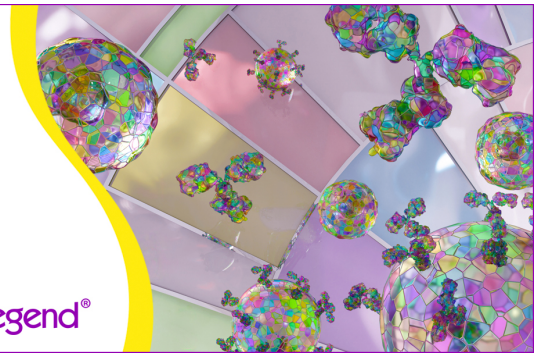


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Laurien H. Ulfman; ... et. al

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# Differences in Potency of CXC Chemokine Ligand 8-, CC Chemokine Ligand 11-, and C5a-Induced Modulation of Integrin Function on Human Eosinophils<sup>1</sup>

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The hypothesis was tested that different chemoattractants have different effects on the activity of integrins expressed by the human eosinophil. Three chemoattractants, CXCL8 (IL-8), CCL11 (eotaxin-1), and C5a were tested with respect to their ability to induce migration and the transition of eosinophils from a rolling interaction to a firm arrest on activated endothelial cells under flow conditions. CCL11 and C5a induced a firm arrest of eosinophils rolling on an endothelial surface, whereas CXCL8 induced only a transient arrest of the cells. The CXCL8- and CCL11-induced arrest was inhibited by simultaneously blocking  $\alpha_4$  integrins (HP2/1) and  $\beta_2$  integrins (IB4). In contrast, the C5a-induced arrest was only inhibited by 30% under these conditions. The potency differences of C5a > CCL11 > CXCL8 to induce firm adhesion under flow condition was also observed in migration assays and for the activation of the small GTPase Rap-1, which is an important signaling molecule in the inside-out regulation of integrins. Interestingly, only C5a was able to induce the high activation epitope of  $\alpha_M\beta_2$  integrin recognized by MoAb CBRM1/5. The C5a-induced appearance of this epitope and Rap activation was controlled by phospholipase C (PLC), as was shown with the PLC inhibitor U73122. These data show that different chemoattractants are able to induce distinct activation states of integrins on eosinophils and that optimal chemotaxis is associated with the high activation epitope of the  $\alpha_M\beta_2$  integrin. Furthermore, PLC plays an important role in the inside-out signaling and, thus, the activation status of integrins on eosinophils. *The Journal of Immunology*, 2005, 175: 6092–6099.

The migration of leukocytes to inflammatory sites occurs in multiple steps. In short, cells first roll along the endothelium and when a chemoattractant is present firm adhesion is induced and migration through the endothelium starts (1). Firm adhesion is a result of activation of integrins that under resting conditions are not able to bind to their ligands expressed by activated endothelial cells. VLA-4/ $\alpha_4\beta_1$  is an exception in that it binds its endothelial ligand VCAM-1 without prior activation (2). This chemoattractant-induced activation of integrins is generally referred to as “inside-out” signaling and results in 1) a conformational change of the integrin that enhances affinity and/or 2) a clustering of integrins that enhances avidity of the integrins (3).

Chemoattractants are divided into several classes. The classical peptide chemoattractants, like C5a (4), act on many leukocyte subtypes and are known to be very potent. These are also called “end-target” chemoattractants because they are present at the site where (bacterial) infections are located. More cell subset-specific chemoattractants are the chemokines that are divided as CXC, CC, C,

and CX3C families based on the position of a cysteine motif (reviewed in Ref. 5). These are also called host-derived chemoattractants. CXC chemokines are most active on polymorphonuclear cells, T cells, and B cells, whereas CC chemokines exert their action more on eosinophils, monocytes, basophils, T cells, and NK cells. CXCL8 (IL-8), a CXC chemokine, is a very potent chemoattractant for neutrophils and far less active for eosinophils and monocytes (6). In contrast, CCL11 (eotaxin-1) (7), a CC chemokine, acts predominantly on eosinophils and basophils but not on neutrophils (8, 9). Although there is specificity, no inflammatory chemokine is uniquely active on one population of leukocytes.

Chemoattractant receptors belong to the family of seven-transmembrane or serpentine receptors that generally signal through heterotrimeric G proteins. Upon receptor activation phospholipase C (PLC)<sup>3</sup> is activated with concomitant production of inositol phosphates and diacylglycerols, resulting in the enhancement of the intracellular free  $Ca^{2+}$  concentration and activation of protein kinase C, respectively. Despite this general paradigm, differences are present in the mediation of signals by different chemoattractant receptors expressed by a single cell. Indeed, it has been shown that neutrophils migrate to gradients of end-target chemoattractants like C5a and fMLP despite the presence of high concentrations of host-derived chemoattractants like CXCL8 and leukotriene B<sub>4</sub> (10). Little is known regarding subtle differences in signaling between different chemoattractant receptors on eosinophils.

Chemoattractants induce activation of integrins on leukocytes by inside-out signaling (11). For human eosinophils, early work has shown that mediators such as platelet-activating factor, IL-5, and GM-CSF induce the expression and activation of MAC-1

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<sup>3</sup> Abbreviations used in this paper: PLC, phospholipase C; HSA, human serum albumin; RBD, Rap-binding domain; DAG, diacylglycerol.

