CHAPTER 4

IL-8 induces a transient arrest of rolling eosinophils on human endothelial cells

Laurien H. Ulfman, Dianne P.H. Joosten, Jan A.M. van der Linden, Jan-Willem J. Lammers, Jaap Jan Zwaginga*, Leo Koenderman
Dept. of Pulmonary Diseases, University Medical Center, Utrecht, The Netherlands
*Present address: Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands
Abstract

Eosinophils exhibit a rolling interaction with E-selectin-expressing endothelium and need to be activated by inflammatory mediators to firmly adhere to this surface. This study shows that IL-8 induces a transient arrest of unprimed eosinophils that roll on E-selectin present on tumor necrosis factor α-activated human umbilical vein endothelial cells in an in vitro flow chamber. This process was antagonized by neutralising antibodies directed against IL-8, showing the specificity of the IL-8 effect. Furthermore, blocking antibodies against both α4- and β2-integrins inhibited the IL-8-induced transient arrest while these antibodies had no effect when they were added separately. The IL-8-induced arrest was Pertussis Toxin (PTX) sensitive. Studying the effect of IL-8 in more detail we evaluated putative changes in [Ca2+]i in eosinophils induced by IL-8. We showed that IL-8 induces a transient increase in [Ca2+]i in ~40% of the cells, provided that the eosinophils are interacting with endothelial cells or fibronectin-coated surfaces. Together these data show that resting eosinophils respond to IL-8 provided that the cells adhere on physiological surfaces. The induction of a transient arrest provides a new level of chemokine-induced regulation of leukocyte adhesion under flow conditions.

Introduction

Eosinophils play an important role in allergic inflammatory diseases like asthma. Infiltrates of these cells are present in the structures of the airway wall and the lumen of the bronchi of allergic asthmatic patients (1). To enter the site of inflammation, eosinophils must leave the bloodstream and pass the endothelium. A widely accepted paradigm for leukocyte extravasation is referred to as the multistep model (2). In this model selectins and their carbohydrate-bearing ligands mediate rolling interactions between leukocytes and the endothelium. In this respect E-selectin, which is present on activated endothelium, has been shown to mediate rolling of neutrophils (3) and eosinophils (4). Subsequently, cells can be activated upon interaction with inflammatory mediators, resulting in the activation of integrins which bind to their ligands expressed on the endothelium. In this way, firm adhesion of the cells to the endothelium is established (5;6).

An important class of inflammatory mediators involved in the arrest of inflammatory cells are chemokines which are released at the site of inflammation. Chemokines can be divided in 4 different groups: CL, CCL, CXCL, and CX3CL in which X is the number of amino acids in between cysteine residues at the NH2-terminal site of the molecule. Of the CL and CX3CL families, only one member of each group has been described,
namely lymphotactin (7) and fraktalkine (8), respectively. CCL chemokines, including eotaxin, RANTES and MCP-3 have been reported to be mainly chemotactic for monocytes, lymphocytes and eosinophils. CXC chemokines, including IL-8 and GRO-α, have been shown so far as more specific for neutrophils (see 9;10 for reviews). However, several clinical studies indicate that expression of the CXCL chemokine IL-8 is enhanced both at the level of mRNA and protein in pulmonary “eosinophilic” diseases such as asthma (11-13). IL-8 has been shown to be produced by bronchial epithelium cells of asthmatic patients (11). Other sources for IL-8 include endothelial cells, fibroblasts, macrophages and mast cells (see 12 for review). These cells play a key role in the pathogenesis of allergic asthma. Many studies have been designed to link the production of different chemokines to the occurrence of inflammatory cells in the tissues. As allergic asthma is characterized by a clear eosinophilic inflammation in the bronchial tissue, several studies evaluated the role for IL-8 in eosinophil activation and migration processes in vitro and in vivo. No clear consensus exists concerning this issue. Some in vitro studies on IL-8 induced eosinophil chemotaxis failed to show an IL-8 dependent effect on eosinophil migration (14), whereas other reports did: some studies were performed with cytokine-activated or “primed” eosinophils (15;16) and other studies were performed with cells from allergic asthmatic (17) or eosinophilic (18) subjects. In most of these studies the effect of IL-8 on eosinophils have been determined by the use of chemotaxis assays such as the Boyden chamber. Subtle differences in experimental set up (e.g. choice of filters, medium etc.) might explain differences in outcome between the different studies. In addition to these in vitro studies the effect of IL-8 on eosinophils has also been suggested by an in vivo study that showed that provocation with IL-8 induces eosinophilia in the nasal epithelium (19).

