

T-helper 2 cytokines attenuate senescent eosinophil activation by the CXCR4 ligand stromal-derived factor-1 α (CXCL12)

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Summary

Background Different chemokine receptors have been suggested to play a pivotal role in allergic diseases and therefore to be relevant for the activation of effector cells and propagation of the inflammatory response. The CXC chemokine receptor CXCR4 has recently been found on the surface of eosinophils implicating a role in allergic diseases.

Objective The aim of this study was to investigate the functional expression of CXCR4 on senescent eosinophils. Moreover, we questioned whether the cytokine profile – T-helper (Th)1 and Th2 cytokines – affect the activation of eosinophils via the CXCR4 that could be important for the different phases of the allergic reaction.

Methods CXCR4 expression on human eosinophils was analysed by flow cytometry and RT-PCR. Functional analyses of intracellular calcium fluxes, actin polymerization, release of reactive oxygen species and, chemotaxis were carried out using spectrofluorometry, flow cytometry, chemiluminescence and modified Boyden chamber technique.

Results Whole blood and freshly isolated eosinophils weakly express CXCR4 surface protein. Incubation in culture medium without addition of cytokines for 24 h always lead to strong CXCR4 surface expression that paralleled with stromal-derived factor-1 α (CXCL12)-induced eosinophil activation. Stimulation of eosinophils with CXCL12 leads to an internalization of CXCR4, which could be prevented by phenylarsine oxide. Co-incubation of eosinophils with Th2 cytokines such as IL-3, IL-4, IL-5, IL-13, and granulocyte macrophage-colony stimulating factor prevented the expression of CXCR4 and affected eosinophil activation after stimulation with the CXCR4 ligand CXCL12. From these cytokines, IL-3 was the only cytokine completely inhibited intracellular calcium fluxes and chemotaxis of eosinophils in response to CXCL12.

Conclusion Senescent eosinophils express functional CXCR4 receptors, which are prevented by Th2 cytokines that are found in the early phase of allergic reaction. Therefore, CXCR4 activation of eosinophils seems to be important in the chronic phase of allergic reaction, which is dominated by a Th1 cytokine profile.

Keywords chemokine receptor, chemokine, cytokines, eosinophils, receptor regulation, transcription

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Introduction

Allergic diseases such as allergic asthma, allergic rhinitis, and atopic eczema are characterized by a complex interaction of different cell types and mediators [1, 2]. In atopic dermatitis it could be argued that T-helper (Th)1- and Th2-type cytokines contribute to different stages of the development of skin lesions in atopic eczema. Among this, a key element is the sequential activation of the Th2-cell subsets characterized by cytokines such as IL-4, IL-5, and IL-13 in the initiating or early phase, followed by the Th1-cell subset such as IFN- γ and IL-12 to account for the persistence of the inflammatory or chronic response [3–5]. Patients with allergic asthma show

increased numbers of eosinophils contributed to elevated protein level of the Th2 cytokine IL-5 24 h after segmental allergen provocation [6]. Without treatment the number of eosinophils remains elevated and are also found in patients with chronic asthma [7].

Locally generated chemokines and cytokines regulate the accumulation of eosinophils in the allergic inflamed tissue [8]. Different chemokine receptors have been suggested to play a pivotal role in allergic diseases and therefore to be relevant for the activation of effector cells and propagation of the inflammatory response [9]. In this context, the chemokine receptors CCR5 [10], CCR6, [11] and CCR8 [12] have been controversially discussed to play a role in allergic diseases. However, until now, the chemokine receptor CCR3 seems to be the most important receptor responsible for the recruitment of effector cells in allergic diseases [13, 14].

Besides the CC chemokine receptors the CXC chemokine receptors CXCR1 and CXCR2 have been postulated to play

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a role in allergic diseases because eosinophils may express these receptors under certain circumstances [15]. The finding of CXCR4 on the surface of eosinophils has focused the interest of this receptor on allergic diseases [16]. The CXCR4 also termed as LESTR or fusin is a seven-transmembrane domain glycoprotein and has been shown to respond to the CXC chemokine stromal-derived factor-1 α (CXCL12). Furthermore, CXCR4 has been shown to be an important co-receptor for human immuno-deficiency virus-1 (HIV-1) infection [17]. The CXC chemokine receptor 4 is predominantly expressed on inactivated naïve T lymphocytes [18], B lymphocytes [19], dendritic cells [20], endothelial cells, [21] and basophils [22]. Previously, intense expression of CXCR4 in long-term medium-incubated human neutrophils and eosinophils has been described [16, 23–25]. However, no data exist about constitutive expression of CXCR4 surface protein in freshly isolated human eosinophils.

The role of CXCR4 in allergic diseases has recently been underlined in a mouse model of asthma [26]. Treatment of allergic mice with the CXCR4 antagonist, AMD3100, results in a significantly reduced airway hyper-reactivity and peribronchial eosinophilia. In addition, there was a shift in the cytokine profile that was observed in the AMD3100-treated animals with a significant reduction in IL-4 and, IL-5 and significant increase in IL-12 and IFN- γ levels within the lungs of treated allergic mice [26]. Since it has been demonstrated that Th2 cytokines prevented the expression of CXCR4 on human eosinophils [16] no data exist about the functional consequence of cytokine-mediated CXCR4 activation of eosinophils. In this study, we focused therefore our interest on the regulation and functional CXCR4 expression in human eosinophils in dependence of the different cytokine milieu in allergic reaction.

Materials and methods

Reagents and anti-CXCR4 monoclonal antibodies

The following reagents were purchased as indicated: recombinant human CXCL12 and Eotaxin (PeproTech, London, UK); C5a (Sigma Chemicals, Deisenhofen, Germany); IL-3, IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-13, IFN- γ , and anti-CXCR4-Phycoerythrin (PE)-conjugated monoclonal antibody (mAb) and anti-CD16-flourescein isothiocyanate (FITC) mAb (R&D Systems, Wiesbaden, Germany); anti-CXCR4 mAb (PharMingen, Heidelberg, Germany); isotype control mAb IgG2a (Immunotech, Hamburg, Germany); anti-CD16-PE mAb (clone 3G8; Immunotech).

Eosinophil and peripheral blood mononuclear cell isolation

Human granulocytes and peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated venous blood from normal non-atopic healthy or atopic donors using Ficoll (Pharmacia, Erlangen, Germany) density gradient centrifugation as described previously [27]. The resulting eosinophil purity was 99% determined by microscopic examination with Kimura staining and flow cytometric analysis (FACScan[®], Becton Dickinson, Heidelberg, Germany) using

Table 1. Co-incubation of eosinophils with PBMCs prevented CXCR4 expression on eosinophils

	Ratio CXCR4	Medium ratio CXCR4
Serum ($n = 8$)	8.6 ± 2.5 , NS	5.2 ± 0.7
PBMC ($n = 12$)	4.3 ± 0.6 , $P = 0.02$	6.7 ± 0.9

Eosinophils were co-incubated either with serum or with the PBMCs from the same donor as described in Materials and methods. Thereafter, eosinophils were stained with anti-CXCR4 and the isotype control and analysed by flow cytometry. Data are expressed as the ratio of specific anti-CXCR4 and isotype control. Paired t -test was performed between ratio CXCR4 and medium ratio CXCR4. PBMC, peripheral blood mononuclear cell; NS, not significant.

anti-CD16. There were no differences in viability of the cells (>95%) between the fractions, as judged by trypan blue exclusion.

