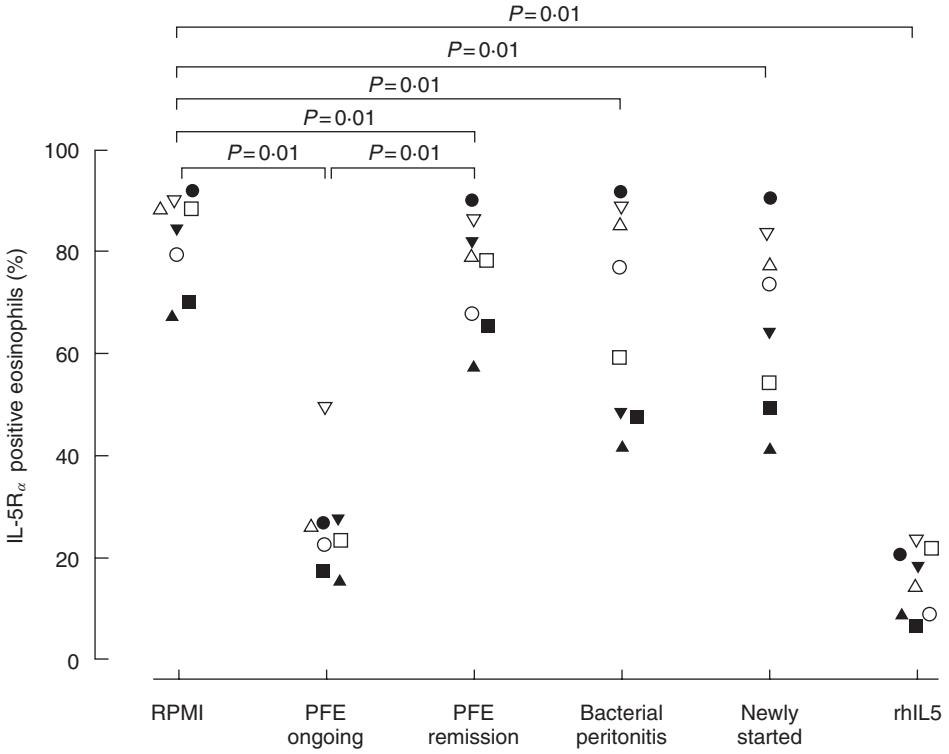

Fig. 4. Purified peripheral blood eosinophils ($0{\cdot}4 \times 10^6$/ml) from healthy subjects ($n = 8$) were incubated with PF from the CAPD-patient with PFE, both during and after remission of the disease, or PF from a bacterial peritonitis patient or a newly started patient, for 120 min at +37°C. RhIL-5 (10 ng/ml) was used as a control. Results are presented as the proportion of IL-5Rα positive eosinophils. Statistics are based on the comparison with the RPMI control and the comparison between during and after remission of PFE.

from the newly started CAPD-patient, or the patient with bacterial peritonitis (all values < 4·5%).

Effect of addition of neutralizing antibodies to PF from the patient with PFE

The PF-induced decrease in the proportion of IL-5Rα positive eosinophils, 28·3% ± 1·2, was almost abolished by the addition of anti-IL-5 antibodies, 70·1% ± 5·9, compared to RPMI, 89·1% ± 0·6, whereas no change was observed by the addition of anti-GM-CSF antibodies, 28·9% ± 1·6 ($n = 3$) (Fig. 5). When both anti-IL-5 and anti-GM-CSF antibodies were added, 67·2% ± 6·0, the inhibition was as marked as when only anti-IL-5 was added ($n = 3$) (Fig. 5).

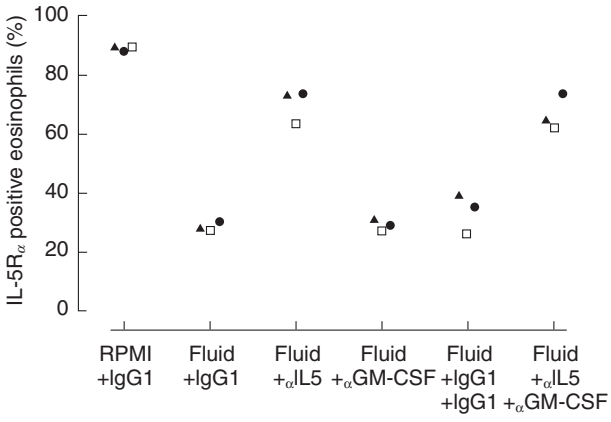

Fig. 5. RPMI or PF (500 μl) from the patient with PFE was preincubated with anti-IL-5 (5 μg/ml), anti-GM-CSF (5 μg/ml) or the IgG1 isotypic control (5 μg/ml), for 60 min at +37°C. Thereafter purified peripheral blood eosinophils ($0{\cdot}4 \times 10^6$/ml) from healthy subjects ($n = 3$) were added. When both antibodies were used in PF (5 μg/ml each), the isotypic control antibody was used at 10 μg/ml. Eosinophils were subsequently cultured for 120 min. Results are presented as the proportion of IL-5Rα positive eosinophils.