Chemokines are ligands for G-protein-coupled serpentine receptors. On neutrophils, two high-affinity G-protein-coupled receptors for IL-8 have been described, CXCR1 and CXCR2 (20;21). When chemokines bind to their receptor, an increase in intracellular free Ca^{2+} ions [Ca^{2+}]_i is elicited. Increased [Ca^{2+}]_i leads to multiple downstream signaling events, and these have been correlated with a number of cellular functions (22). It has been questioned whether eosinophils can increase [Ca^{2+}]_i upon IL-8 stimulation, because these changes in [Ca^{2+}]_i were very small (15). Indeed, Petering et al. contributed these small changes to contamination of neutrophils. Remarkably, all studies that failed to show the effect of IL-8 on [Ca^{2+}]_i mobilization in eosinophils were performed on cells in suspension. Migration studies, on the other hand, showed that eosinophils can respond to IL-8, and in these experiments cells adhered to substrates. We hypothesize that eosinophils that adhere to physiological substrates are more susceptible for IL-8 stimulation compared to cells in suspension. Therefore, the effect of IL-8 on eosinophils adherent to physiological relevant surfaces was evaluated.
Materials and Methods

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma Chemicals (St. Louis, MO, USA). Human serum albumin (HSA) was purchased from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNFα was purchased from Boehringer Mannheim (Germany). IL-8 was purchased from Peprotech (72aa, Rocky Hill, NJ, USA) and Eotaxin-1 was purchased from R&D Systems (Minneapolis, MN, USA). Pertussis Toxin (50 μg/ml, Sigma, St. Louis, Missouri, USA). Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4, supplemented with 5 mM glucose, 1.0 mM CaCl2, and 0.5 % (w/v) HSA (Human Serum Albumin). All other materials were reagent grade.

Antibodies

The MoAb HP2/1 (anti VLA-4, CD49d) was purchased from Immunotech (Marseille, France). MoAb IB4 was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). The antibodies we used against α4 integrins (HP2/1) and β2 integrins (IB4) have been described as functional blocking antibodies (23-25;26) Therefore, antibody-induced differences in function of the eosinophils caused e.g. by crosslinking of integrins, seem to be unlikely. Control antibody W6/32 (anti HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). Activating antibody 8A2 (26) was kindly provided by dr. J.M. Harlan (University of Washington, Seattle, USA) and dr. T.W Kuijpers (Central Laboratory for Blood Transfusion (CLB), Amsterdam, The Netherlands). Anti-CXCR1 and anti-CXCR2 antibodies, 5A12 and 6C6 (Pharmingen, San Diego, USA), respectively, have been described as blocking MoAbs of neutrophil migration (27). Anti-E-selectin MoAb BBIG-E4 (5D11) was purchased from R&D systems (Abingdon, UK). MoAbs were incubated with eosinophils (4x10^6 cells/ml) at 10 μg/ml during 15 minutes before the experiments. The cell suspensions were diluted once with incubation buffer (final concentration of 5 μg/ml MoAb at 2x10^6 cells/ml in incubation buffer), and the coverslips were placed directly in the system. Anti-IL-8 (clone B-K8, Biosource International, California, USA) was added to IL-8 10^{-8} M in a final concentration of 20 μg/ml.
Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy-coat of 500 ml blood anti-coagulated with 0.4 % (w/v) trisodium citrate (pH 7.4), as previously described (28). Mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice-cold NH₄Cl solution, the granulocytes were washed and resuspended in RPMI 1640 (Gibco, Paisley, UK) with 0.5 % (w/v) HSA. Granulocytes were incubated for 30 min at 37°C to restore the initial density of the cells. Thereafter, the cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 0.5 % HSA and 13 mM trisodium citrate, and incubated with fMLP (10 nM) for 10 min at 37°C, to decrease the specific gravity of the neutrophils, but not that of the eosinophils. Subsequently, eosinophils were obtained by centrifugation (20 min, 1000 g) over isotonic Percoll (density 1.082 g/ml, layered on Percoll with a density of 1.1 g/ml), washed and resuspended in incubation buffer. Purity of eosinophils was >95 %. This procedure leads to the isolation of relatively unprimed eosinophils compared to conventionally used isolation procedures with immunomagnetic beads (29).

Endothelial cells

Human umbilical vein endothelial cells were isolated from human umbilical cord veins according to Jaffe et al. (30), with some minor modifications (31). The cells were cultured in RPMI 1640 containing 20 % (vol./vol.) heat-inactivated human serum, 200 µg/ml penicillin/streptomycin (GIBCO, Life Technologies, Breda, The Netherlands) and fungizone (GIBCO, Life Technologies, Breda, The Netherlands). Cell monolayers were grown to confluence in 5-7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVEC was activated by TNFα (100 U/ml, 7 hours, 37°C) prior to the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber (32) as previously described by Van Zanten et al. (31). This micro-chamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with confluent HUVEC was mounted. Immediately after mounting the HUVEC, which were activated with
TNFα for 7 hours, the flow chamber was flushed with HEPES buffer during 2 minutes to wash out residual TNFα.