Immunofluorescence of eosinophils

Immunofluorescence of eosinophils was carried out as described previously [27]. Aliquots (100 μ L) containing 1×10^5 highly purified eosinophils were incubated at 4 °C for 30 min with the anti-CXCR4 mAb, stained in a second step by a FITC-conjugated mouse anti-human antibody (Immunotech) and analysed by flow cytometry.

Whole blood samples were taken from different donors (atopic and non-atopic). After double staining with anti-CXCR4-PE mAb and anti-CD16-FITC mAb according to standard techniques, erythrocytes were lysed and remaining cells were fixed simultaneously with Simultest[™] IMK-Lymphocyte⁺ Lysing solution (Becton Dickinson). Cells were analysed by flow cytometry and granulocytes were discriminated on the basis of different forward/side scatter properties and electronic gates on CD16-negative cells were set to eliminate neutrophils. Data are expressed as original plot (specific mAb vs. isotype control), ratio of fluorescence intensity of specific mAb (median) and isotype control (median) or fluorescence intensity (%) calculated as: (median channel fluorescence [stimulus] – median channel fluorescence [isotype control]) / (median channel fluorescence [medium] – median channel fluorescence [isotype control]) $\times 100\%$.

Toxin treatment

Cycloheximide (2500 ng/mL) and Actinomycin D (1000 ng/mL) treatment (Sigma Chemicals) was performed by incubation of 1×10^6 freshly isolated cells/mL for 24 h at 37 °C with and without toxins as described previously [28]. The cells were then washed and resuspended in test medium. Cytochalasin B (Sigma Chemicals), treatment was performed by incubation of 1×10^6 freshly isolated cells/ml for 15 min at 37 °C with different concentrations of the toxin (1, 5, 10 μ g/mL). The cells were then washed, resuspended in test medium and incubated for 30 min with C5a (Sigma Chemicals) 10^{-7} and 10^{-8} M, respectively. After a further wash step immunofluorescence staining with anti-CXCR4 mAb was carried out as described above.

Co-incubation of eosinophils with serum or peripheral blood mononuclear cell

Freshly isolated human eosinophils (0.2×10^6 cells in 100 μ L RPMI medium) were maintained in a humidified CO₂

environment in 96-well plates with 100 μ L serum of the same donor for 24 h at 37 °C. Eosinophils were washed and CXCR4 expression was analysed by flow cytometry. Coculturing of human eosinophils and PBMCs was performed by transferring 0.5×10^6 eosinophils (in 500 μ L RPMI) in a 24-well plate. Eosinophils were then separated by a tissue insert (Anapore Membran, 0.2 μ m, Nunc GmbH & Co. KG, Wiesbaden, Germany) from PBMCs (0.5×10^6 cells in 500 μ L RPMI). After 24 h incubation time at 37 °C PBMCs were removed, eosinophils were washed and CXCR4 expression was analyzed by flow cytometry.

Internalization of CXCR4

These experiments were carried out as described in detail previously [29]. Purified eosinophils were incubated in a total volume of 100 μ L of RPMI for different time-points at 37 °C in an incubator with CXCL12 or medium as a control. Aliquots were taken at the times indicated and stained for receptor surface expression as described above. To inhibit CXCR4 internalization eosinophils were treated for 5 min with 8 μ M phenylarsine oxide (PAO) (Sigma Chemicals) at 37 °C. Thereafter, cells were further incubated for 30 min in the presence of PAO with CXCL12 or medium as control. After washing immunofluorescence staining was carried out as described above. There were no differences in viability of the cells (>95%) between the fractions, as judged by trypan blue exclusion.

Lucigenin-dependent chemiluminescence

Lucigenin-dependent chemiluminescence (CL) was measured using a single-photon imaging system with a two-dimensional photon counting system (MTP reader, Hamamatsu Photonics, Herrsching, Germany) as described [27]. In brief, eosinophils were suspended to a density of 5×10^4 cells/mL in HBSS+BSA, respectively, containing 200 μ M lucigenin (Sigma Chemicals). Aliquots (100 μ L) containing 5×10^3 eosinophils were placed into flat-bottom white microtitre plates (Microfluor, Dynatech, Denkendorf, Germany). Cells were stimulated by the indicated stimuli and medium. Measurements were performed in triplicate at 37 °C. Integral counts from 0 to 30 min incubation intervals after addition of the stimuli to the cell sample were measured and expressed as intensity integral counts. Data are expressed as original plot or ratio between stimulus- and medium-induced intensity integral counts [27].

Assessment of actin polymerization in eosinophils

Nitrobenzoxadiazole (NBD)-phalloidin (Molecular Probes, Eugene, OR, USA) staining of granulocytes was carried out by modification of the method described by Howard and Meyer [30, 31]. Briefly, purified cells were re-suspended at a concentration of 2×10^5 cells/mL in phosphate-buffered saline (PBS)-buffer lacking Ca^{2+} and stimulated with the indicated chemotaxin or PBS buffer at 37 °C. Following stimulation, cells were fixed using 3.7% formaldehyde for 60 min. Lysophosphatidylcholine (20 μ g/mL) and 3.7×10^{-7} M NBD-phalloidin were added to the sample and incubated for a period of 60 min in the dark. NBD-phalloidin-stained cells were analyzed on a Becton Dickinson FACScan®

with a linear fluorescence channel (FL1) where the fluorescence is proportional to F-actin content [30, 31]. Relative F-actin content is expressed as the ratio of the mean channel fluorescence (= integrated fluorescence) between chemotaxin-stimulated and non-stimulated cells.

Measurement of $[\text{Ca}^{2+}]_i$

Eosinophils were loaded with 2 μ M Fura-2 (Molecular Probes, Leiden, the Netherlands) and $[\text{Ca}^{2+}]_i$ was measured at 37 °C in an Aminco Bowman Series 2 spectrofluorometer (SLM-Aminco, Urbana, IL, USA) as described [27]. Autofluorescence represented approximately 10% of the total fluorescence of cells loaded with fura-2. In addition autofluorescence was higher at 340 nm than at 380 nm. To minimize these effects the fluorescence of unloaded cells was subtracted from an equivalent density of cells loaded with fura-2 to obtain fluorescent signals that were solely representative of intracellular fura-2. The autofluorescence of cells was virtually unchanged upon addition of stimulus. Calibration was carried out after addition of the stimuli. Thus, in all experiments, the fluorescence of untreated and unloaded cells was used for subtraction of autofluorescence. Dual excitation spectra were collected at 340 and 380 nm; emission was determined at 510 nm. Absolute $[\text{Ca}^{2+}]_i$ was calculated automatically by AB2 series 2 software (SLM-Aminco) according to the equation described by Grynkiewicz et al. [32].

Chemotaxis of eosinophils

Before performing the chemotaxis experiments, purified eosinophils were incubated for 24 h with different dosages of IL-3 and medium. Chemotactic activity of eosinophils was performed as described previously using the modified Boyden chamber technique [33]. Briefly, Boyden chambers (Nuclepore GmbH, Tübingen, Germany) were filled with the stimuli and covered with polycarbonate filters (pore size, 3 μ m; Nuclepore, GmbH). One hundred microlitres of a human eosinophil suspension at a concentration of 5×10^5 /mL was added to each chamber. After incubation for 1 h at 37 °C migrated cells in the lower part of the Boyden chambers were lysed by adding 0.1% Triton X-100, and β -glucuronidase activity in the lysates was determined photometrically using *p*-nitrophenyl β -D-glucuronide as a substrate (all by Sigma chemicals). For calculation of the number of migrated cells, equivalent to β -glucuronidase activity determined in the lower part of the Boyden chamber, values were calculated by a computer-assisted technique from a standard curve using known numbers of unchallenged eosinophils. Chemotactic activity was expressed as chemotactic index: ratio of the number of migrating cells in presence of stimulus/migrating cells in presence of medium.