DISCUSSION

The main finding in the present study is that IL-5 and GM-CSF down-regulate the membrane-bound IL-5Rα on peripheral blood eosinophils from healthy individuals. In addition, extravascular fluid from a pro-eosinophilic milieu has the potential to down-regulate the IL-5Rα, and the effect is mediated through the action of IL-5. The kinetics for IL-5-induced down-regulation of IL-5Rα is rapid, beginning within 30 min and reaches its lowest expression at 120 min.

Interleukin-5 is the main actor in the activation and survival of mature eosinophils and its action is mediated through binding to the IL-5Rα [25,26]. Previous data suggest that there is an increase in the IL-5Rα expression on bone marrow-derived CD34+ cells from asthmatic subjects [31] and that IL-5 is able to up-regulate its own receptor on progenitors [29]. However, the mechanisms involved in the regulation of the IL-5Rα on mature eosinophils and the potential role of IL-5 in this respect, are poorly known.

In this study the membrane-bound IL-5Rα was expressed on the majority of isolated peripheral blood eosinophils from healthy subjects. *In vitro* exposure to recombinant IL-5 for 120 min induced an extensive dose-dependent down-regulation of IL-5Rα positive eosinophils. Recombinant GM-CSF down-regulated the IL-5Rα in a dose-dependent manner but to a less extent than recombinant IL-5. The IL-5-induced down-regulation of the IL-5Rα was initiated within 30 min and continued gradually for up to 120 min. These results are supported by Wang *et al*. [34] who demonstrated that the membrane-bound IL-5Rα mRNA is down-regulated by IL-5 and GM-CSF after incubation for four hours. The combination of IL-5 and GM-CSF induced a significant less down-regulation of the IL-5Rα than IL-5 alone and a plausible explanation is a competitive inhibition of the beta-chain which is shared between the IL-5, IL-3 and GM-CSF receptors [25–27]. In contrast to these results, the proportion of CD69 positive eosinophils, which was used as a marker for eosinophil activation [35], was significantly up-regulated to the same level by IL-5, GM-CSF as well as the combination of both cytokines. Our interpretation of data is that different concentrations of IL-5 are needed to achieve CD69 up-regulation and IL-5Rα down-regulation, respectively. When both IL-5 and GM-CSF are added the amount of IL-5 engaging β-chains is sufficient for maximal up-regulation of CD69, but not for maximal down-regulation of IL-5Rα. We therefor suggest that activation of eosinophils, judged by CD69 up-regulation, is not mandatory linked to IL-5Rα down-regulation.

Since both GM-CSF and IL-5 can be produced by eosinophils [36,37], we tested the hypothesis that IL-5 and GM-CSF-induced IL-5Rα down-regulation was mediated through autocrine action of released GM-CSF and IL-5, respectively. However, our results do not support this mode of action, and data point towards a direct effect of IL-5 and GM-CSF on IL-5Rα expression.

To further extend our *in vitro* observations, we addressed the question whether a pro-eosinophilic milieu has the propensity to generate an IL-5Rα-low phenotype. Indeed, an IL-5Rα-low/CD69-high phenotype was generated when eosinophils were incubated with cell-free peritoneal dialysis fluid from a CAPD-patient with on-going peritoneal fluid eosinophilia. Our data also indicate that IL-5 in the fluid was the principal cytokine responsible for this effect. These data support our results using recombinant cytokines and are also in concordance with our previous data showing down-regulated IL-5Rα expression on peritoneal eosinophils from patients with peritoneal fluid eosinophilia [32]. However, since dialysis fluids from CAPD-patients with no peritoneal fluid eosinophilia had low levels of IL-5 and GM-CSF but still induced a down-regulation of the IL-5Rα on eosinophils, we can not exclude the possibility that other unidentified factors in the fluid act on IL-5Rα-expression. The existence of tissue eosinophils with an IL-5Rα-low phenotype is supported by data from a murine model, which demonstrated that BAL eosinophils stained negative for IL-5Rα [38].

The potential physiological consequence of a down-regulated IL-5Rα on extravasated eosinophils is not fully delineated. We raise the hypothesis that a down-regulated IL-5Rα implies a reduced responsiveness to IL-5 in tissues. The rational for this hypothesis is based on our notion that eosinophils with a down-regulated IL-5Rα have a reduced *in vitro* responsiveness to recombinant IL-5 [39]. This, however, does not exclude other stimuli like eotaxin and GM-CSF to exert action on tissue-dwelling eosinophils.

In conclusion our data indicate that IL-5, as opposed to its proposed action on eosinophil progenitors, down-regulates the IL-5Rα chain on mature eosinophils. In addition, we suggest that tissue-dwelling eosinophils have a weaker expression of the IL-5Rα, as compared to blood eosinophils, rendering the eosinophils a reduced responsiveness towards IL-5. Collectively, these data suggest that the net outcome of IL-5 on IL-5Rα expression is critically dependent on maturation stage as well as localization of eosinophils and their progenitors. These aspects of IL-5 action on IL-5Rα expression have to be taken into account, when evaluating the effects of specific anti-eosinophilic therapies, such as anti-IL-5.

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