**Eosinophil perfusion and evaluation**

Eosinophil perfusions were performed as described (4). In short, eosinophils in suspension (2x10^6 cells/ml in incubation buffer) were aspirated from a reservoir through plastic tubing and the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, MA). The individual runs all occurred under specific shear conditions in a 37°C temperature box. Perfusion experiments were recorded on video tape. The eosinophil suspension was perfused during 3 minutes at a shear stress of 0.8 dyn/cm² to obtain an endothelial surface with firmly adhering and rolling eosinophils (4). After these 3 minutes buffer was added and the shear stress was increased to 2 dyn/cm². After 20 seconds at a shear stress of 2 dyn/cm², recording of the images on video was started. Subsequently, HEPES buffer containing IL-8 (10^-8 M) or eotaxin (10^-8 M) was added to the flow chamber. During the whole perfusion experiment antibodies (IB4; 10 µg/ml, HP2/1; 10 µg/ml, anti IL-8; 50 µg/ml or W6/32; 10 µg/ml) were present. After ~30 seconds the cytokine buffer reached the cells and the cells arrested. Subsequently it took ~30 seconds to ~2 minutes for the cells to start rolling again. Therefore the percentage rolling cells were determined at the following points: “before” stimulation: this is at time point 30 sec before addition of the cytokine-containing buffer; “during” stimulation: this is at the time point that cytokine-containing buffer has reached the cells and the cells arrest; “after” stimulation: this is at time point 1 minute after the cytokine-containing buffer reached the cells. One randomly chosen image per experiment was recorded. Cells which started rolling again after the IL-8-induced arrest rolled out of the recorded image, but also cells at the front of the image rolled into the recorded image. Therefore we calculated the total amount of adherent cells present in the field “before”, “during” and “after” stimulation which did not differ significantly in all tested circumstances. More than 90 % of the firmly adherent cells under baseline conditions were stable throughout the whole observation period when no cytokine was added (results not shown). Also, of the stable stationary cells in the “before” period, less than 5 % of the cells started rolling again after addition of IL-8.

To automatically determine the percentage of rolling cells custom-made software was developed in Optimas 6.1 (4). In short, a sequence of 50 frames, representing an adjustable time interval, was digitally captured. The velocity of each cell was calculated. The cut-off value to distinguish between rolling and static adherent cells was set at 1 µm/sec. With this method, static adherent, rolling and freely flowing cells (which were not in focus) could be clearly distinguished.
IL-8 INDUCES A TRANSIENT ARREST OF ROLLING EOSINOPHILS ON HUMAN ENDOTHELIAL CELLS

**Flow cytometry**

Flow cytometry analysis was carried out as described before (33). As primary MoAbs were used antibodies against β2 integrins (IB4), CXCR1 (5A12), CXCR2 (6C6) or a control antibody (5D11 anti-E-selectin). Granulocytes were analyzed with a FACSVantage flowcytometer (Becton Dickinson immunocytometry systems, Mountain View, CA).

**Imaging of intracellular free Ca$^{2+}$**

Imaging of intracellular free Ca$^{2+}$ was performed with a custom-built setup, consisting of a computerized excitation filterswitcher (Lambda-10, Sutter Instrument Company, Novato, California, USA) with excitation filters (D340/10 and D380/13, Chroma Technology Corporation, McHenry, Illinois, USA) coupled to a Leica (Wetzlar Germany) Leitz DMIL inverted microscope, which was equipped with light filters appropriate for Fura-2 (D510/40 and 400 DCLP, Chroma Technology Corporation, McHenry Illinois, USA), high n.A Immersion objective (UV-F 40x nA 1.30 Glycerol Imm., Nikon Corporation, Tokyo, Japan) and a Xenon arc lamp (XBO 75 W/2, Osram, Berlin, Germany). Series of 50 image pairs (512 x 512, ~1 second apart) were sequentially grabbed with a black and white framegrabber (Pulsar, Matrox Electronic Systems Ltd., Dorval, Quebec, Canada) from an intensified video camera (LI-μCAM, Lambert Instruments, Leutingewolde, The Netherlands) directly into computer memory. Ratios and calcium values were computed off line with Image Analysis software (Optimas 6.1, Media Cybernetics, Silver Spring Maryland, USA) and custom-made macros (A.L.I.). Calcium levels were calculated with the standard calibration formula of Grynkiewics et al. (34):

$$[\text{Ca}^{2+}]_i = \frac{K_d \times \beta \times (R_{\text{min}})/(R_{\text{max}} - R)}{\beta}$$

with parameters $K_d=225 \text{nM}$, $\beta=2.4$, and were displayed in false colors (blue-red ramp). Cell calcium was separated from background by calculating the threshold mask from the mean of the two ratio images for each of the 50 ratio images in the series. Threshold level was obtained by choosing the first and the last level in the series, and then by linear interpolation for the rest of the series, thereby counteracting the effect of bleaching over time. Intracellular [Ca$^{2+}$] levels are determined by calculating the mean [Ca$^{2+}$]$_i$ of all pixels in a cell in each of the 50 ratios taken. The cut-off value to distinguish between a responding and a non responding cell was set at a mean value of 200 nM. Figure 4 and 6 show the mean [Ca$^{2+}$]$_i$ of all cells exceeding this cut-off value. In the experiments investigating the IL-8-induced increase in [Ca$^{2+}$]$_i$, eotaxin was given after the IL-8. The cells which did not respond to eotaxin (likely to be neutrophils) were excluded from analyses.