Real-time quantitative reverse transcriptase-polymerase chain reaction assay

RT-PCR analysis of eosinophil mRNA was performed as described previously [34]. In brief, poly (A⁺) RNA was isolated from 10^6 eosinophils using a mRNA isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). The isolated mRNA was used for cDNA synthesis with the first strand cDNA synthesis kit (Roche Molecular Biochemicals)

following the instruction manual as described previously [34]. Specific PCR primers for human mRNA of CXCR4 and β -Actin were designed and conventional PCR was performed under primer-specific conditions. The direct and reverse oligo primers for CXCR4 used were: sense, 5'-CCACGCCACCAACAGTC-3' and antisense, 5'-AATTTGGCTCCAAGGAAGCA-3' (expected bp length 507; GI no. 2565335). The β -actin genes were generated by connecting the following sequences of the specific primers: sense, 5'-GAGCGG-GAAATCGTGCCTGACATT-3' and antisense, 5'-GAAGGTAGTTTCGTGGATGCC-3' (expected bp-length 204; GI No. 28251). The standards and unknown samples were added in a total volume of 20 μ L/reaction as described in detail previously [34]. The standards used in this experiment were obtained from a serial dilution of cDNA from medium-stimulated eosinophils (24 h). The following experimental protocol was adapted to use CXCR4 and β -actin: After an initial denaturation step at 95 °C for 30 s, 40 (CXCR4) and 30 cycles (β -actin) of amplification were performed, respectively. Beginning with a denaturation step at 95 °C, followed by primer-specific annealing (65 °C for CXCR4; 60 °C for β -actin), the elongation was carried out at 72 °C with an amplicon-depending incubation time (20 s for CXCR4; 9 s for β -actin). Finally, the standard curve allowed calculation of the template concentrations in the unknown samples using LightCyclerTM software.

Statistical analysis

Unless otherwise stated, the data in the text and figures were expressed as mean $\pm$ standard error of the mean (SEM) as determined by SigmaStatTM (Jandel Scientific Corp., Erkrath, Germany) analysis. Values of $P < 0.05$ were accepted as significant using Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Kinetics of CXCR4 expression paralleled with CXCL12-induced eosinophil cell functions

Since previous studies demonstrated enhanced surface expression of CXCR4 on human eosinophils after long-term incubation in medium, we first focused our interest on the time dependence of this process. To determine whether surface expression of CXCR4 occurred because of the long-term Ficoll separation steps, expression of CXCR4 was examined in whole-blood eosinophils in place of Ficoll-separated eosinophils. As shown in Fig. 1A, eosinophils in fresh whole blood expressed significant ($P < 0.001$) amounts of CXCR4 on their surface when compared with the isotype control ($n = 20$, median channel fluorescence (MCF) IgG2a = 3.6 ± 0.2 , MCF CXCR4 = 5.5 ± 0.4). The expression pattern of CXCR4 of whole-blood eosinophils and freshly isolated eosinophils show a heterogeneous expression of CXCR4. In these cases most eosinophils are CXCR4 negative, however, using the mean of positive and negative CXCR4 there is a significant expression pattern of CXCR4 (Figs 1A and B). From the data of our study we could detect approximately 15% of blood eosinophils that express low levels of CXCR4. However, after 2 h of incubation almost all eosinophils do express CXCR4 (Fig. 1B).

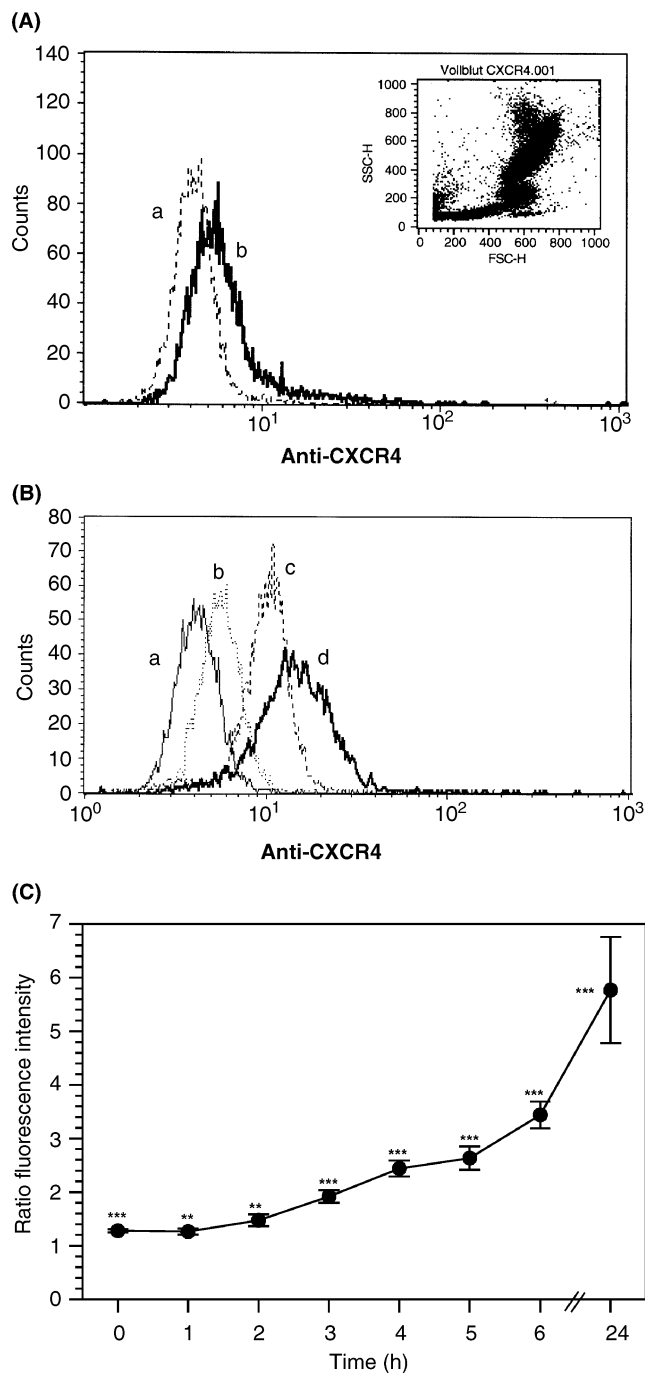


Fig. 1. Time course of CXCR4 expression on human eosinophils. (A) Whole-blood eosinophils in place of Ficoll-separated eosinophils were double-stained with anti-CXCR4-Phycoerythrin (PE) and anti-CD16-fluorescein isothiocyanate (FITC) and analysed by flow cytometry. Granulocytes were discriminated on the basis of different forward/side scatter properties (inset) and electronic gates on CD16-negative cells were set to eliminate neutrophils. Interrupted line represents the isotype control and bold line represents CXCR4⁺ eosinophils. Data are expressed as original histogram or dot blot and are representative of 20 separate experiments. (B) Purified eosinophils were immediately (b), after 2 h (c) and after 24 h (d) stained with anti-CXCR4 monoclonal antibody (mAb) or isotype control (a) and analysed by flow cytometry as described in Materials and methods. Data are expressed as original plot and are representative of six separate experiments as shown in (B). (C) Time course of surface CXCR4 expression is shown. Data ($n = 6$) are expressed as the ratio of fluorescence intensity of specific mAb (median) and isotype control (median) $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. each control (control IgG).