(CD11b/CD18) via different signal transduction pathways (12, 13). Furthermore, several chemoattractants such as RANTES, MCP-3, and C5a were shown to induce actin-dependent  $\beta_1$  integrin avidity changes parallel to actin-independent  $\beta_2$  integrin changes on eosinophils. This suggests that two integrins on the same cell are differentially regulated by the same chemoattractant (14). Signal transduction molecules such as H-Ras and cPLA<sub>2</sub> have been described to play a role in inside-out activation of  $\beta_2$  integrins on eosinophils and eosinophilic cell lines, respectively (15, 16). Recently, much attention has been given to the role of the small GTPase Rap-1 in activating  $\beta_1$  and  $\beta_2$  integrins (17–19). Until now, Rap-1 activity has not been studied in eosinophils.

In this study we compared the effects of CXCL8, a weak eosinophil activator (20, 21), CCL11, an eosinophil/basophil-specific host-derived chemoattractant, and C5a, an end-target chemoattractant on eosinophil inside-out signaling and subsequent integrin activation. We show that these chemoattractants have different effects on 1) the morphology of the cells, 2) the migratory behavior of eosinophils, 3) activation of  $\alpha_4$  and  $\beta_2$  integrins, 4) the activation of the small GTPase Rap-1, and 5) the involvement of PLC in integrin regulation, rolling arrest, and migration.

## Materials and Methods

### Reagents

CD16, CD14, and CD3 beads and isolation tools were purchased from Miltenyi Biotec. Human serum albumin (HSA) and pasteurized plasma solution was purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Recombinant human TNF- $\alpha$  was purchased from Boehringer Mannheim. Isolation buffer contained PBS supplemented with pasteurized plasma solution (10%) and trisodium citrate (0.4% (w/v)). CXCL8 (72 aa) was obtained from PeproTech, CCL11 from R&D Systems, and C5A from Sigma-Aldrich. Calcein-AM was obtained from Molecular Probes. The PLC inhibitor U73122 (1-{{6-[[17 $\beta$ -3-methoxestra-1,3,5(10)trien-17- $\gamma$ ]amino]hexyl}}-1H-pyrrole-2,5-dione) and its inactive congener U73343 (1-{{6-[[17 $\beta$ -3-methoxestra-1,3,5(10)trien-17- $\gamma$ ]amino]hexyl}}-2,5-pyrroline-dione) were from Calbiochem. Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, supplemented with 5 mM glucose, 1.0 mM CaCl<sub>2</sub>, and 0.5% (w/v) HSA. All other materials were reagent grade.

### Ab agents

The mAb HP2/1 (anti-VLA-4, CD49d) was purchased from Immunotech. Monoclonal Ab IB4 (anti- $\beta_2$  integrin) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (ATCC). As mentioned mAbs are functionally blocking Abs. Control Ab W6/32 (anti-HLA-A, HLA-B, and HLA-C) was isolated from the supernatant of a hybridoma obtained from the ATCC. The mAb CBRM1/5 (against the high activation epitope of MAC-1) was provided by Dr. T. A. Springer (Center for Blood Research, Harvard Medical School, Boston, MA) (22). In the perfusion experiments mAbs were preincubated with eosinophil ( $4 \times 10^6$  cells/ml) at 10  $\mu$ g/ml during 15 min. The cell suspensions were diluted twice with incubation buffer (final concentration of 5  $\mu$ g/ml mAb at  $2 \times 10^6$  cells/ml in incubation buffer), and the coverslips were placed directly in the system.

### Isolation of eosinophils

Trisodium citrate (0.4% (w/v) (pH 7.4)) anticoagulated blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands) and Donor Service of the University Medical Center Utrecht. Mixed granulocytes were isolated as previously described (23). In short, mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice-cold NH<sub>4</sub>Cl solution, the granulocytes were washed and resuspended in isolation buffer. Eosinophils were purified from granulocytes by negative immunomagnetic selection using anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec) (24). To avoid mononuclear cell contamination also anti-CD3- and CD14-conjugated microbeads were added to the granulocyte suspension. Purity of eosinophils was >97%.

### Endothelial cells

HUVEC were isolated from human umbilical cord veins according to Jaffe et al. (25) with some minor modifications (26). The cells were cultured in endothelial cell growth medium-2 (BioWhittaker). 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 TNF- $\alpha$  (100 U/ml, 5–7 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 as previously described by van Zanten et al. (26). 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  $\times$  18 mm) with confluent HUVEC was mounted.