Freshly isolated eosinophils adhere strongly to some glass substrates. Under these circumstances attachment induces cells to flatten out with activation and generation of
calcium signals (unpublished observations). To circumvent these problems we let eosinophils adhere to fibronectin-coated surfaces with the use of MoAb 8A2 or to 7h TNF-α-activated HUVEC. Addition of this MoAb leads to freezing of β1 integrins in a high-affinity state. By this procedure, the cells strongly attach to fibronectin, but otherwise remain deactivated, keeping a round shape (35), thus allowing clear ratio imaging. Eosinophils attached in this way display low intracellular free Ca²⁺ levels under control conditions for long periods of time, up to 30 minutes. Hereby large numbers of cells could be imaged while being treated with IL-8 and eotaxin.

All washing and incubation steps were performed in incubation buffer. Eosinophils (5x10⁶/ml) were loaded with 2.5 μM Fura-2 AM (Molecular Probes, Eugene, Oregon, USA) for 15 minutes. Hereafter, the cells were incubated in the absence or presence of the antibody 8A2 (10 μg/ml) for 15 minutes at 37°C in an agitated water bath. After washing, 8A2 treated cells (1 x10⁶ to 2 x10⁶) were plated onto fibronectin (0.1 mg/ml in Hepes, 15 min, 37°C) coated 24 mm cover slips. 7h TNF-α-activated HUVEC cultured on 24 mm cover slips were extensively washed with PBS and non-treated, Fura-2 AM loaded eosinophils were plated on the endothelium. The eosinophils were allowed to settle for 15 minutes on the substrate, and non-adherent cells were removed by washing the cover slips. Cover slips were mounted in an open chamber and placed in a warmed (37°C) metal ring for Calcium Imaging. Stimulants were added from the top after two pre-stimulus image ratios had been obtained. Stimulants (37°C, at two times the concentration) were added to an equal volume (250 μl) of incubation buffer already present in the chamber to obtain a homogenous mixture at the start of the ratio imaging.

**Measurement of oxygen consumption**

Oxygen consumption was measured at 37°C with an oxygen electrode as described previously (36). In short, eosinophils were resuspended in incubation buffer (2x10⁶ cells/ml). PMA (100 ng/ml) was added and oxygen consumption was measured for 5 minutes.

**Statistical Analysis.** Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using a Student T test for paired data; p values < 0.05 were considered to be significant.
Results

Eosinophils exhibit a transient arrest upon IL-8 addition during rolling adhesion along TNFα-activated endothelium.

To investigate the effect of IL-8 on rolling eosinophils, freshly isolated eosinophils were perfused over 7h TNFα-activated confluent HUVEC at a shear stress of 2 dyn/cm². When eosinophils were treated with a control anti-HLA-class-I antibody (W6/32, figure 1a, 2a) the percentage of rolling cells compared to the total number of adherent cells (rolling and firmly adherent cells) was 48 ± 3 %. Upon stimulation with IL-8 (10⁻⁸ M), the percentage of rolling cells decreased to 12 ± 3 %. After 1 minute, the percentage of rolling cells increased to 28 ± 5 % (movie of IL-8-induced transient arrest is available on CD-ROM). Addition of anti-IL-8 antibody (10 μg/ml) to the IL-8 suspension prevented this transition from rolling to a stationary arrest of W6/32 treated (control) eosinophils (figure 1a). When eotaxin (10⁻⁸ M), a potent chemokine for eosinophils, was added to the rolling, W6/32-treated eosinophils, the percentage rolling cells decreased from 56 ± 3 to 7 ± 3 %. The cells now bound stably and longterm because after 1 minute the percentage of rolling cells was still very low (8 ± 3 %, figure 1a, 2b) (movie of eotaxin induced firm arrest is available on CD-ROM). In addition, the eosinophils flattened upon eotaxin treatment, while this was not observed upon addition of IL-8. We also tested whether eotaxin could induce a stable and longterm arrest when applied to cells which started rolling again after the IL-8-induced arrest. Indeed, more than 97 % of the cells were longterm arrested after addition of eotaxin to W6/32-treated eosinophils which were rolling after the IL-8-induced transient arrest.
IL-8 INDUCES A TRANSIENT ARREST OF ROLLING EOSINOPHILS ON HUMAN ENDOTHELIAL CELLS