Incubation of eosinophils in RPMI medium without further supplements resulted in an increase of CXCR4 expression when compared with freshly isolated eosinophils (Fig. 1B). Surface expression of CXCR4 was detectable when freshly isolated eosinophils were immediately stained with anti-CXCR4 mAb (Fig. 1B). A *t*-test between mean fluorescence of the isotype control and mean fluorescence of CXCR4 mAb demonstrated a highly significant difference ($P < 0.001$, Fig. 1C). Furthermore, constitutive CXCR4 expression was independent of the state of health of the patient (atopic or non-atopic). Relevant surface expression of CXCR4 in all donors was observed within 3 h of incubation at 37°C, and the level of expression increased linearly with a maximum at 24 h of incubation (Fig. 1C). To exclude the possibility that the lack of an inhibitor present in the blood may be responsible for the weak expression of CXCR4 on human eosinophils, we incubated freshly isolated eosinophils with the serum or PBMCs from the same donor. Whereas serum did not significantly alter CXCR4 expression on eosinophils, PBMCs significantly ($P = 0.021$) prevented eosinophils from expressing high amounts of CXCR4 (Table 1).

To address the question whether the CXCR4 expression has functional relevance, release of reactive oxygen species (ROS) and $[Ca^{2+}]_i$ was measured at different time-points from 0 min to 24 h after isolation. Therefore eosinophils were stimulated with the specific ligand for CXCR4, CXCL12. In freshly isolated eosinophils no CXCL12 activation, leading to the release of ROS, could be detected at this time-point (Fig. 2a). In contrast to these findings, CXCL12 elicited a small but apparent Ca^{2+} influx in these cells (Fig. 2c). However, in eosinophils incubated for a longer time (2 h) the ROS release after stimulation with CXCL12 began and increased continuously (Fig. 2a). In cells incubated for 24 h at 37°C, much stronger ROS release, actin polymerization and influx of Ca^{2+} were observed (Figs 2a, b and d). Therefore, the expression pattern of CXCR4 correlates with the functional activity of aged eosinophils following stimulation with CXCL12.

CXCR4 was not detected in intracellular compartments of freshly isolated eosinophils but could be abolished by the transcription inhibitor actinomycin D

Next we investigated whether CXCR4 may be localized in intracellular compartments before intense surface protein expression is detectable. Stimulation of freshly isolated

eosinophils with C5a in the presence of cytochalasin B, which is a well-known granule-interrupting agent, did not result in a significant expression of CXCR4 (Fig. 3). Moreover, intracellular staining of freshly isolated eosinophils with

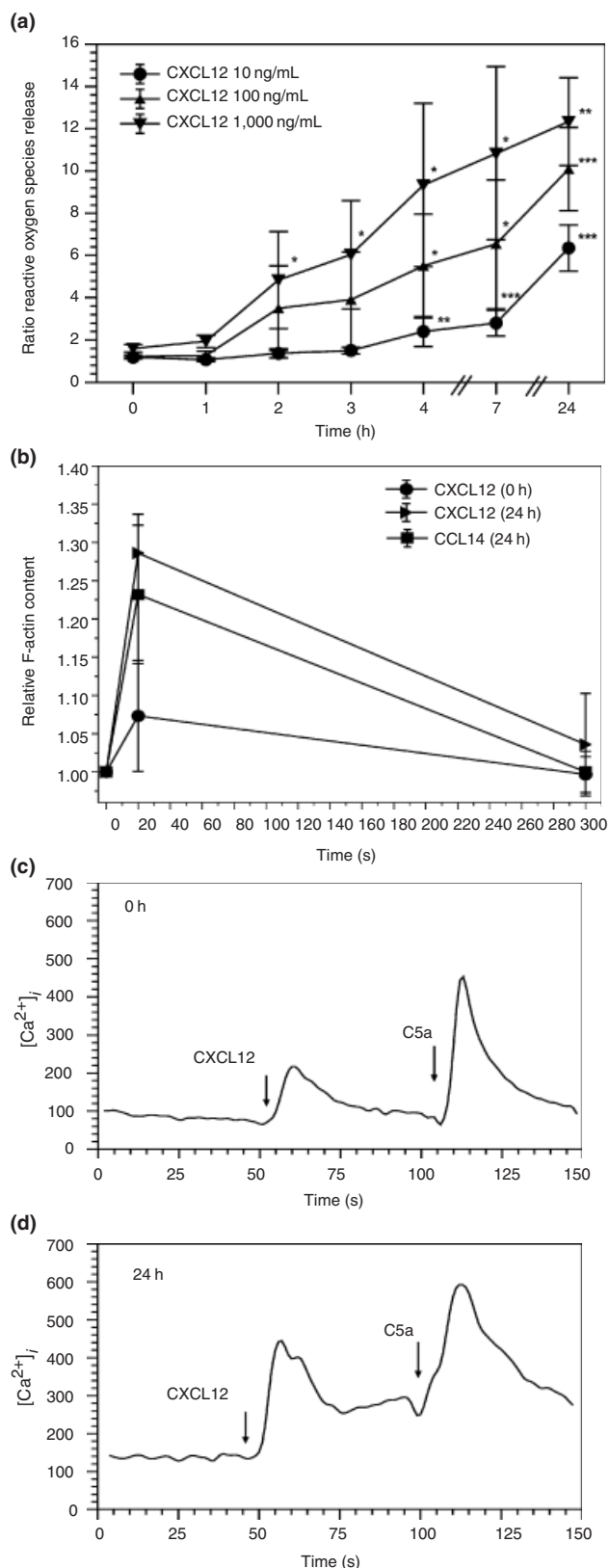


Fig. 2. Kinetics of CXCR4 expression paralleled with CXCL12 induced reactive oxygen species (ROS) release and intracellular Ca^{2+} influxes. (a) The release of ROS was measured using lucigenin-dependent chemiluminescence. Human eosinophils were stimulated with different concentrations of CXCL12. Data ($n = 6$) are expressed as relative ROS release that is expressed as the ratio of stimulus- and medium-stimulated cells. Values of $P < 0.05$ were accepted as significant using Student's *t*-test as described in Materials and methods. (b) Actin polymerization in freshly (0 h) and overnight-incubated (24 h) eosinophils was measured using nitrobenzoxadiazole (NBD)-phalloidin staining and flow cytometry as described in Materials and methods. Eosinophils were stimulated either with 10^{-7} M CXCL12 or CCL14 as a control. Data ($n = 3$) shows relative F-actin content at different points of time after chemokine stimulation. (c, d) Spectrofluorometric measurement of $[Ca^{2+}]_i$ in Fura-2-loaded human eosinophils was carried out as described in Materials and methods. Eosinophils were incubated with medium at 37°C for 0 h (c) and 24 h (d) to induce enhanced CXCR4 surface expression. Cells were stimulated with CXCL12 (1.25×10^{-8} M) and C5a (10^{-8} M). Data are expressed as original plot of one representative experiment out of six.