### Eosinophil perfusion and evaluation

In vitro flow chamber experiments were performed as described (21). In short, eosinophils in suspension ( $2 \times 10^6$  cells/ml in incubation buffer) were aspirated from a reservoir through the perfusion chamber. Eosinophil perfusions were performed as individual runs under specific shear conditions by 37°C. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE; Leica), which was equipped with a B/W CCD Video camera (Sanyo), coupled to a VHS video recorder. Perfusion experiments were recorded on videotape. Video images were evaluated for the percentage of rolling cells using dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics). The eosinophils that were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The adhering (rolling or firmly attached) cells on the HUVEC were detected by the image analyzer. The eosinophil suspension was perfused during 3 min at shear stress 0.8 dyn/cm<sup>2</sup> to obtain an endothelial surface with firmly adhering and rolling eosinophils (27). Shear stress was increased to 2 dyn/cm<sup>2</sup> and recording of the images on video was started. Subsequently, the cytokines CXCL8 ( $10^{-8}$  M), CCL11 ( $10^{-8}$  M), or C5A ( $10^{-8}$  M) were added in the presence or absence of Abs (IB4, HP2/1, or W6/32; 10  $\mu$ g/ml) or in the presence of the PLC inhibitor U73122 (10  $\mu$ M) or the control analog U73343 (10  $\mu$ M). To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. For determining percentage rolling cells and rolling velocity a sequence of 50 frames representing an adjustable time interval,  $\Delta t$ , with a minimal time interval of 80 ms ( $\sim$ 30 s before, during and 1 min after stimulus addition) was digitally captured. At each frame, the position of every cell was detected and for all subsequent frames the distance moved by each cell and the number of images in which a cell appears in focus was measured. The velocity of a cell in micrometers per sec was calculated from the equation:  $v = L/\Delta t(x - 1)$ , in which  $L$  is the covered distance (in micrometers),  $\Delta t$  is the time interval between images (seconds), and  $x$  the number of images in which a cell appears. The cutoff value to distinguish between rolling and static adherent cells was set at 1  $\mu$ m/sec. With this method, static adherent, rolling, and freely flowing cells (which were not in focus) could be clearly distinguished.

### Migration assay using the Boyden chamber

Eosinophil migration was measured in the modified Boyden chamber assay exactly as described (28). Cellulose nitrate filters (pore width 8  $\mu$ m, thickness 150  $\mu$ m; Sartorius) were soaked in 0.5% HSA. The assays were performed in HEPES-buffered RPMI 1640 (Invitrogen Life Technologies) supplemented with 0.5% HSA for 2.5 h at 37°C in a CO<sub>2</sub> incubator. Filters were fixed, stained with hematoxylin (Weigert's method), and embedded in malinol. Analysis of the filters was done by an image analysis system (Quantimet 570 C; Leica) and an automated microscope to score the number of cells at 15 intervals of 10  $\mu$ m in the  $z$ -direction of the filters. The results are expressed as the chemotactic index, indicating the mean migrated distance (in micrometers), excluding cells with migration 0.

### Transendothelial migration

Endothelial cells (EC-RF24) were plated in Transwell chambers (pore width 8  $\mu$ m; diameter 6 mm; Costar) coated with fibronectin. When confluence was reached, purified eosinophils ( $1 \times 10^5$ /well) were calcein-AM loaded according to the manufacturer, washed, resuspended in RPMI 1640-HEPES and placed in the upper wells. The lower wells were filled with RPMI 1640-HEPES containing the chemoattractant. The chambers were placed for 1 h at 37°C in a CO<sub>2</sub> incubator. The inserts were then placed in a separate well, and the cells still present in the inserts were taken out. The migrated cells in the lower wells, the inserts as well as the cells from the

upper wells were lysed in 1% Triton X-100-containing buffer. Fluorescence intensities were measured using a FluorImager. Standard curves using fixed amounts of calcein-AM-loaded cells were prepared each time. The number of cells still present in the filter was negligible. Values are given as a percentage migrated cells of total cells loaded.

#### Time lapse image analysis of eosinophil migration on activated HUVEC

HUVEC were grown as described and activated with TNF- $\alpha$  for 5–7 h. Just before each experiment the coverslip with HUVEC were washed twice with PBS. Eosinophils ( $1 \times 10^6$ /ml in HEPES incubation buffer) were put on the HUVEC for 10 min to settle down (37°C). Incubation buffer was removed and replaced by incubation buffer containing no stimulus, CXCL8, CCL11, or C5a ( $10^{-8}$  M). The coverslip was placed on an object glass containing a window that prevented the cells from crushing. Time lapse microscopy was performed and at least 30 images were taken with a time interval of 20 s. Offline, the migration speed of the cells was calculated using Optimas 6.1.

#### Rap-1 activation assay

Rap-1 activation was determined as described before (19). One milliliter of eosinophils ( $2\text{--}5 \times 10^6$ /ml) was preincubated without or with U73343 or U73122 (2, 5, or 10  $\mu$ M) for 15 min at 37°C as indicated. Cells were stimulated with CXCL8, CCL11, or C5a ( $10^{-8}$  M) for 10 s and then 0.5 ml of ice-cold  $3\times$  GST lysis buffer (final concentration of  $1\times$  GST lysis buffer: 200 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 2 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM PMSF, 1 mM benzamide, 100 kIE/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1 mM vanadate, 1 mM DTT) was added and cell lysates were shaken vigorously. Lysates were kept on ice for 10 min and clarified by centrifugation (10 min, 14,000 rpm, 4°C). As a loading control for total Rap-1, 1/30ste (50  $\mu$ l) of cell lysate was taken and supplemented with sample buffer and boiled (5 min). GST-RalGDS-Rap-binding domain (RBD) containing bacterial lysate was precoupled to 50  $\mu$ l of glutathion beads per sample and incubated for 30 min on a rotating wheel at 4°C. After coupling, GST-RalGDS-coated beads were washed three times with GST lysis buffer, added to the cell lysate and incubated for 1 h at 4°C. Samples were washed three times with GST lysis buffer and 20  $\mu$ l of sample buffer was added and boiled (5 min). The samples (Rap-1 pulldown and loading controls) were run on SDS-PAGE and transferred to polyvinylidene difluoride membranes (NEN). Rap-1 was detected by a polyclonal rabbit Ab (sc-65; Santa Cruz Biotechnology) and HRP-coupled swine anti-rabbit using ECL plus (Amersham Biosciences) and detected using the Typhoon 9410 (Amersham Biosciences). Quantification was performed using Image Quant TL v2003.02 (Amersham Biosciences).