Figure 1. Effect of perfusion of cytokines on percentage of rolling eosinophils on 7h TNFα-stimulated HUVEC. The percentage of rolling cells per field for W6/32-, HP2/1- and HP2/1-, IB4-treated eosinophils are depicted. The percentage of rolling cells was determined before (white bars), during (black bars) and 1 minute after (grey bars) stimulation. The effect of A) IL-8 (10^{-8} M), IL-8 (10^{-8} M)/ anti-IL-8 and eotaxin (10^{-8} M) on control MoAb W6/32-treated rolling eosinophils, B) IL-8 (10^{-8} M), IL-8 (10^{-8} M)/ anti-IL-8 and eotaxin (10^{-8} M) on MoAb HP2/1-treated rolling, C) IL-8 (10^{-8} M) and eotaxin (10^{-8} M) on anti-β_2 integrin integrin-treated and both anti-α_4 and β_2 integrin-treated eosinophils. Percentage rolling cells are plotted for 3 to 6 experiments ± SEM, except for W6/32 / IL-8 (n=10) and HP2/1 / IL-8 (n=16). The statistically significant effects of the different treatments against the situation before treatment were determined by paired Student T test (*: p<0.05).

To investigate whether α_4 integrins played a role in this IL-8-induced arrest, eosinophils were treated with anti-α_4 integrin antibody (HP2/1, figure 1b, 2c). The percentage of rolling cells of HP2/1-treated eosinophils on TNFα-activated HUVEC was 52 ± 5 % which was significantly higher than W6/32-treated eosinophils, as shown in a previous study (4). The rolling percentages of all groups are higher in this study compared to an earlier study (4). This is caused by the increase in the shear stress used in this study (2 dyn/cm^2) whereas the shear stress in the former study was 0.8 dyn/cm^2. Upon stimulation with IL-8 (10^{-8} M) the percentage of rolling cells decreased to 14 ± 3 %. After 1 minute, the percentage of rolling cells increased from 14 ± 3 % to 33 ± 5 %. Within 2 minutes almost all arrested cells (both W6/32- and HP2/1-treated) started rolling again. Addition of anti-IL-8 antibody (10 µg/ml) prevented the IL-8-induced transition from
IL-8 INDUCES A TRANSIENT ARREST OF ROLLING EOSINOPHILS ON HUMAN ENDOTHELIAL CELLS

Figure 2. Computerized analyses of the effect of perfusion with cytokines on the percentage of rolling eosinophils on 7h TNFα-stimulated HUVEC. Eosinophils (2x10^6/ml) were incubated with 10 μg/ml of the indicated MoAbs before the assay. Lines indicate the rolling tracks of the cells in one field during 2 seconds. Cells are positioned at the beginning of the track. Images depict rolling tracks before, during and 1 minute after indicated cytokine stimulation. Data are depicted as representative experiments (see also figure 1).

rolling to stationary arrest of HP2/1-treated eosinophils (figure 1b). When eotaxin (10^-8 M) was added to the rolling HP2/1-treated eosinophils, all cells bound stably and longterm (figure 1b). The experiment with the W6/32- and HP2/1-treated cells show that the IL-8 effect on eosinophils can occur independently of the interaction between α4β1 integrin with its ligand VCAM-1 on activated endothelium.

To investigate whether β2 integrins are implicated in the IL-8-induced arrest, eosinophils were incubated with an anti-β2 integrin MoAb IB4. When β2 integrins were blocked, the percentage of rolling cells per field was 66 ± 6%. Upon IL-8 stimulation, the percentage of rolling cells decreased to 28 ± 6 % and after 1 minute increased to 40 ± 6 % rolling cells again (figure 1c). When eotaxin was added to rolling IB4-treated
eosinophils, the percentage of rolling cells decreased from 64 ± 8 % to 9 ± 3 %. After 1 minute the cells were still firmly adhered to the endothelial cells.

Finally, we investigated the effect of blocking of β2 integrins and α4 integrins simultaneously. When both β2 integrin and α4 integrins were blocked, the percentage of rolling cells was 84 ± 4 %. Upon addition of IL-8 all cells kept rolling (80 ± 5 %). After 1 minute upon addition of IL-8 the percentage of rolling cells was still 85 ± 5 %. When eotaxin was added to IB4 and HP2/1 treated eosinophils, no significant changes in the percentage of rolling cells were observed (figure 1c). These data show that the IL-8-induced transient arrest and the eotaxin induced stable arrest can be mediated by either β2 integrins or α4 integrins.