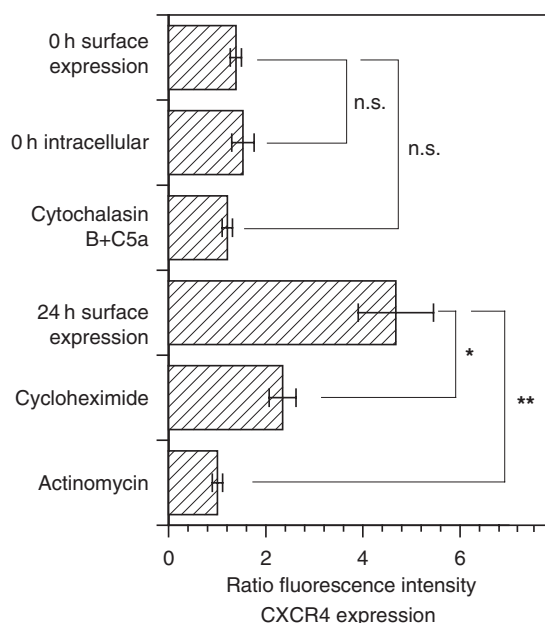


Fig. 3. Intracellular staining of CXCR4 and surface expression after toxin treatment. Eosinophils were treated with cytochalasin B, C5a, cycloheximide or actinomycin, respectively, as described in detail in Materials and methods. Data ($n = 6$) are expressed as the ratio of fluorescence intensity of specific anti-CXCR4 monoclonal antibody (median) and isotype control (median) $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. each control (0 or 24 h medium-incubated eosinophils); n.s., not significant.

anti-CXCR4 mAb did not reveal significant expression of CXCR4 (Fig. 3). Therefore, the expression of CXCR4 on the surface of human eosinophils probably cannot be explained by the release of intracellular preformed receptors.

To further determine the mechanism leading to CXCR4 surface expression we treated cells with cycloheximide and actinomycin, respectively, as described in Materials and methods. As shown in Fig. 3, cycloheximide as a biosynthesis inhibitor significantly inhibited the increase in CXCR4 expression up to app. 50%. These data indicate that protein biosynthesis is one regulative step for induction of CXCR4 surface expression. Furthermore, we tested the effect of the transcription inhibitor actinomycin D on CXCR4 expression. Again CXCR4 expression was abolished by pre-incubation of eosinophils with actinomycin D (Fig. 3). In contrast to cycloheximide surface expression of CXCR4 was extremely suppressed (up to approximately 80%) indicating that transcriptional down- and up-regulation, respectively, is an important factor contributing to increased CXCR4 pathway activity in these cells.

Expression of CXCR4 mRNA and protein in eosinophils and modulation by cytokines

To prove whether transcription is the crucial regulative step leading to an enhanced or reduced surface CXCR4 expression, we examined the mRNA expression of freshly, medium- and cytokine-stimulated eosinophils using real-time quantitative RT-PCR. The melting curves in Fig. 4a indicate that mRNA of CXCR4 was present in non-stimulated freshly isolated human eosinophils as well as in eosinophils incubated for 24 h in medium alone or with

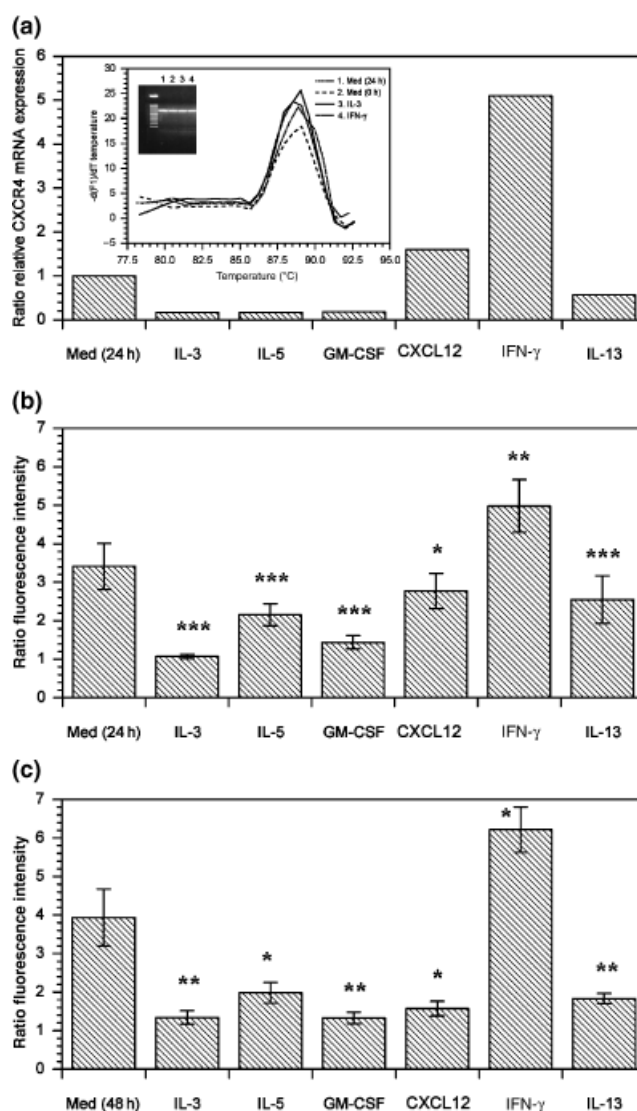


Fig. 4. Effect of cytokines on CXCR4 mRNA and surface protein expression analysed by real-time quantitative RT-PCR assay and flow cytometry. (a) Real-time quantitative RT-PCR assay and melting curve analysis revealed a specific denaturation profile of CXCR4 cDNA. Eosinophils were cultured for 24 h in medium either with no cytokine or with indicated cytokine (10^{-9} M). Isolation of mRNA and RT-PCR were performed as described in detail in Materials and methods. The standard curve allows calculation of the template concentrations in the unknown samples using LightCycler™ software. CXCR4 mRNA expression is calculated by the ratio of concentration of CXCR4 mRNA and concentration of β -actin mRNA in untreated or cytokine-treated eosinophils, respectively. One representative experiment out of three is shown. Inset: melting curves of the indicated PCR products are illustrated (T_m , $88.4 \pm 0.5^\circ\text{C}$) and electrophoresis of RT-PCR products and visualization with ethidium bromide indicated the predicted size for CXCR4 cDNA (M: 50 bp DNA size marker; bright band: 350 bp). (b) Eosinophils were treated with medium or different cytokines for 24 h at 37°C . Thereafter cells were stained with anti-CXCR4 monoclonal antibody (mAb) and analysed by flow cytometry. Data ($n = 13$) are expressed as the ratio of fluorescence intensity of specific mAb (median) and isotype control (median) $\pm$ SEM. (c) Eosinophils were incubated with medium at 37°C for 24 h to induce enhanced CXCR4 surface expression. Thereafter cells were stimulated for another 24 h with the indicated cytokines and CXCR4 surface expression was measured by flow cytometry. Data ($n = 13$) are expressed as the ratio of fluorescence intensity of specific mAb (median) and isotype control (median) $\pm$ SEM.

different cytokines. Agarose gel electrophoresis of the probes obtained by the LightCycler™ revealed the predicted size for CXCR4 (Fig. 4a).

