IL-8 Induces a Transient Arrest of Rolling Eosinophils on Human Endothelial Cells[1]

Laurien H. Ulfman, Dianne P. H. Joosten, Jan A. M. van der Linden, Jan-Willem J. Lammers, Jaap Jan Zwaginga, and Leo Koenderman[2]

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 TNF-α-activated HUVEC in an in vitro flow chamber. This process was antagonized by neutralizing Abs directed against IL-8 showing the specificity of the IL-8 effect. Furthermore, blocking Abs against both α_4 and β_2 integrins inhibited the IL-8-induced transient arrest while these Abs had no effect when they were added separately. The IL-8-induced arrest was pertussis toxin sensitive. Studying the effect of IL-8 in more detail, we evaluated putative changes in intracellular Ca^{2+} concentration in eosinophils induced by IL-8. We could show that IL-8 induces a transient rise in intracellular Ca^{2+} concentration 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. ***The Journal of Immunology,* 2001, 166: 588–595.**

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 these patients (1). To enter the site of inflammation, eosinophils have to leave the bloodstream and pass the endothelium. A widely accepted paradigm for leukocyte extravasation is referred to as the multi-step 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 the rolling of neutrophils (3) and eosinophils (4). Subsequently, cells can be activated upon interaction with inflammatory mediators resulting in the activation of integrins that 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 that are released at the site of inflammation. Chemokines can be divided in four different groups: C, CC, CXC, and CX_3C in which X is the number of amino acids in between cysteine residues at the NH_2-terminal site of the molecule. Of the C and CX_3C families only one member of each group is described, lymphotactin (7) and fraktalkine (8), respectively. CC chemokines, including eotaxin, RANTES, and monocyte chemoattractant protein-3, have been reported to be mainly chemotactic for monocytes, lymphocytes, and eosinophils. CXC chemokines, including IL-8 and growth-related oncogene-α, have been shown so far as more specific for neutrophils (see Refs. 9 and 10 for reviews).

However, several clinical studies indicate that expression of the CXC 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 Ref. 12 for review). These cells play a key role in the pathogenesis of allergic asthma. Many studies are 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 is present 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. These latter studies were performed with cytokine-activated or "primed" eosinophils (15, 16) or cells from allergic asthmatic (17) or eosinophilic (18) subjects. In most of these studies, the effect of IL-8 on eosinophils has 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 2 (20, 21). When chemokines bind to their receptor, an increase of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)[3] is elicited. Increased $[Ca^{2+}]_i$ leads

Department of Pulmonary Diseases and Haematology, University Medical Center, Utrecht, The Netherlands

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[2] Address correspondence and reprint requests to Dr. Leo Koenderman, Department of Pulmonary Diseases, F02.333, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. E-mail address: L.Koenderman@hli.azu.nl

[3] Abbreviations used in this paper: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; HSA, human serum albumin; PTX, pertussis toxin.

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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.(41) contributed these small changes to the 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, in contrast, show that eosinophils can respond to IL-8, and in these experiments cells adhere to substrates. We hypothesized that eosinophils that adhere to physiological substrates are more susceptible for IL-8 stimulation compared with cells in suspension. Therefore, the effect of IL-8 on eosinophils adhering to physiological relevant surfaces was evaluated.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). fMLP was purchased from Sigma (St. Louis, MO). Human serum albumin (HSA) was purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNF-α was purchased from Boehringer Mannheim (Mannheim, Germany). IL-8 (72 aa) was obtained from PeproTech (Rocky Hill, NJ) and eotaxin-1 was obtained from R&D Systems (Minneapolis, MN). Pertussis toxin (PTX; 50 μg/ml) was obtained from Sigma. Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) HSA. All other materials were reagent grade.

Antibodies

The mAb HP2/1 (anti-very late Ag-4, CD49d) was purchased from Immunotech (Marseille, France). mAb IB4 was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD). The Abs we used against α_4 integrins (HP2/1) and β_2 integrins (IB4) are described as functional blocking Abs (23–26) Therefore, Ab-induced differences in function of the eosinophils (e.g. by crosslinking of integrins) seem to be unlikely. Control Ab W6/32 (anti-HLA-A, -B, -C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection. Anti-CXCR1 and 2 Abs, 5A12 and 6C6 (PharMingen, San Diego), respectively, are described as blocking mAbs of neutrophil migration (27). Anti-E-selectin mAb BBIG-E4 (5D11) was purchased from R&D Systems (Abingdon, U.K.). mAbs were incubated with eosinophils (4×10^6 cells/ml) at 10 μg/ml for 15 min before the experiments. The cell suspensions were diluted twice with incubation buffer (final concentration of 5 μg/ml mAb at 2×10^6 cells/ml in incubation buffer), and the coverslips were placed directly in the system. Anti-IL-8 (clone B-K8, BioSource International, Camarillo, CA) 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 of blood anti-coagulated with 0.4% (w/v) trisodium citrate (pH 7.4) as previously described (28). Mononuclear cells were removed by centrifugation over 1.077 g/ml isotonic Ficoll. After lysis of the erythrocytes with an isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in RPMI 1640 (Life Technologies, Paisley, U.K.) 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 PBS supplemented with 0.5% HSA and 13 mM trisodium citrate, and incubated with 10 nM fMLP 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 with conventionally used isolation procedures with immunomagnetic beads (29).