#### Immunofluorescent staining of integrins

Eosinophils ( $1 \times 10^6$ /ml, in indicated experiments cells pretreated with 10  $\mu$ M PLC inhibitor U73122 or control compound U73343 for 10 min at 37°C) were plated on 0.01% poly-L-lysine coated coverslips (5 min, 37°C). CXCL8, CCL11, or C5a ( $10^{-8}$  M) was added during 15 min in the presence of Abs against  $\beta_2$  integrins (IB4, 10  $\mu$ g/ml) for the total  $\beta_2$  integrin pool and CBRM1/5 (10  $\mu$ g/ml) for the high activation  $\alpha_M\beta_2$  pool. Coverslips were washed once with warm HEPES incubation buffer and fixed with 1% paraformaldehyde. Cells were washed three times with PBS. Additional staining of primary Abs IB4 and CBRM1/5 were performed during 30 min. Cells were washed three times with PBS and secondary Ab goat anti-mouse Alexa Fluor 488 was added (1 h). Cells were washed three times with PBS and coverslip was embedded in Mowiol. Cells were imaged on a confocal microscope (LSM510 Meta Confocal microscope; Zeiss).

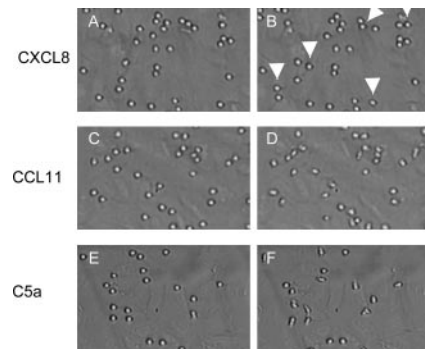
#### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Where indicated, statistical analysis was performed using the Student's *t* test or one-way ANOVA with Bonferroni correction. A value of  $p < 0.05$  was considered to be significant.

## Results

### CXCL8-, CCL11-, and C5a-induced arrest of rolling eosinophils

Previous results have shown that CXCL8 induced a transient arrest of eosinophils on TNF- $\alpha$ -activated HUVEC that is  $\alpha_4$ - and  $\beta_2$ -integrin-dependent (21). Cells stayed round upon CXCL8-induced arrest (Fig. 1, A and B) and remarkably most cells started rolling again after 1 min of CXCL8 exposure (Fig. 1, arrows). In contrast



**FIGURE 1.** Effect of CXCL8, CCL11, and C5a on the transition from rolling to arrest of eosinophils on TNF- $\alpha$ -activated HUVEC under flow conditions (2 dyn/cm<sup>2</sup>). A, C, and E, Effects of CXCL8 (A), CCL11 (C), and C5a (E) at the moment the cells arrest. Cells are round. B, D, and F, Effects of CXCL8 (B), CCL11 (D), and C5a (F) 1 min after the chemoattractants induced the arrest. B, For CXCL8, the cells stay round and some started rolling again as is shown by the different positions the cells have compared with A (arrows indicate rolling cells). For CCL11 (D) and C5a (F), the cells spread are at the exact same place as in C and E, respectively.

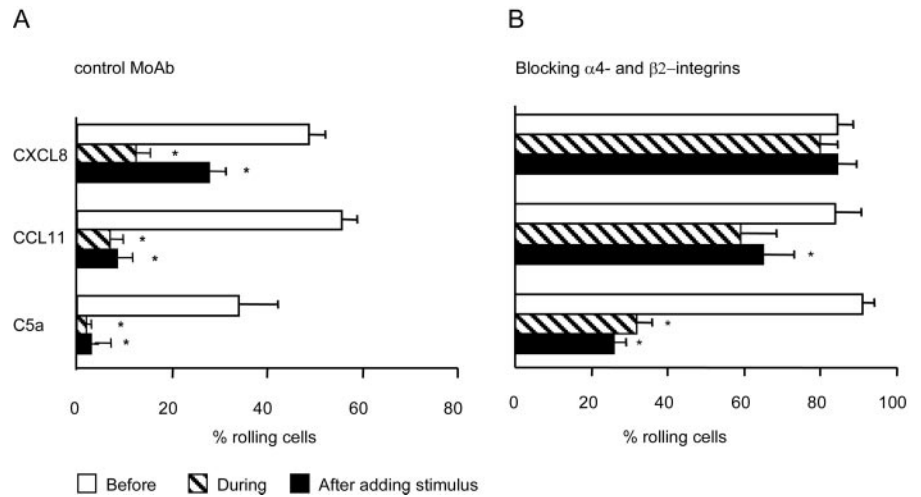
to CXCL8, CCL11 (Fig. 1, C and D) and C5a (Fig. 1, E and F) induced a stable firm arrest of eosinophil to activated HUVEC followed by spreading of the cells. To address the involvement of integrins induced by these chemoattractants we next focused on the two main integrin types, the  $\alpha_4$  (CD49d) and the  $\beta_2$  (CD18) integrins.