To investigate whether the known IL-8 receptors CXCR1 and CXCR2 mediated the IL-8-induced response, eosinophils were incubated with antibodies against CXCR1 and CXCR2 (6C6 and 5A12, respectively). IL-8-induced transient arrest was not inhibited and the percentage of rolling cells decreased from 46 ± 10 % to 16 ± 5 % upon IL-8 stimulation (figure 3a). The IL-8 induced arrest was transient and the percentage rolling cells increased from 16 ± 5 to 27 ± 11 %. The functionality of these antibodies on IL-8 (10^{-8} M)-induced neutrophil chemotaxis in a Boyden chamber assay was confirmed by their ability to block the IL-8 induced migration by 74 %, as was also shown by others (27). In figure 3b and 3c it is shown that CXCR1 and CXCR2 are not present on isolated eosinophils whereas they are present on neutrophils (40). To address whether the IL-8-induced arrest was mediated by G-protein-coupled receptors, eosinophils were incubated with solvent (0.5 % glycerol), or PTX (100 or 500 ng/ml) for 2 hours. Control, glycerol-treated eosinophils arrested transiently upon IL-8 perfusion, comparable to W6/32- and HP2/1-treated eosinophils. In contrast, eosinophils treated with PTX showed a dose-dependent inhibition of IL-8-induced arrest (figure 3a). To control for possible negative effects of PTX on the normal physiology of the eosinophils, we performed respiratory burst experiments. Eosinophils incubated with glycerol (0.5 %) or PTX (500 ng/ml) for 2 h at 37°C were tested for the induction of the respiratory burst upon activation with PMA. No differences were found in the PMA-induced oxidative burst of glycerol- vs. PTX-treated eosinophils (not shown).
IL-8 INDUCES A TRANSIENT ARREST OF ROLLING EOSINOPHILS ON HUMAN ENDOTHELIAL CELLS

Figure 3. A) The effect of inhibition of serpine receptors on the percentage of rolling eosinophils on 7h TNFα-stimulated HUVEC. The effect of IL-8 on anti-CXCR1 and anti-CXCR2 antibodies (5A12 and 6C6, 15 min, 37°C), control solvent (glycerol 0.5 %, 2h, 37°C), and Pertussis Toxin (100 and 500 ng/ml, 2h, 37°C)-treated eosinphils was determined before (white bars), during (black bars) and 1 minute after (grey bars) stimulation. Percentages of rolling cells are plotted for 3 to 6 experiments ± SEM. The statistically significant effects of the different treatments compared to the situation before treatment were determined by paired Student T test (*: p<0.05). In B) and C) granulocytes were stained for β2 integrins (IB4, blue line), CXCR1 (5A12, green line), CXCR2 (6C6, red line) or an irrelevant antibody (black line) by FACS. B) and C) show the fluorescence for eosinophils and neutrophils, respectively. The experiment shown is representative for 3 independent experiments.

Eosinophils bound to fibronectin and endothelium show significant calcium responses upon IL-8 addition

To investigate the changes in intracellular free Ca²⁺ of adherent eosinophils upon IL-8 and eotaxin stimulation, cells were incubated with 8A2 and loaded on fibronectin-coated cover slips or non-treated cells were loaded on 7h TNFα-stimulated HUVEC (see Materials and Methods 35). After stimulation with IL-8 (10⁻⁸ M) a clear increase in [Ca²⁺]i (>200 nM) was observed in 42 ± 7 % and 30 ± 7 % of the cells adherent to fibronectin and activated HUVEC, respectively (figure 4a and 4b showing a representative experiment). The increase in [Ca²⁺]i of cells adherent to fibronectin is depicted in figure 5a. When neutralizing antibodies for IL-8 (clone B-K8) were added to the IL-8 solution before addition to the cells, the change in [Ca²⁺]i response was blocked (figure 4a and 5b). A second IL-8 stimulation given did not elicit a [Ca²⁺]i response, indicating homologous desensitization of the receptor (data not shown). Upon eotaxin (10⁻⁸ M) stimulation 97 ± 0.5 % and 98 ± 1.7 % of the cells adherent to fibronectin and activated HUVEC, respectively, increased their intracellular free Ca²⁺ concentration (figure 4 and 5c).
Figure 4. Effect of adding cytokines to adhering eosinophils on fibronectin. Eosinophils were loaded with 2.5 μM Fura-2 AM, incubated with MoAb 8A2 (35) and put on A) fibronectin-coated glasses for 15 minutes or put on B) 7h TNFα-activated HUVEC for 15 minutes. Non-adherent cells were washed away. IL-8 (open squares), eotaxin (solid diamonds) or IL-8/anti-IL-8 (open triangles) was added after 2 blanco images (arrow) and 48 images were taken at 340 and 380 nm after addition of cytokines. [Ca^{2+}]_i was measured by calculating 340/380 ratios (see Materials and Methods). The figure is representative for 3 independent experiments.
To investigate whether the IL-8-induced $[Ca^{2+}]_i$ responses were sensitive to PTX, eosinophils were incubated with control solution (glycerol 0.5 %) or 500 ng/ml PTX for 2 hours at 37°C. Figure 6 shows that the $[Ca^{2+}]_i$ responses were blocked in PTX-treated cells adherent to fibronectin (figure 6a) or 7h TNF-α-activated HUVEC (figure 6b). Also, the positive control C5a induced a $[Ca^{2+}]_i$ response which was completely blocked by PTX (500 ng/ml).