Endothelial cells

HUVEC 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% (v/v) heat-inactivated human serum, 200 μg/ml penicilin/streptomycin (Life Technologies, Breda, The Netherlands), and Fungizone (Life Technologies). 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 100 U/ml TNF-α (7 h, 37°C) before 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 microchamber 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 × 18 mm) with confluent HUVEC was mounted. Immediately after mounting the HUVEC, which was activated with TNF-α for 7 h, the flow chamber was flushed with HEPES buffer for 2 min to wash out residual TNF-α.

Eosinophil perfusion and evaluation

Eosinophil perfusions were performed as described (4). In short, eosinophils in suspension (2×10^6 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 Natick, MA). The individual runs occurred under specific shear conditions all in a 37°C temperature box. Perfusion experiments were recorded on video tape. The eosinophil suspension was perfused during 3 min at shear stress 0.8 dyn/cm^2 to obtain an endothelial surface with firmly adhering and rolling eosinophils (4). After these 3 min, buffer was added and shear stress was increased to 2 dyn/cm^2. After 20 s at shear stress 2 dyn/cm^2 recording of the images on video was started. Subsequently, HEPES buffer containing 10^{-8} M IL-8 or 10^{-8} M eotaxin was added to the flow chamber. During the whole perfusion experiment, Abs (10 μg/ml IB4, 10 μg/ml HP2/1, 20 μg/ml anti-IL-8, or 10 μg/ml W6/32) were present. After ~30 s, the cytokine buffer reached the cells and the cells arrested. Subsequently it took 30 s to ~2 min for the cells to start rolling again. Therefore, the percentage of rolling cells was determined at the following points: "before" stimulation, at time point 30 s before addition of the cytokine-containing buffer; "during" stimulation, at the time point that cytokine-containing buffer has reached the cells and the cells arrest; and "after" stimulation, at the time point 1 min after the cytokine-containing buffer reached the cells. Only one randomly chosen image per experiment was recorded. Cells that 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 adhering 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, <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.

Flow cytometry

Flow cytometry analyses was conducted as described before (33). β_2 integrins (IB4), CXCR1 (5A12), CXCR2 (6C6), or a control Ab (5D11 anti-E-selectin) were used as primary mAbs. Granulocytes were analyzed using a FACSvantage flow cytometer (Becton Dickinson, 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 filter switcher (Lambda-10; Sutter Instruments, Novato, CA) with excitation filters (D340/10 and D380/13; Chroma Technology, McHenry, IL) coupled to a Leica (Wetzlar, Germany) Leitz DMIL inverted microscope, which was equipped with light filters appropriate for fura-2 (D510/40 and 400DCLP; Chroma Technology), a high immersion objective (UV-F ×40 nA 1.30 glycerol immersion; Nikon, Tokyo, Japan), and a Xenon arc lamp (XBO 75 W/2; Osram, Berlin, Germany). A series of 50 image pairs (512 × 512, ~1 s apart) were sequentially grabbed with a black and white framegrabber (Pulsar, Matrox Electronic Systems, 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, MD) using custom-made macros (Arithmatic Language for Images). Calcium levels were calculated with the standard calibration formula of Grynkiewics et al. (34)as follows with parameters of K_d = 225 nM and β = 2.4, and displayed in false colors (blue-red ramp): $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$. Cell calcium was separated from background 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 nonresponding cell was set at a mean value of 200 nM. Figs. 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 that did not respond to eotaxin (likely to be neutrophils) were excluded from analyses.