Comparing the amplification of standard DNA template (24 h medium-incubated eosinophils) and long-term (24 h) cytokine-treated cDNA with the cDNA of a housekeeping gene (β -actin), showed the fivefold amount of CXCR4 cDNA in IFN- γ -stimulated eosinophils (Fig. 4a). On the other hand, we could detect the fifth of gene copies in IL-3-, IL-5-, and GM-CSF-stimulated eosinophils compared with medium-incubated eosinophils. Whereas IL-13 slightly reduced CXCR4 mRNA, CXCL12 incubation of eosinophils slightly enhanced CXCR4 mRNA expression (Fig. 4a). Therefore, cytokine treatment of human eosinophils led to an altered CXCR4 mRNA expression indicating a regulative transcriptional mechanism for CXCR4 influenced by cytokines.

To examine the modulation of CXCR4 surface expression on eosinophils after cytokine treatment during 24 h of incubation, we tested the effects of different cytokines on CXCR4 expression by flow cytometry in comparison with the specific CXCR4 ligand CXCL12. Freshly isolated eosinophils were incubated either with medium or with IL-3, IL-5, GM-CSF, IL-4 and IL-13 for 24 h in various doses. IFN- γ enhanced significantly and dose dependently ($P < 0.01$ at 10^{-9} and 10^{-10} M) the up-regulation of CXCR4 surface protein compared with medium (Figs 4b and 5a). In contrast to IFN- γ , IL-3 ($P \leq 0.001$), IL-5 ($P \leq 0.001$), GM-CSF ($P \leq 0.001$), IL-4 ($P < 0.001$), and IL-13 ($P \leq 0.001$) significantly prevented the surface expression of CXCR4 compared with medium-incubated cells (Figs 4b and 5b). Other cytokines, like TNF- α and TGF- β , tested had no significant effect on the expression (data not shown).

To prove the effect of the cytokines on eosinophils with stronger CXCR4 surface expression, we cultured eosinophils for 24 h in RPMI medium alone. Thereafter, we treated the cells with different cytokines for 24 h. As shown in Fig. 4c IL-3 ($P < 0.01$), IL-5 ($P < 0.05$), GM-CSF ($P < 0.01$), and IL-13 ($P < 0.05$) again significantly decreased and IFN- γ ($P < 0.05$) significantly increased the surface expression of CXCR4, when added to 24 h medium-incubated CXCR4⁺ eosinophils. Therefore, these data emphasize that type 2 cytokines prevent and type 1 cytokines enhance the expression of CXCR4 on the surface of human eosinophils as well as on mRNA level.

CXCL12 induces an internalization of CXCR4 on human eosinophils

To assess whether the CXCR4 of 24 h senescent eosinophils could be regulated by an internalization mechanism, we stimulated eosinophils with CXCL12 at 37 °C in the absence and presence of PAO, an inhibitor of receptor internalization [29]. Two of the donors, which have been incorporated into this series of experiments show only few basal level of CXCR4 after 24 h (Fig. 6). If the basal expression of a receptor is low the internalization experiment is not as imposing as compared with the donors that show high CXCR4 expression at basal level. However, as indicated from the other three donors, which express high amount CXCR4 after 24 h, we could clearly demonstrate that CXCR4 is down-regulated after stimulation with CXCL12. Moreover, even when all donors are incorporated into the statistics there is a significant difference ($P < 0.05$) of CXCR4 internalization after 30 min in response to CXCL12 (Fig. 6). Since PAO inhibited significantly the CXCL12-induced down-regulation

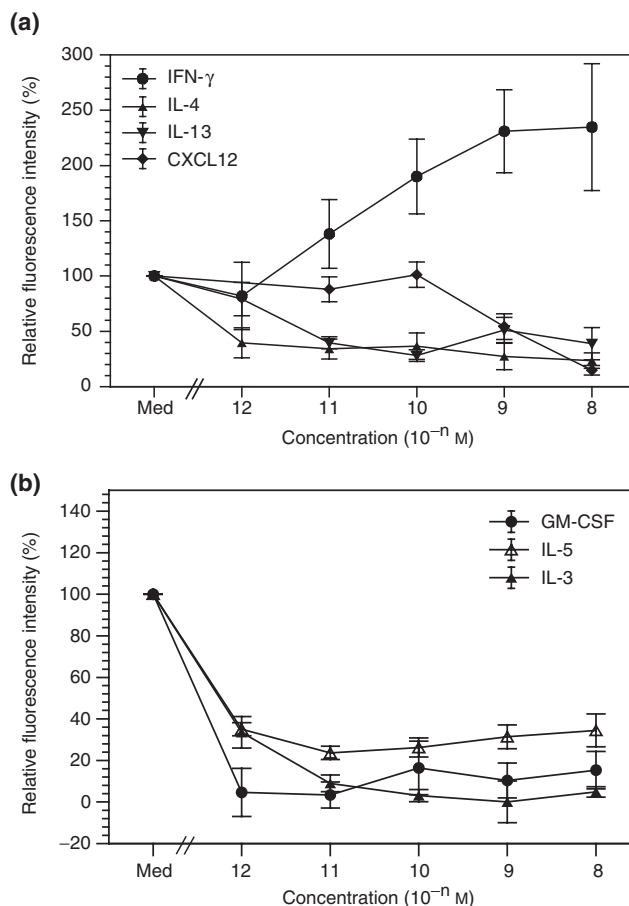


Fig. 5. Dose-dependent modulation of CXCR4 surface expression in eosinophils by cytokines. Eosinophils were treated with different concentrations of IFN- γ , IL-4, IL-13 and CXCL12 (a) or IL-3, IL-5, and granulocyte macrophage-colony stimulating factor (GM-CSF) (b) for 24 h at 37 °C. Thereafter cells were stained with anti-CXCR4 monoclonal antibody (mAb) and analysed by flow cytometry. Data ($n = 13$) are expressed as relative fluorescence intensity (%), calculated as described in detail in Materials and methods. Significances are as follows: * $P < 0.05$ (IFN- γ 10^{-8} M; CXCL12, 10^{-8} and 10^{-9} M); ** $P < 0.01$ (IFN- γ , 10^{-9} and 10^{-10} M); *** $P < 0.001$ (IL-3, IL-5, GM-CSF all concentrations; IL-13, 10^{-8} – 10^{-11} M) vs. each control (100%).

of CXCR4, it could be argued that the down-regulation of CXCR4 is because of an internalization mechanism.

T-helper 2 cytokines induce an attenuation of CXCR4 activation in response to CXCL12

To find out the functional consequences of CXCR4 cytokine modulated down-regulation intracellular Ca^{2+} fluxes and chemotaxis have been investigated. For this purpose eosinophils were cultured for 24 h with different cytokines (IL-3, IL-5, GM-CSF, and IFN- γ) and intracellular Ca^{2+} fluxes measured after stimulation with CXCL12. Interestingly, incubation with IL-3, which induced the strongest CXCR4 down-regulation on protein level, completely abolished the increase of Ca^{2+} fluxes after treatment with CXCL12 as shown in Fig. 7a. In contrast to IL-3, IL-5 and GM-CSF weakened but did not completely inhibit the response of eosinophils to CXCL12 when incubated for 24 h with these cytokines (Fig. 7a). IFN- γ which increased CXCR4 surface protein level, did not lead to an enhanced influx of intracellular