Fig. 2A shows the quantitative results of Fig. 1. In the presence of a control Ab (W6/32) the addition of CXCL8 resulted in a decrease in the percentage of rolling eosinophils from  $48 \pm 3\%$  to  $12 \pm 3\%$ . However, 1 min after addition of CXCL8 the percentage of rolling cells increased again to  $28 \pm 5\%$ . CCL11 induced a decrease in the percentage of rolling eosinophils from  $56 \pm 3\%$  to  $7 \pm 3\%$  in the presence of control Ab. One minute after the addition of CCL11, all cells were still firmly adhered ( $8 \pm 3\%$  rolling cells). C5a induced a decrease in the percentage of rolling cells, which was similar to the CCL11-induced arrest (from  $34 \pm 8\%$  to  $2 \pm 1\%$  rolling cells). Fig. 2B shows the percentage of rolling eosinophils before, during, and after activation of the cells in the presence of blocking Abs against  $\alpha_4$  integrin (HP2/1) and  $\beta_2$  integrin (IB4). An important note is that inhibition of these integrins results in an increase in the basal (or “before stimulus”) percentage of rolling cells, which was due to the inhibition of  $\alpha_4$  integrins as we published previously (21). The CXCL8-induced arrest of eosinophils was totally inhibited by blocking  $\alpha_4$  and  $\beta_2$  integrins, and the CCL11-induced arrest was largely but not completely inhibited by blocking these integrins (Fig. 2B). However, the C5a-induced arrest of eosinophils was only partially inhibited by blocking both integrin subtypes; the percentage rolling cells decreased from  $91 \pm 3$  (before C5a) to  $32 \pm 4\%$  (during C5a) to  $26 \pm 3\%$  (after C5a).

### Differences between CXCL8, CCL11, and C5a in their ability to induce chemotaxis

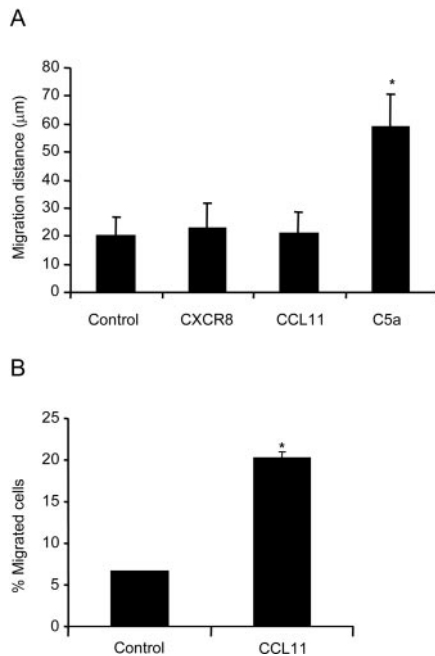
CXCL8, CCL11, and C5a were compared with respect to their ability to induce chemotaxis in a Boyden chamber assay using 150- $\mu$ m thick filters. This is a stringent migration assay because many groups use filters only 10-mm thin (comparable to one cell body) or use transendothelial migration assays (29–31). C5a induced an average migration distance of  $59 \pm 12$  mm in 150-mm thick filters, whereas CCL11 and CXCL8 did not increase chemotaxis of eosinophils above background levels under these conditions (buffer alone,  $20 \pm 7$   $\mu$ m; CXCL8,  $23 \pm 9$   $\mu$ m; CCL11,

**FIGURE 2.** Chemoattractant-induced arrest of rolling eosinophils on TNF- $\alpha$ -activated HUVEC under flow conditions (2 dyn/cm<sup>2</sup>). Eosinophils were preincubated with control Moab (W6/32) (A) and blocking Abs (B) against  $\alpha_4$  (HP2/1) and  $\beta_2$  (IB4) integrins. Eosinophils ( $2 \times 10^6$ /ml) were perfused over activated HUVECs for 3 min, and subsequently chemoattractant was perfused over the cells. The percentage of rolling cells was determined before ( $\square$ ), during ( $\square$ ) and 1 min after ( $\blacksquare$ ) stimulation with indicated chemoattractants. The average of three to six experiments is depicted, and the statistically significant effects of the different treatments against the situation before chemoattractant was determined by a paired Student *t* test (\*,  $p < 0.05$ ).