Freshly isolated eosinophils adhere strongly to some glass substrates. In these circumstances, attachment induces cells to flatten out with activation and generation of calcium signals (our unpublished observations). To circumvent these problems we let eosinophils adhere to fibronectin-coated surfaces with the use of mAb 8A2 or to 7-h TNF-α-activated HUVEC. Addition of this mAb leads to freezing of β_1 integrins in a high-affinity state and thereby cells strongly attached to fibronectin, but otherwise stay deactivated, keeping a round shape (35) allowing clear ratio imaging. Eosinophils attached in this way display low intracellular free Ca^{2+} levels under control condition for long periods of time up to 30 min. In this way, 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 (5×10^6/ml) were loaded with 2.5 μM fura 2-AM (Molecular Probes, Eugene, OR) for 15 min. Hereafter, the cells were incubated in the absence or presence of 10 μg/ml of the Ab 8A2 for 15 min at 37°C in an agitated water bath. After washing, 8A2-treated cells (1–2 $\times 10^6$) were plated onto fibronectin (0.1 mg/ml in HEPES, 15 min, 37°C)-coated 24-mm cover slips. Seven-hour TNF-α-activated HUVEC cultured on 24-mm cover slips was extensively washed with PBS, and nontreated fura 2-AM-loaded cells were plated on the endothelium. They were allowed to settle for 15 min on the substrate, and the nonadherent 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 prestimulus image ratios. Stimulants (37°C, at 2 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 (2×10^6 cells/ml). PMA (100 ng/ml) was added and oxygen consumption was measured for 5 min.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis of the data was performed using a Students *t* test for paired data. Values of $p < 0.05$ were considered to be significant.

Results

Eosinophils exhibit a transient arrest upon IL-8 during rolling adhesion along TNF-α-activated endothelium

To investigate the effect of IL-8 on rolling eosinophils, freshly isolated eosinophils were perfused over 7-h TNF-α-activated confluent HUVEC at shear stress 2 dyn/cm^2. When eosinophils were treated with a control anti-HLA class I Ab (W6/32) (Figs. 1*A* and 2*A*), the percentage of rolling cells of the total number of adhering 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 min, the percentage of rolling cells increased to 28 ± 5%. Addition of 20 μg/ml anti-IL-8 Ab to the IL-8 suspension prevented this transition from rolling to a stationary arrest of W6/32-treated (control) eosinophils (Fig. 1*A*). When

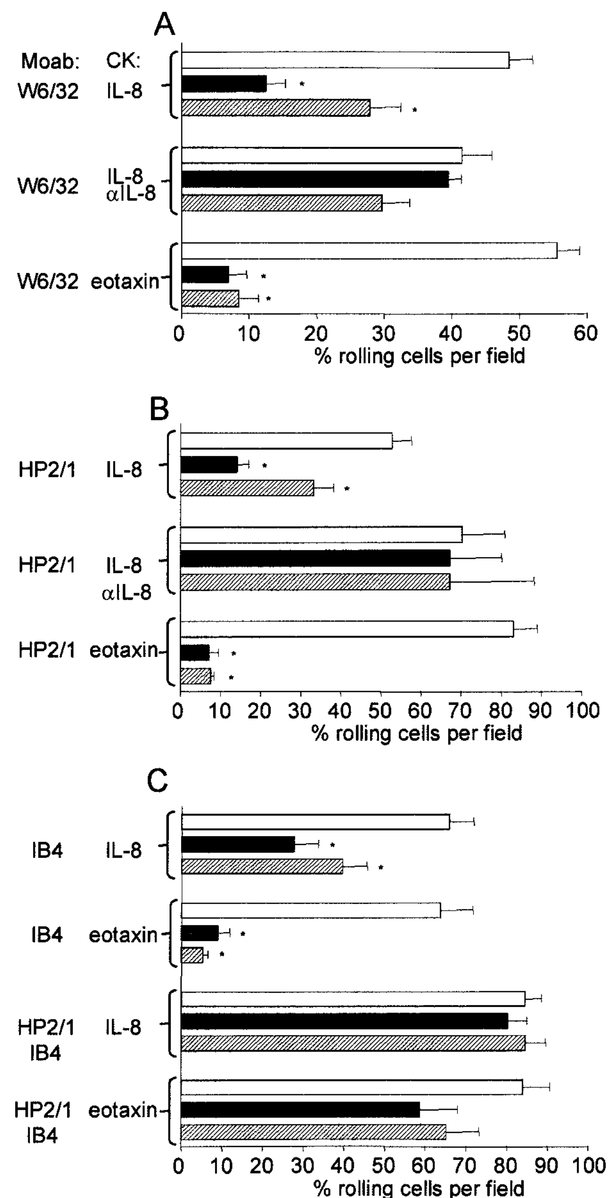

FIGURE 1. Effect of perfusion of cytokines on the percentage of rolling eosinophils on 7-h TNF-α stimulated HUVEC. The percentage of rolling cells per field for W6/32-, HP2/1-, IB4-, and HP2/1+IB4-treated eosinophils are depicted. The percentage of rolling cells was determined before (□), during (■), and 1 min after (▨) stimulation. The effect of 10^{-8} M IL-8, 10^{-8} M IL-8/anti-IL-8, and 10^{-8} M eotaxin on control mAb W6/32-treated rolling eosinophils (*A*); 10^{-8} M IL-8, 10^{-8} M IL-8/anti-IL-8, and 10^{-8} M eotaxin on mAb HP2/1-treated rolling eosinophils (*B*); and 10^{-8} M IL-8 and 10^{-8} M eotaxin on anti-β_2 integrin-treated and both anti-α_4 and β_2 integrin-treated eosinophils (*C*). The percentage of rolling cells are plotted for three to six 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's *t* test (*, $p < 0.05$).