Figure 5. Computerised analyses of the effect of adding cytokines to fibronectin-adhering eosinophils. Eosinophils were loaded with 2.5 μM Fura-2 AM, incubated with 8A2 and put on fibronectin-coated glasses for 15 minutes. A) shows effect of IL-8 before, during and after addition. B) shows the effect of IL-8/anti-IL-8 before, during and after addition. C) shows the effect of eotaxin before, during and after addition. Data are depicted as a representative of 3 experiments (see also figure 4A).
Discussion

In this article, the hypothesis was tested whether IL-8 can activate eosinophils when interacting with a physiological relevant substrate such as endothelial cells and/or fibronectin. Therefore, we first performed in vitro flow chamber experiments and evaluated the effect of IL-8 on rolling, non-stimulated eosinophils. We showed that IL-8 induced a transient arrest of eosinophils which were rolling on 7h TNFα-activated HUVEC even when α4- or β2-integrins were blocked. Only in the presence of blocking antibodies against both α4- and β2-integrins the IL-8-induced arrest was prevented. This shows that IL-8 can transduce signals leading to activation of α4- as well as β2-integrins. Furthermore we conclude that the chemokines eotaxin and IL-8 both act on α4- and β2-integrins. In contrast to IL-8 activation, eotaxin-induced activation of the integrins leads to firm adhesion and spreading. Also, Weber et al. concluded from static adhesion assays that chemoattractants, like RANTES, regulate the avidity of both β1- and β2-integrins expressed on the same eosinophil (37).

This is the first report showing I) that IL-8 affects resting, unprimed eosinophils in the transition from rolling to firm adhesion (figure 1 and 2) and II) that a chemokine can induce a transient arrest for a period of 0.5 to 2 minutes in the presence of the stimulus.

Recently, Gerzten et al. (38) showed that monocytes, which are typical C-C chemokine

**Figure 6.** Effect of Pertussis Toxin on IL-8 (10^{-8} M) and C5a (10^{-8} M) induced [Ca^{2+}]_i responses in eosinophils. Eosinophils were incubated with glycerol (0.5 %) or Pertussis Toxin (500 ng/ml, 2h, 37°C) and loaded with 2.5 μM Fura-2 AM. A) 8A2-treated cells were put on fibronectin-coated glasses or B) 8A2-untreated cells were put on 7h TNFα-activated HUVEC for 15 minutes. IL-8 (open squares) and C5a (solid diamonds) were added (arrow) to glycerol treated cells and IL-8 (open circles) and C5a (solid line) were added to Pertussis-Toxin-treated cells. Images were taken at 340 and 380 nm. [Ca^{2+}]_i was measured by calculating 340/380 ratios (see Materials and Methods). The figure is representative for 3 independent experiments.
IL-8 INDUCES A TRANSIENT ARREST OF ROLLING EOSINOPHILS ON HUMAN ENDOTHELIAL CELLS

responder, firmly adhere to endothelium upon stimulation with the CXC chemokine IL-8. In contrast to eosinophils, monocytes adhered longterm to the endothelium upon IL-8 stimulation. These results suggest that the reaction to IL-8 is not restricted to the neutrophil lineage and can have different effects on different leukocyte subsets. We cannot exclude that activation of the endothelium by TNFα induces IL-8 secretion or presentation which could influence the eosinophil function. However, this seems unlikely because I) treatment of the endothelium by anti-IL-8 did not influence rolling velocity (data not shown) and II) the HUVEC was washed extensively before every experiment.

Our results also suggest that at least for unprimed eosinophils an additional stimulus besides IL-8 is needed to induce long term adhesion. This could be cytokines/chemokines which are associated with allergic inflammation, like IL-5, IL-4 or eotaxin. Indeed, it is known from in vitro (15;16) studies that cytokine-activated eosinophils migrate in response to IL-8 in contrast to unactivated eosinophils. When eosinophils, which started rolling after the IL-8-induced transient arrest, were subsequently activated by eotaxin, the cells adhered firmly and longterm to the endothelium and spreading was visible. This indicates that IL-8 does not cross desensitise or modulate the eotaxin induced response. Longterm adhesion and spreading was also visible when eotaxin was administered directly to rolling cells (figure 1b, 1d, 2b). This transition between transient and longterm adhesion allows the control of leukocyte extravasation by integration of different signals induced by multiple cytokines. The effect of IL-8 on the arrest of cytokine-primed eosinophils cannot be addressed because cytokine-primed eosinophils showed static adhesion on activated endothelium, i.e. they did not roll because β2 integrins were activated (39).