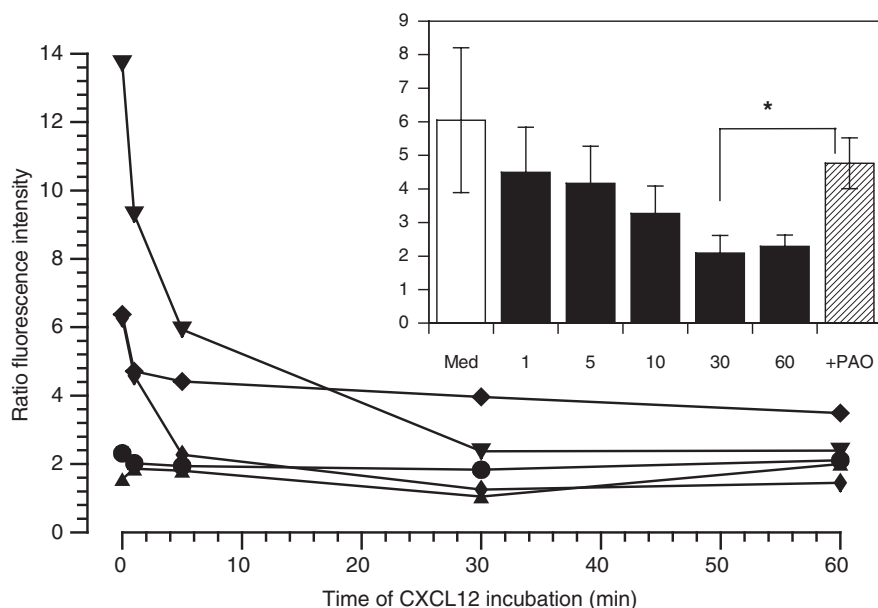


Fig. 6. CXCL12 induces an internalization of CXCR4 from the surface of senescent eosinophils. Purified eosinophils were incubated for 24 h in RPMI medium as described in Materials and methods leading to increased expression of CXCR4. Thereafter, cells were stimulated with CXCL12 (10^{-8} M) or medium as a control for different time-points. Graph shows five different experiments. Inset: Eosinophils were incubated in the presence or absence of 8 μ M phenylarsine oxide (PAO) (inhibitor of internalization). * $P < 0.05$.

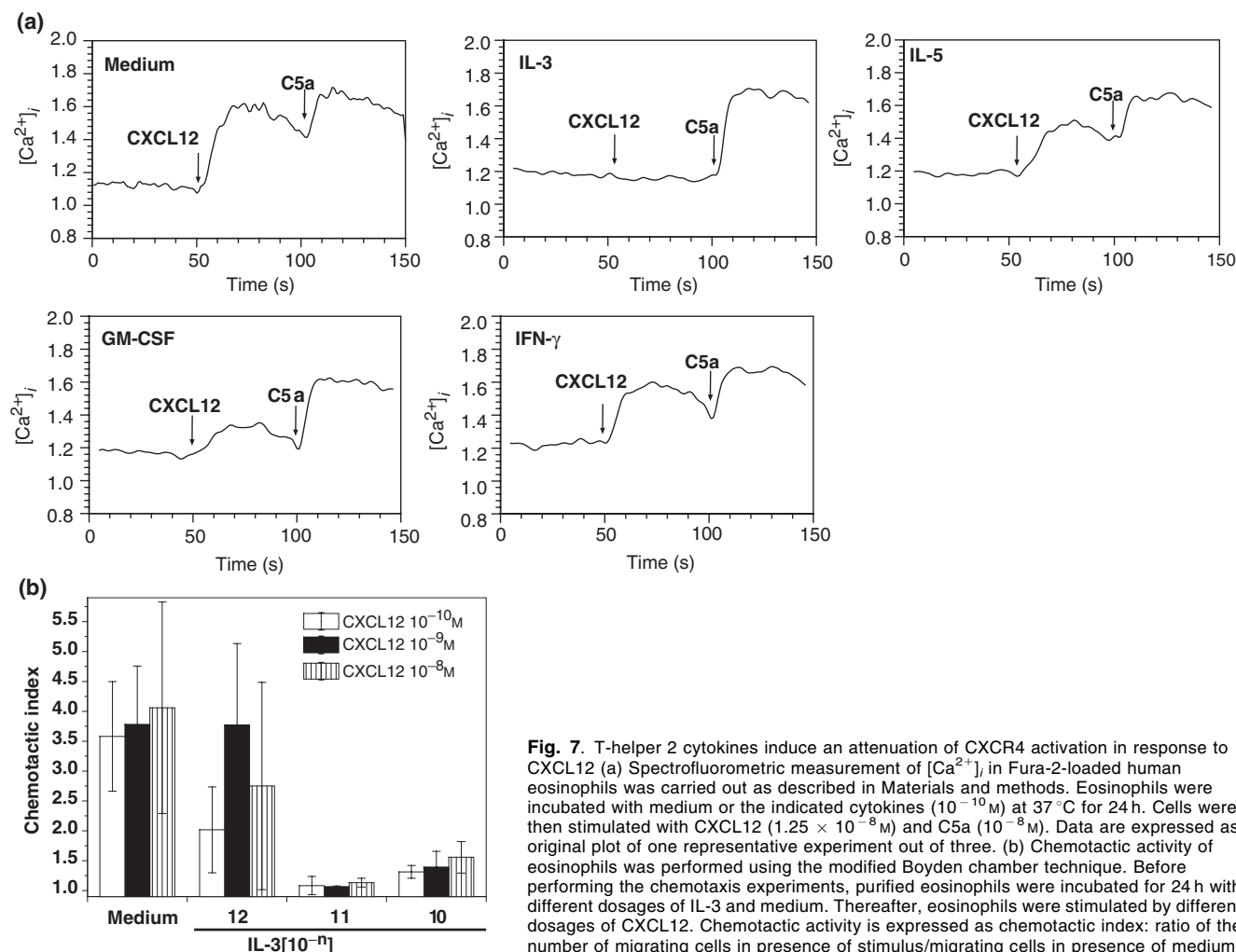


Fig. 7. T-helper 2 cytokines induce an attenuation of CXCR4 activation in response to CXCL12 (a) Spectrofluorometric measurement of $[Ca^{2+}]_i$ in Fura-2-loaded human eosinophils was carried out as described in Materials and methods. Eosinophils were incubated with medium or the indicated cytokines (10^{-10} M) at 37°C for 24 h. Cells were then stimulated with CXCL12 (1.25×10^{-8} M) and C5a (10^{-8} M). Data are expressed as original plot of one representative experiment out of three. (b) Chemotactic activity of eosinophils was performed using the modified Boyden chamber technique. Before performing the chemotaxis experiments, purified eosinophils were incubated for 24 h with different dosages of IL-3 and medium. Thereafter, eosinophils were stimulated by different dosages of CXCL12. Chemotactic activity is expressed as chemotactic index: ratio of the number of migrating cells in presence of stimulus/migrating cells in presence of medium.

calcium (Fig. 7a). Since IL-3 was the only cytokine inducing a complete desensitization of CXCR4, we investigated the effect of IL-3 on CXCL12-induced eosinophil chemotaxis.

IL-3 prevented dose dependently the chemotaxis response of human eosinophils in response to CXCL12 (Fig. 7b). Taken together, our data demonstrate that Th2-cytokines are able to

modulate not only CXCR4 mRNA and protein expression, but also influence eosinophil cell functions.