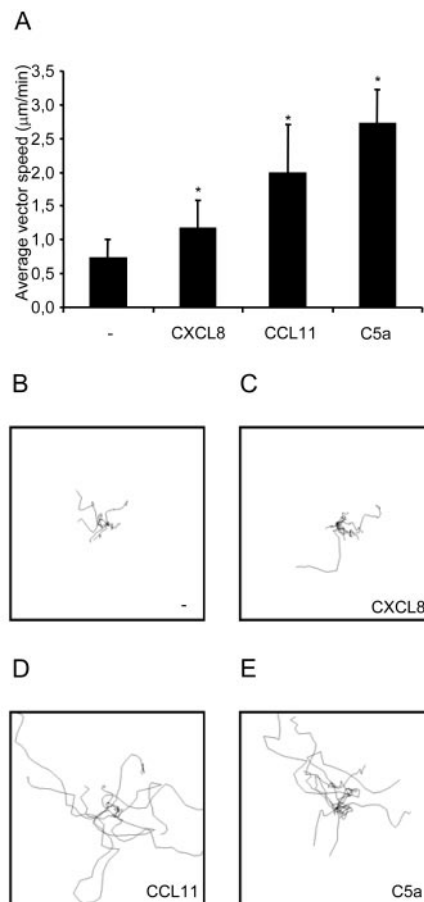


$21 \pm 8 \mu\text{m}$ ) (Fig. 3A). In addition, eotaxin-2 was tested but did not induce chemotaxis either, whereas the classical chemoattractant platelet-activating factor did (data not shown). In contrast, in a transendothelial migration assay CCL11 induced chemotaxis of  $20.3 \pm 0.6\%$  of the cells compared with a control value of  $6.6 \pm 0.1\%$  (Fig. 3B). In the latter assay C5a induced chemotaxis as well (data not shown). This result suggests that CCL11 is able to induce chemotaxis in the context of endothelial cells, whereas C5a is more potent and active even under stringent conditions of thick filters.

Next, CXCL8, CCL11, and C5a were compared in their ability to induce migration of eosinophils on activated endothelial cells (Fig. 4). Fig. 4A shows the average vector speed (in micrometers per minute) for eosinophils. CXCL8 induced a slight but significant



**FIGURE 3.** Migration of eosinophils toward chemoattractants in a Boyden chamber assay (A) and a Transwell migration assay (B). A, Eosinophils ( $2 \times 10^6$ /ml) were put in the upper chamber of a Boyden chamber and were allowed to migrate through 150- $\mu\text{m}$  thick filters toward indicated chemoattractants CXCL8 ( $10^{-8}$  M), CCL11 ( $10^{-8}$  M), and C5a ( $10^{-8}$  M) for 2.5 h (37°C). Cells were fixed, stained, and quantified by an image analysis system (Quantimet 570 C). Depicted is the average migration distance of all the migrating cells in the filter. B, Eosinophils ( $1 \times 10^5$ /well) were allowed to migrate through confluent endothelial cells toward buffer or CCL11 in a Transwell migration assay (1 h, 37°C). The average of at least three experiments is depicted, and the statistically significant effects of the different treatments against the buffer control was determined by a paired Student *t* test (\*,  $p < 0.05$ ).



**FIGURE 4.** Migration of eosinophils on TNF- $\alpha$ -activated HUVEC. Eosinophils ( $1 \times 10^6$ /ml) were put on activated endothelial cells in the presence of buffer, CXCL8, CCL11, or C5a (all stimuli  $10^{-8}$  M) and during 10 min the cells were tracked (A). The average vector speed (in micrometers per minute) was calculated offline with the image analysis system Optimas 6.1. Significant analysis of the different treatments against the buffer control was performed using a paired Student *t* test (\*,  $p < 0.05$ ). Representative migration tracks of eosinophils on activated endothelial cells are shown in B–E.

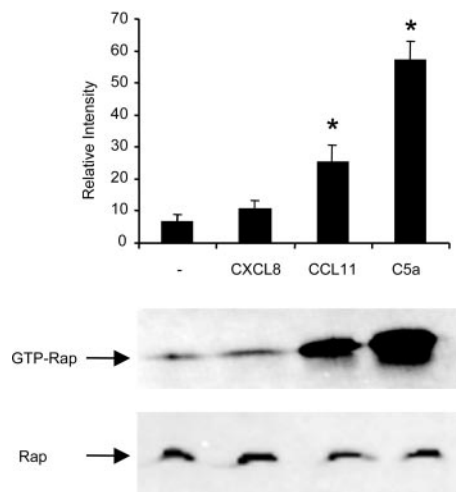
increase in vector speed ( $1.2 \pm 0.4 \mu\text{m}/\text{min}$ ) compared with control cells ( $0.7 \pm 0.3 \mu\text{m}/\text{min}$ ). The increase in vector speed of eotaxin-1 ( $2.0 \pm 0.7 \mu\text{m}/\text{min}$ ) and C5a ( $2.7 \pm 0.5 \mu\text{m}/\text{min}$ ) compared with the buffer control was more pronounced. Fig. 4, B–E, shows the centered tracks of eosinophils moving on activated endothelial cells during 15 min of a representative experiment.

*The CXCL8-, CCL11-, and C5a-induced integrin function parallels Rap-1 activity*

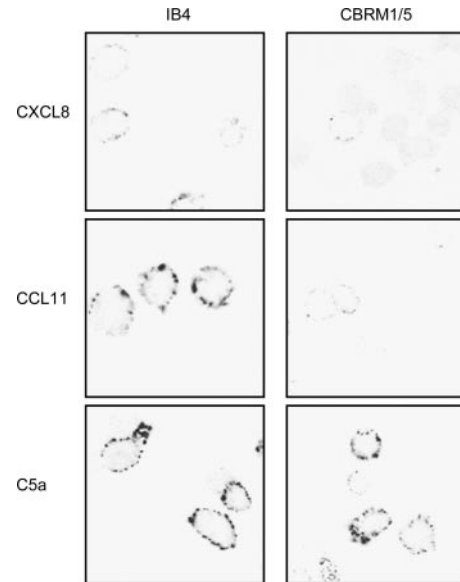
Recently, the Rap-1-GTPase has been described to play a key role in the activation of integrins on several cell types (32). We addressed the question of whether the hierarchy of C5a>CCL11>CXCL8 on adhesion and migration processes is mirrored by the regulation of this small GTPase. Activation of Rap-1 was analyzed using a pull-down assay of GTP-bound Rap from eosinophil cell lysates by its substrate the RalGDS-RBD GST fusion protein. Fig. 5 shows the active pool of Rap-1 after 10 s of stimulation with CXCL8, CCL11, or C5a. CXCL8 stimulation resulted in a slight but not significant increase in Rap-1 activity (relative intensity of control, nonstimulated  $7 \pm 2$  vs CXCL8  $11 \pm 3$ ). CCL11 (relative intensity  $25 \pm 5$ ) induced a strong Rap-1 activation and C5a (relative intensity  $57 \pm 6$ ) was even more potent. Thus, the chemoattractant-induced Rap-1 activation by CXCL8, CCL11, and C5a parallels the hierarchy in adhesion and migration function.