Activation of leukocytes by chemoattractants is often associated with an increase in the intracellular free Ca²⁺ concentration. However, many reports have only shown a small (if any) increase in [Ca²⁺]i upon IL-8 stimulation of eosinophils (15;40;41). These studies measured the mean increase in [Ca²⁺]i of a large population of cells in suspension. Indeed, Petering et al. showed increasing [Ca²⁺]i responses in eosinophil suspensions to which increasing concentrations of neutrophils were added, suggesting that eosinophils in suspension do not raise [Ca²⁺]i upon IL-8 stimulation (41). From a point of view it is more relevant to study changes in [Ca²⁺]i in eosinophils adherent to natural relevant surfaces for several reasons: I) adhesion changes signaling in granulocytes (42), and II) chemokines are often presented by large carbohydrate structures on the surface of endothelial cells (43). Therefore, we investigated if IL-8 would elicit a change in [Ca²⁺]i in eosinophils when attached to fibronectin or activated endothelium. We showed that ~40 % of the eosinophils adherent to fibronec-
tin (induced by \(\beta_1\) integrin freezing antibody 8A2) and \(\sim 30\%\) of the eosinophils adherent to activated HUVEC exhibited an increase in \([Ca^{2+}]_i\) in response to IL-8. Petering et al. (41) concluded that contaminating neutrophils in the eosinophil suspensions caused the IL-8-induced increase in total \([Ca^{2+}]_i\). However, contaminating neutrophils cannot explain our results for varying reasons (I) our eosinophil populations contained less than 5\% neutrophils while 30 to 40\% of the adherent cells showed a \([Ca^{2+}]_i\) response upon IL-8 stimulation, (II) the contaminating effects of neutrophils were excluded by using single-cell measurement on adherent cells. Moreover, the eosinophil-specific eotaxin was added at the end of every experiment to show that the IL-8-responsive cells were indeed eosinophils.

In the static \(Ca^{2+}\) experiments only 30 and 40\% of the eosinophils adherent to activated HUVEC and fibronectin, respectively, were activated by IL-8. In contrast, almost all of the rolling eosinophils responded upon IL-8 in the flow chamber experiments. This discrepancy is consistent with the hypothesis that the subpopulation of IL-8-responsive cells are prone for an interaction with cytokine-activated endothelial cells under flow conditions.

We were not able to block the IL-8 induced transient arrest of eosinophils by antibodies against CXCR1 and CXCR2 (5A12 and 6C6, respectively) although the functionality of these antibodies was confirmed in migration assays. Using 5A12 and 6C6, the IL-8 receptors CXCR1 and 2 were not detected by FACS on eosinophils (figure 3b, c). This is analogous to the data of Petering et al. (41). Therefore, it is tempting to hypothesize the existence of an unknown IL-8 receptor on eosinophils. To show that a G-protein-coupled receptor is involved, PTX was added to the eosinophils, and this inhibited the IL-8-induced transient arrest and also the IL-8- and C5a-induced increase in \([Ca^{2+}]_i\). This indicates that a PTX sensitive G-protein-coupled receptor is mediating the effects of IL-8 on eosinophils.

Our experiments were performed on physiological surfaces expressing several integrin ligands, which might lead to cross-linking of integrins on the cell surface and concomitant cross-talk between these proteins (44). This putative cross-talk between integrins is not necessary per se for this transient arrest, because blockade of either Mac-1 or VLA-4 did not affect the IL-8 induced arrest in our flow chamber experiments. However, this does not mean that cross-talk did not occur. Interestingly, our experiments shown in figures 4 and 6 seem to indicate that possibly cross-linking of integrins by their ligands expressed by different surfaces influenced the kinetics of the IL-8- and/or C5a-induced changes in \([Ca^{2+}]_i\). The IL-8-induced changes in \([Ca^{2+}]_i\) are remarkably slow compared to eotaxin and C5a. These latter agonists were in contrast to
IL-8, very active in increasing $[\text{Ca}^{2+}]_i$ in eosinophils in suspension (41;15). Therefore, adhesion mediated by cross-linking of integrins might initiate a permissive signal for the IL-8-induced increase in $[\text{Ca}^{2+}]_i$ in adherent eosinophils. In addition, the C5a response had an unexpected sustained behaviour in eosinophils adherent on a surface, e.g. TNFα-activated endothelium, which is rich in different integrin ligands. Again, the kinetics of this response in adherent cells is different compared to the situation in suspension.

Summarizing, this study shows that resting, rolling eosinophils on 7h TNFα-stimulated HUVEC arrest transiently upon IL-8 stimulation at a shear rate of 2 dyn/cm². This $\alpha_4$- and $\beta_2$-integrin-dependent process was probably not mediated by the known IL-8 receptors CXCR1 or CXCR2. In addition, ~40 and ~30 % of the adherent eosinophils (to fibronectin and activated endothelium respectively) increased their $[\text{Ca}^{2+}]_i$ in response to IL-8 stimulation. Our findings are consistent with a model that IL-8 can only transiently activate eosinophils, provided that they adhere to physiologically relevant surfaces. Transient arrest can easily be shifted to firm long-term arrest by additional chemokines. The transient arrest of eosinophils upon IL-8 exposure increases the time of contact between the cell and the endothelial lining, which potentiates the immunological surveillance.

Acknowledgements

We would like to acknowledge Annemarie Hoeven van den Kaiser and Glenda Heijnen-Snyder for growing the HUVEC. The 8A2 antibody was kindly provided by dr. J.M. Harlan (University of Washington, Seattle).
References


IL-8 INDUCES A TRANSIENT ARREST OF ROLLING EOSINOPHILS ON HUMAN ENDOTHELIAL CELLS


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