Discussion

Different chemokine receptors have been suggested to play a pivotal role in allergic diseases and therefore to be relevant for the activation of effector cells and propagation of the inflammatory response [9]. Among this, the extraordinary role of CCR3 is widely accepted [8]. Recent studies emphasizes, however, that the CXC chemokine receptor CXCR4 is also involved in allergic diseases. In a mouse model of allergic asthma, an antagonist against CXCR4 reduced a number of pathological parameters related to asthmatic-type inflammation [26]. Functional CXCR4 receptors have been found on the surface of senescent neutrophils and eosinophils [16, 23–25]. Nagase et al. [16] found intense expression of CXCR4 surface protein after 24 h culturing of human eosinophils that could be up- and down-regulated by cytokines. However, the underlying mechanism for CXCR4 expression and regulation could not be fully elucidated. Therefore, the aim of this study was to investigate the functional expression of CXCR4 on senescent eosinophils. Moreover, we questioned whether the cytokine profile – Th1 and Th2 cytokines – affect the activation of eosinophils via the CXCR4 that could be important for the different phases of the allergic reaction.

In contrast to Nagase et al. [16], we observed that surface CXCR4 protein was constitutively and significantly detectable in whole-blood and freshly isolated eosinophils indicating that long-term Ficoll separation steps did not alter CXCR4 surface expression. To investigate whether a lack of an inhibitor present in the blood may be responsible for the weak expression of CXCR4 on human eosinophils, we incubated freshly isolated eosinophils with the serum or PBMCs from the same donor. While serum did not significantly alter CXCR4 expression on eosinophils, PBMCs significantly prevented eosinophils to express high amounts of CXCR4. Next, we investigated whether the CXCR4 expression level goes along with cell functions of eosinophils after stimulation with CXCL12. After a 24 h incubation of eosinophils in medium, fluorescence signal of CXCR4 mAb increased and was finally one log step higher than in freshly isolated cells. These findings are in accordance with the fact that CXCL12 elicited a small but apparent Ca^{2+} influx in some freshly isolated eosinophils, and the CXCL12 responsiveness increased after medium incubation. In contrast to the early outset of Ca^{2+} fluxes, significant amounts of reactive oxygen species after CXCL12 stimulation could be first detected after 2 h of incubation in medium. Therefore, we hypothesized that this delayed reaction is because of the higher sensitiveness of the Ca^{2+} measuring procedure. When CXCR4 receptor signalling is measured in B cells a strong Ca^{2+} response is detectable on a cell population with as few as 11% of cells being CXCR4 positive [35]. Based on this analysis, we speculate that the magnitude of the response to CXCL12 is related to either the frequency of CXCR4 positive cells or the number of CXCR4 receptor molecules expressed on eosinophils.

Whereas CXCR4 is weakly detectable in freshly isolated eosinophils, surface expression increased gradually during

incubation at 37 °C in medium. Jourdan et al. [36] reported that freshly isolated peripheral blood T cells express low levels of cell surface CXCR4 that are strongly enhanced following incubation of the cells in medium only. It has been reported that freshly isolated Langerhans cells contain intracellular CXCR4 protein that is rapidly transported to the cell surface during *in vitro* culture [37]. The observed spontaneous up-regulation of CXCR4 on human eosinophils might be because of a different mechanism since we could not detect intracellular CXCR4 in freshly isolated eosinophils. In monocytes and lymphocytes surface expression of CXCR4 seems not to be regulated at the mRNA level but mainly at the protein level [38]. The process leading to the expression of CXCR4 in eosinophils was almost completely (up to 80%) inhibited by actinomycin D, which binds to DNA and blocks the movement of RNA polymerase. Our results indicate that transcriptional control is the important regulative step for CXCR4 expression. In contrast to actinomycin D, the biosynthesis inhibitor cycloheximide inhibited the increase in CXCR4 expression only up to approximately 50% indicating that protein biosynthesis is also but not the crucial regulative step for induction of CXCR4 surface expression.

Increased surface expression of CXCR4 has recently reported of medium-incubated human neutrophils. The authors explained this observation by an aging process of neutrophils [23, 24]. Moreover, CXCR4 seems to induce a homing of neutrophils leading to a clearance of senescent neutrophils from the circulation [24]. Because CXCR4 is already homogeneously expressed after 2 h on the surface of human eosinophils and apoptosis of eosinophils occurred after 24 h in only 20% of eosinophils [39, 40], it is unlikely that CXCR4 expression is a feature of apoptotic eosinophils. Since eosinophil apoptosis does not correlate with CXCR4 expression [16], we postulate a similar mechanism for eosinophils as for neutrophils in which increased CXCR4 expression parallels with the lifetime of eosinophils.

After binding of a chemokine to its corresponding receptor, the receptor is internalized and cells are not able to respond to the same ligand [41]. Incubation of leucocytes with CXCL12 induces down-regulation of up to 60% of surface-expressed receptors in a pertussis toxin-insensitive manner [42]. However, no data exist about a possible internalization of CXCR4 from the surface of purified human eosinophils. Herein, we could clearly demonstrate that CXCR4 is down-regulated within 30 min after stimulation with CXCL12. Because the internalization inhibitor PAO [29] inhibited this CXCR4 down-regulation it could be argued that CXCL12 induces an internalization of CXCR4 on senescent eosinophils.

The allergic reaction can be distinguished between an early and a late phase, which is characterized by a distinct cytokine profile. Increased levels of Th2 cytokines such as IL-3, IL-4, IL-5 and IL-13 are found in acute lesions of atopic eczema [3–5]. In contrast to the early phase, the chronic phase of allergic reaction is dominated by Th1 cytokine profiles (e.g. IFN- γ), which has been well documented in skin lesion of patients with chronic atopic eczema [3–5]. In this study, we found an up-regulation of CXCR4 protein and mRNA induced by Th1 cytokine IFN- γ and a down-regulation by Th2 cytokines (IL-3, IL-5, IL-13 and GM-CSF) which is in accordance to the previously described data of Nagase et al. [16]. To get in more detail about the functional consequence of the CXCR4

cytokine regulation we focused on relevant eosinophil functions. Herein, we could demonstrate that the Th2 cytokines attenuate the calcium response of human eosinophils in response to CXCL12 but IFN- γ does not modulate the calcium mobilization. From the tested Th2 cytokines IL-3 completely abolished the calcium response as well as the chemotaxis of human eosinophils indicating its extraordinary role of chemokine receptor regulation, which has recently shown for IL-3 on the CCR3 [29]. Therefore, Th2 cytokines are able to prevent surface expression of CXCR4 and its activation. From the data presented it could not be concluded that only senescent eosinophils are attracted into allergic tissues in response to CXCL12. However, we speculate that during the late allergic response the switch from Th2 to Th1 cytokine profile [5] may result in an increased expression of CXCR4. Since eosinophils expressing higher amounts of CXCR4 respond to a higher extent to CXCL12 it is likely that in the chronic allergic response eosinophils are more susceptible to CXCL12.

In summary, we have demonstrated constitutive expression of CXCR4 mRNA and surface protein in human eosinophils. Th2 cytokines down-regulated and the Th1 cytokine IFN- γ up-regulated CXCR4 mRNA and surface protein expression. The discovery that CXCL12 activated effector functions of senescent eosinophils is of interest, since blocking of CXCR4 by the antagonist AMD3100 reduced a number of pathological parameters related to asthmatic-type inflammation in a murine model of allergy [26]. Moreover, our study reveals that regulation of CXCR4 expression by cytokines has functional relevance as shown by reduced responsiveness of the senescent eosinophils to CXCL12 in calcium assays and chemotaxis. Therewith, it seems necessary to pay attention to the cytokine milieu when estimating the role of CXCR4 expression on eosinophils in the chronic phase of allergic diseases such as asthma and atopic eczema.

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