eotaxin (10^{-8} M), a potent chemokine for eosinophils, was added to the rolling W6/32-treated eosinophils, the percentage of rolling cells decreased from 56 ± 3 to 7 ± 3%. The cells bound stable and long term because after 1 min the percentage of rolling cells was still very low (8 ± 3%) (Figs. 1*A* and 2*B*). In addition, the eosinophils flattened upon eotaxin treatment, while this was not seen upon addition of IL-8. Also, we tested whether eotaxin could induce a stable and long-term arrest when applied to cells that started

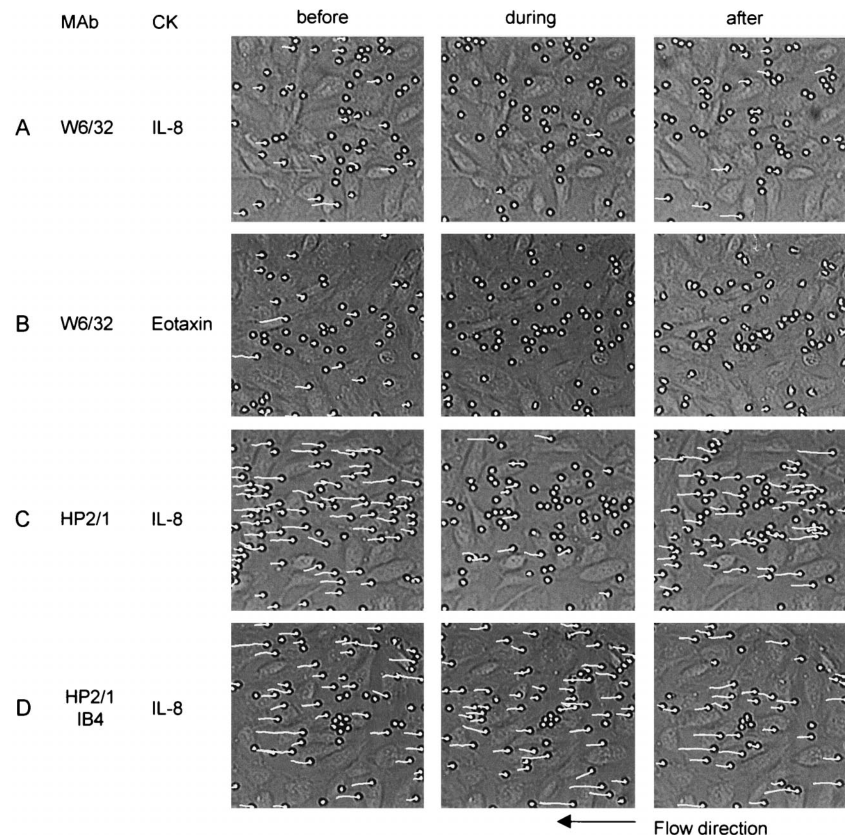

FIGURE 2. Computerized analyses of the effect of perfusion of cytokines on the percentage of rolling eosinophils on 7-h TNF-α-stimulated HUVEC. Eosinophils (2×10^6/ml) were incubated with 10 μg/ml of the indicated mAbs before the assay. Lines indicate the rolling tracks of the cells in one field during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

rolling again after the IL-8-induced arrest. Indeed, >97% of the cells were long-term arrested after addition of eotaxin to W6/32-treated eosinophils, which were rolling after the IL-8-induced transient arrest.