*C5a but not CXCL8 and CCL11 induces a high activation epitope of  $\alpha_M\beta_2$  on eosinophils*

Because we showed clear differences in the functionality of the distinct chemoattractants under flow conditions and in the chemotaxis assays, we focused on the ability of these mediators to activate the functionality of integrins. For this we used the Abs CBRM1/5 (22) that recognizes the high activation epitope of  $\alpha_M\beta_2$  (MAC-1) on eosinophils and IB4 (CD18) to visualize the total  $\beta_2$  integrin pool. The experiments were performed on poly-L-lysine coated glass to avoid effects of outside-in signaling upon  $\beta_2$  integrin binding or cross-linking.  $\beta_2$  integrin expression is shown in the Fig. 6 on the left. CXCL8 treatment of the cells resulted in a



**FIGURE 5.** Effects of CXCL8, CCL11, and C5a on Rap-1-GTP loading in eosinophils. Eosinophils ( $2\text{--}3 \times 10^6/\text{point}$ ) were treated with indicated chemoattractants ( $10^{-8}$  M) for 10 s and cells were lysed immediately on ice. Using GST-RalGDS-RBD fusion protein, active Rap-1-GTP was pulled down. The average of three independent experiments is depicted using a volume quantification in Image Quant, and a representative experiment with its loading control is shown.



**FIGURE 6.**  $\beta_2$  integrin expression on eosinophils in response to CXCL8, CCL11, and C5a. Eosinophils were plated on poly-L-lysine-coated coverslips and activated with indicated chemoattractants for 15 min. Staining was performed for total  $\beta_2$  integrin surface expression (IB4, left) and high activation  $\alpha_M\beta_2$  integrin expression (CBRM1/5, right). Cells were imaged by confocal microscopy. Representative experiment of three conducted is shown.

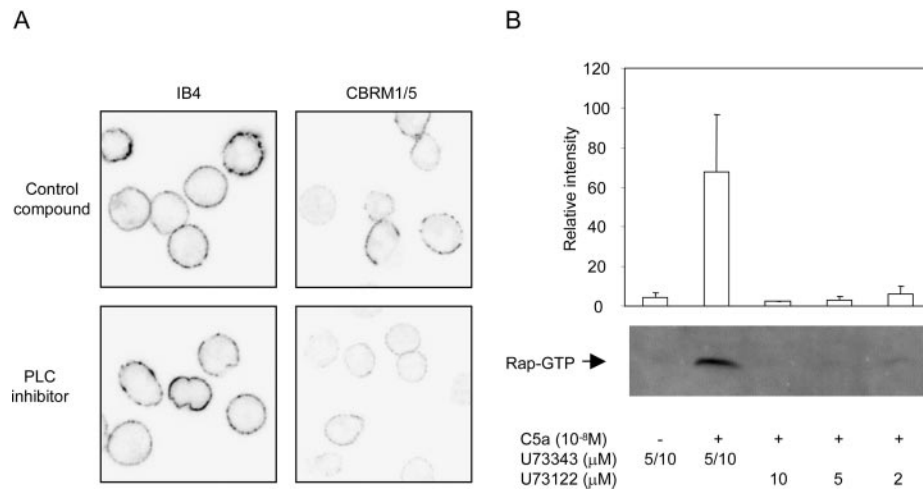
slightly lower expression of  $\beta_2$  integrins on eosinophils compared with CCL11 and C5a treatment. Remarkably, C5a but not CXCL8 or CCL11 induced the high activation epitope of  $\alpha_M\beta_2$  on eosinophils (Fig. 6, right).

*The C5a-induced high activation epitope of  $\alpha_M\beta_2$  and Rap activation is dependent on the activity of PLC*

The three chemokines showed distinct effects on the high activation epitope of  $\alpha_M\beta_2$ . We hypothesized that these chemokines differ in their inside-out signaling pathways toward  $\beta_2$  integrins. An important downstream signaling component that is involved in adhesion of granulocytes is PLC (33). Therefore, we tested whether the induction of the high activation epitope is sensitive to PLC inhibition. The results in Fig. 7A show that the PLC inhibitor U73122 (10  $\mu\text{M}$ ) but not the control compound U73343 (10  $\mu\text{M}$ ) prevented the induction of the high activation epitope of  $\alpha_M\beta_2$  on eosinophils upon C5a stimulation. In Fig. 7B it is shown that C5a induces Rap activity in control compound-treated cells, whereas inhibition of PLC by U73122 (10, 5, or 2  $\mu\text{M}$ ) inhibits the C5a-induced Rap activity to levels comparable to the nonstimulated control compound-treated eosinophils.