To investigate whether α_4 integrins played a role in this IL-8-induced arrest, eosinophils were treated with anti-α_4 integrin Ab (HP2/1) (Figs. 1*B* and 2*C*). The percentage rolling cells of HP2/1-treated eosinophils on TNF-α-activated HUVEC was 52 ± 5%, which was significantly higher than W6/32-treated eosinophils, which was shown in an earlier study (4). The rolling percentages of all groups are higher in this study compared with 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 min, the percentage rolling cells increased from 14 ± 3% to 33 ± 5%. Within 2 min almost all arrested cells (both W6/32 and HP2/1 treated) started rolling again. Addition of 20 μg/ml anti-IL-8 Ab prevented the IL-8-induced transition from rolling to stationary arrest of HP2/1-treated eosinophils (Fig. 1*B*). When 10^{-8} M eotaxin was added to the rolling HP2/1-treated eosinophils, all cells bound stable and long term (Fig. 1*B*). The experiment with the W6/32- and HP2/1-treated cells shows that the IL-8 effect on eosinophils can occur independently of the interaction between the $\alpha_4\beta_1$ integrin with its ligand VCAM-1 on activated endothelium.

To investigate whether β_2 integrins are implicated in the IL-8-induced arrest, eosinophils were incubated with an anti-β_2 integrin mAb IB4. When β_2 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 min increased to 40 ± 6% rolling cells again (Fig. 1*C*). When eotaxin was added to rolling IB4-treated eosinophils, the percentage of rolling cells decreased from 64 ± 8% to 9 ± 3%. After 1 min, the cells were still firmly adhered to the endothelial cells.

Finally, we investigated the effect of blocking β_2 integrins and α_4 integrins simultaneously. When both β_2 integrins and α_4 integrins were blocked, the percentage of rolling cells was 84 ± 4%. Upon addition of IL-8, all cells kept rolling (80 ± 5%). After 1 min, 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 was observed (Fig. 1*C*). These data show that the IL-8-induced transient arrest and the eotaxin-induced stable arrest can be mediated by either β_2 or α_4 integrins.

To investigate whether the known IL-8 receptors CXCR1 and 2 mediated the IL-8-induced response, eosinophils were incubated with Abs against the CXCR1 and 2 (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 (Fig. 3*A*). The IL-8-induced arrest was transient and the percentage of rolling cells increased from 16 ± 5 to 27 ± 11%. The functionality of these Abs 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 Fig. 3, *B* and *C*, it is shown that CXCR1 and 2 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 100 or 500 ng/ml PTX for 2 h. Control glycerol-treated eosinophils arrested transiently upon IL-8 perfusion comparable with W6/32- and HP2/1-treated eosinophils. In contrast, eosinophils treated with PTX showed a dose-dependent inhibition of IL-8-induced arrest (Fig. 3*A*). To control for possible negative effects of PTX on the normal physiology of the eosinophils, we performed respiratory burst experiments. Eosinophils incubated with 0.5% glycerol or 500 ng/ml PTX for 2 h at 37°C were tested for respiratory burst upon activation of PMA. No differences were found in the PMA-induced

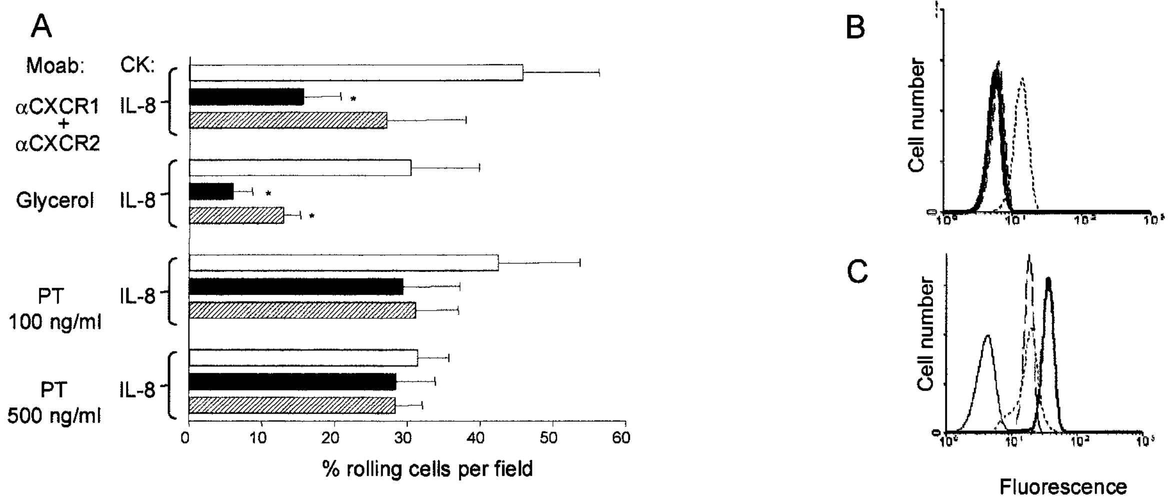

FIGURE 3. *A,* The effect of inhibition of serpentine receptors on the percentage of rolling eosinophils on 7-h TNF-α-stimulated HUVEC is depicted. The effect of IL-8 on anti-CXCR1 and 2 Abs (5A12 and 6C6, 15 min, 37°C), control solvent (glycerol 0.5%, 2 h, 37°C), and PTX (100 and 500 ng/ml, 2 h, 37°C)-treated eosinophils was determined before (□), during (■), and 1 min after (▨) stimulation. The percentage of rolling cells are plotted for three to six experiments ± SEM. The statistically significant effects of the different treatments against the situation before treatment were determined by paired Student's *t* test (*, $p < 0.05$). *B* and *C,* Granulocytes were stained for β_2 integrins (IB4, dotted line), CXCR1 (5A12, thick solid line), CXCR2 (6C6, dashed line), or an irrelevant Ab (thin solid line) by FACS. *B* and *C,* The fluorescence for eosinophils and neutrophils, respectively. The experiment shown is representative for three independent experiments.

oxidative burst of glycerol vs PTX-treated eosinophils (not shown).

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

To investigate the changes in intracellular free Ca^{2+} of adherent eosinophils upon IL-8 and eotaxin stimulation, cells were incubated with 8A2 and loaded on fibronectin-coated cover slips or nontreated cells were loaded on 7-h TNF-α-stimulated HUVEC (see *Materials and Methods* (35)). After stimulation with 10^{-8} M IL-8, a clear increase in $[Ca^{2+}]_i$ (> 200 nM) was observed in 42 ± 7% and 30 ± 7% of the cells adhering to fibronectin and activated HUVEC, respectively (Fig. 4, *A* and *B,* showing a representative experiment). The increase in $[Ca^{2+}]_i$ of cells adherent to fibronectin is depicted in Fig. 5*A*. When neutralizing Abs for IL-8 (clone B-K8) were added to the IL-8 solution before addition to the cells, the change in $[Ca^{2+}]_i$ response was blocked (Figs. 4*A* and 5*B*). A second IL-8 stimulation given did not elicit a $[Ca^{2+}]_i$ 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 adhering to fibronectin and activated HUVEC, respectively, increased their intracellular free Ca^{2+} concentration (Figs. 4 and 5*C*).

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 h at 37°C. Fig. 6 shows that the $[Ca^{2+}]_i$ responses were blocked in PTX-treated cells adhering to fibronectin (Fig. 6*A*) or 7 h TNF-α-activated HUVEC (Fig. 6*B*). Also, the positive control C5A induced a $[Ca^{2+}]_i$ response that was completely blocked by 500 ng/ml PTX.

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, nonstimulated eosinophils. We showed that IL-8 induced a transient arrest of eosinophils, which were rolling on 7-h TNF-α-stimulated HUVEC even when α_4 or β_2 integrins were blocked. Only in the presence of blocking Abs

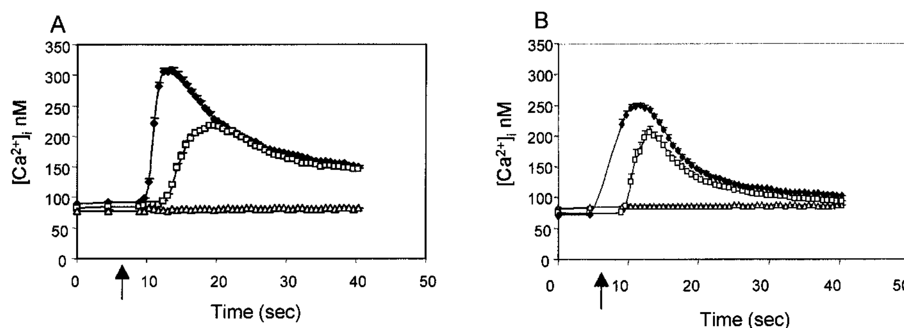

FIGURE 4. Effect of adding cytokines to adhering eosinophils on fibronectin. Eosinophils were loaded with 2.5 μM fura 2-AM, incubated with mAb 8A2 (35), and put on fibronectin-coated glasses for 15 min (*A*) or put on 7-h TNF-α-activated HUVEC for 15 min (*B*). Nonadhering cells were washed away. IL-8 (□), eotaxin (♦), or IL-8/anti-IL-8 (△) was added after 2 blanco images (↑) and 48 images were taken at 340 and 380 nm after the addition of cytokines. $[Ca^{2+}]_i$ was measured by calculating 340/380 ratios (see *Materials and Methods*). The figure is representative for three independent experiments.

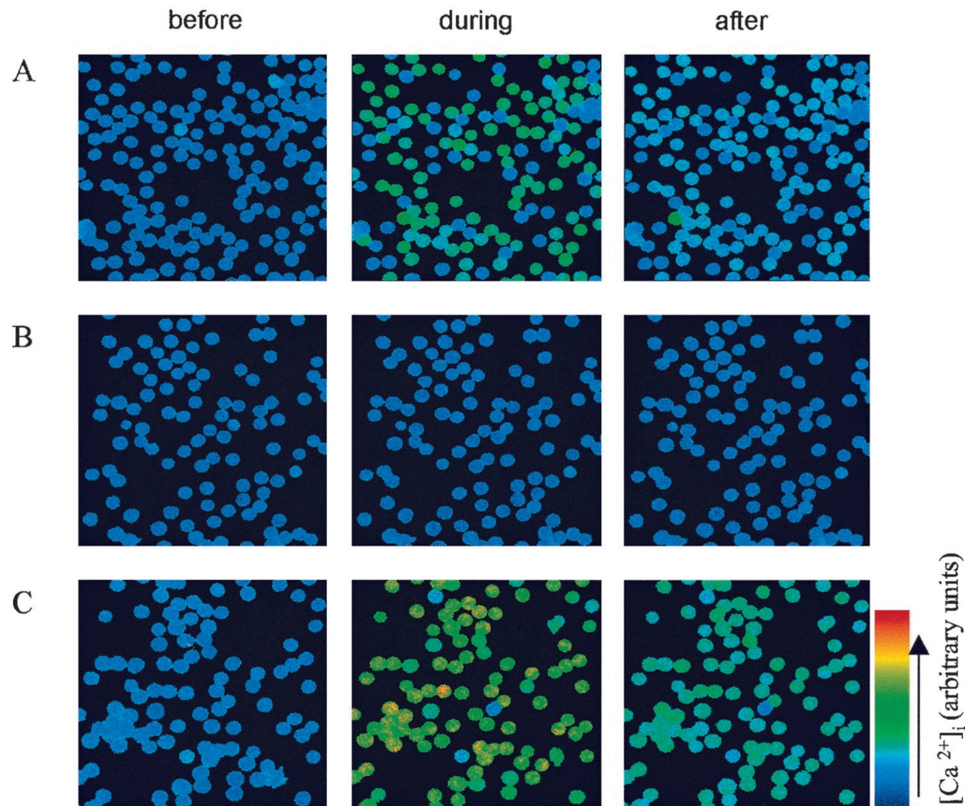

FIGURE 5. Computerized analyses of the effect of adding cytokines to fibronectin-adhering eosinophils. Eosinophils were loaded with 2.5 μM fura 2-AM, incubated with 8A2 and put on fibronectin-coated glasses for 15 min. *A,* The effect of IL-8 before, during, and after addition. *B*, The effect of IL-8/anti-IL-8 before, during, and after addition. *C*, The effect of eotaxin before, during, and after addition. Data are depicted as a representative of three experiments (see also Fig. 4*A*).

against both α_4 and β_2 integrins the IL-8-induced arrest was prevented. This shows that IL-8 can transduce signals leading to activation of α_4 as well as β_2 integrins. Furthermore, we conclude that the chemokines eotaxin and IL-8 act both on α_4 and β_2 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 β_1 and β_2 integrins expressed on the same eosinophil (37).

This is the first report showing that IL-8 affects resting, unprimed eosinophils in the transition from rolling to firm adhesion (Figs. 1 and 2) and that a chemokine can induce a transient arrest for a period of 0.5–2 min in the presence of the stimulus. Recently, Gerszten et al. (38) showed that monocytes, which are typical C-C chemokine responders, firmly adhere to endothelium upon stimulation with the CXC chemokine IL-8. In contrast to eosinophils, monocytes adhered long term to the endothelium upon IL-8 stimulation. These results suggest that IL-8 is not restricted for the neutrophil lineage and can have different effects on different leukocyte subsets. We cannot exclude that activation of the endothelium by TNF-α induces IL-8 secretion or presentation, which could influence the eosinophil function. However, this seems unlikely because treatment of the endothelium by anti-IL-8 did not influence rolling velocity (data not shown) and the HUVEC was washed extensively before every experiment.

Our results also suggest that at least for unprimed eosinophils an additional stimulus aside from IL-8 is needed to induce long-term adhesion. These could be cytokines/chemokines that 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 long term to the endothelium and spreading was visible. This indicates that IL-8 does not cross desensitize or modulate the eotaxin-induced response. Long-term adhesion and spreading was also visible when eotaxin was administered directly to rolling cells (Figs. 1, *B* and *D*, and 2*B*). This transition between transient and long-term 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 show static adhesion on activated endothelium (i.e., they do not roll because β_2 integrins are activated) (39).

Activation of leukocytes by chemoattractants is often associated with an increase in the intracellular free Ca^{2+} concentration. However, many reports have only shown a small, if any, increase in $[Ca^{2+}]_i$ upon IL-8 stimulation of eosinophils (15, 40, 41). These studies measured the mean increase in $[Ca^{2+}]_i$ of a large population of cells in suspension. Indeed, Petering et al. (41) showed increasing $[Ca^{2+}]_i$ responses in eosinophil suspensions to which increasing concentrations of neutrophils were added, suggesting that eosinophils in suspension do not raise $[Ca^{2+}]_i$ upon IL-8 stimulation. From a physiological point of view it is more relevant to study changes in $[Ca^{2+}]_i$ in eosinophils adhered to natural relevant surfaces for several reasons: 1) adhesion changes signaling in granulocytes (42), and 2) chemokines are often presented by large carbohydrate structures on the surface of endothelial cells (43). Therefore, we investigated whether IL-8 would elicit a change in $[Ca^{2+}]_i$ in eosinophils when attached to fibronectin and activated

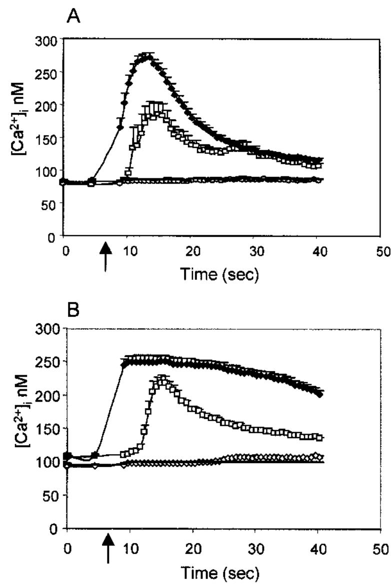

FIGURE 6. Effect of PTX on 10^{-8} M IL-8- and 10^{-8} M C5A-induced $[Ca^{2+}]_i$ responses in eosinophils. Eosinophils were incubated with 0.5% glycerol or 500 ng/ml PTX (2 h, 37°C) and loaded with 2.5 μM fura 2-AM. 8A2-treated cells were put on fibronectin-coated glasses (*A*) or 8A2-untreated cells were put on 7-h TNF-α-activated HUVEC (*B*) for 15 min. IL-8 (□) and C5A (♦) were added (↑) to glycerol-treated cells and IL-8 (◇) and C5A (-) were added to PTX-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 three independent experiments.

endothelium. We showed that ~40% and ~30% of the eosinophils adhering to fibronectin by β_1 integrin freezing Ab 8A2 and activated HUVEC, respectively, 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. Our eosinophil populations consisted of <5% neutrophils while 30–40% of the adhered cells showed a $[Ca^{2+}]_i$ response upon IL-8 stimulation, and the contaminating effects of neutrophils were excluded by using single cell measurement on adhering 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 adhering 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 a 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 Abs against CXCR1 and 2 (5A12 and 6C6 respectively) while the functionality of these Abs was confirmed in migration assays. Using 5A12 and 6C6, the IL-8 receptors CXCR1 and 2 were not detected by FACS on eosinophils (Fig. 3, *b* and *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 that 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 very late Ag-4 does not affect the IL-8-induced arrest in our flow chamber experiments. However, this does not mean that cross-talk does not occur. Interestingly, our experiments shown in Figs. 4 and 6 seem to indicate that possibly cross linking of integrins by their ligands expressed by different surfaces influences 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 with eotaxin and C5a. These latter agonists are in contrast to IL-8 very active in increasing $[Ca^{2+}]_i$ in eosinophils in suspension (41, 15). Therefore, adhesion mediated by cross-linking of integrins might initiate a permissive signal for the IL-8-induced rise in $[Ca^{2+}]_i$ in adherent eosinophils. In addition, the C5a response has a unexpected sustained behavior in eosinophils adherent on a surface (i.e., TNF-activated endothelium) that is rich with different integrin ligands. Again the kinetics of this response in adherent cells is different compared with the situation in suspension.

Summarizing, this study shows that resting rolling eosinophils on 7-h TNF-α-stimulated HUVEC arrest transiently upon IL-8 stimulation at shear rate 2 dyn/cm^2. This α_4 and β_2 integrin-dependent process was not likely to be mediated by the known IL-8 receptors CXCR1 or 2. In addition, ~40 and ~30% of the adhered eosinophils (to fibronectin and activated endothelium, respectively) increased their $[Ca^{2+}]_i$ in response to IL-8 stimulation. Our findings are consistent with a model in which IL-8 can only transiently activate eosinophils provided that they adhere to physiologically relevant surfaces. Transient arrest can be shifted easily into 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.

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