*PLC inhibition prevents the C5a-induced arrest of eosinophils on activated HUVEC*

Next, we tested whether the C5a-induced arrest was sensitive to the PLC inhibitor. In the presence of the noninhibitory control analog U73343 (10  $\mu\text{M}$ ) and blocking Abs against  $\alpha_4$  and  $\beta_2$  integrins  $92 \pm 3.2\%$  of the eosinophils were rolling (Fig. 8). Upon addition of C5a ( $10^{-8}$  M), almost all cells arrested (comparable to Fig. 2B). However, when the cells were incubated with the PLC inhibitor U73122 (10  $\mu\text{M}$ ) together with blocking Abs against  $\alpha_4$  and  $\beta_2$  integrins, no significant change in the percentage of rolling cells was observed (before C5a,  $83 \pm 2.4$ ; during,  $72 \pm 4.4$ ; after,  $68 \pm 8.3$ , not significant).



**FIGURE 7.** Prevention of the C5a-induced high activation MAC-1 epitope and Rap-1 activation by inhibition of PLC in eosinophils. *A*, Eosinophils were pretreated with control compound U73343 (10  $\mu$ M) or PLC inhibitor U73122 (10  $\mu$ M) for 10 min and plated on poly-L-lysine-coated coverslips for 5 min. C5a (10<sup>-8</sup> M) was added during 15 min (in the presence of control compound or inhibitor), washed, and fixed (1% paraformaldehyde) and staining was performed. Cells were imaged by confocal microscopy. A representative experiment of three conducted is shown. *B*, Eosinophils were pretreated with control compound U73343 (10 or 5  $\mu$ M) or PLC inhibitor U73122 (10, 5, or 2  $\mu$ M) for 15 min. Then C5a (10<sup>-8</sup> M) or incubation buffer was added for 10 s and cells were lysed immediately on ice. Using GST-RalGDS-RBD fusion protein, active Rap-1-GTP was pulled down. The average of three independent experiments is depicted using a volume quantification in Image Quant, and a representative experiment is shown.

## Discussion

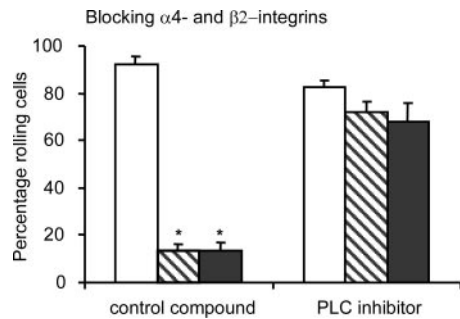
In this study we show that different chemoattractants have different effects on adhesion and migration processes of human eosinophils despite the fact that these chemoattractants make use of pertussis toxin sensitive, G $\alpha_i$  protein-coupled serpentine receptors (21, 34, 35). CCL11, C5a, and CXCL8 all induced arrest of rolling eosinophils but only the first two chemoattractants induced spreading of these cells on endothelial cells. Apparently, signaling induced by CXCL8 is deficient in signals associated with polarization of the cells despite the fact that CXCL8 under these conditions induces a clear increase in intracellular free Ca<sup>2+</sup> concentration (21). Similar differences were found in experiments regarding migration assays using endothelial cells as a substrate (Transwell and 2D migration of eosinophils on activated endothelial cells). Again, CCL11 and C5a were both potent chemotactic factors, whereas CXCL8 was not (see Figs. 3 and 4A). However, when a more stringent migration assay (chemotaxis in thick filters) was used, only C5a was able to induce migration (Fig. 3A). The reason why eotaxin was not able to induce chemotaxis in this assay might be due to the difference in substrate. The thick polycarbonate filters of this assay were soaked in human albumin and did not allow migration toward CCL11, whereas CCL11-induced chemotaxis and chemokines on activated endothelium did occur. All together these results suggest a hierarchy in potency of C5a > CCL11 > CXCL8 in human eosinophils.

Surprisingly little is known regarding differences in chemoattractant-induced signaling in primary granulocytes. Campbell et al. (10) proposed a model in which chemokines (or host-derived chemoattractants, e.g., CXCL8) are less potent in activation of leukocytes than end-target chemoattractants (e.g., fMLP). *N*-formyl peptides are released by invasive bacteria and the dominance of the fMLP receptor on neutrophils over, e.g., CXCR1/2 may ensure that signals from these terminal phagocyte targets overrides host-derived recruitment signals. The differences we observed between CXCL8, CCL11 (cell-derived chemoattractants), and C5a (end-target chemoattractant) on eosinophil adhesion and migration properties fits very well with this theory. Recently, Heit et al. (36) have shown that the signaling pathways underlying the different

outcomes of the effect of fMLP and CXCL8 on migration are the p38/MAPK and PI3K/Akt pathways, respectively. Because these studies on neutrophils compared the strength of the different chemokines in one assay, the term hierarchy was used to address the hierarchy of one chemoattractant over the other. In our study we use "difference in potency" rather than "hierarchy" because we compared the different chemoattractants side to side and not within one assay. Therefore, we can only speculate that a hierarchy of CXCR8 < CCL11 < C5a exist.

The distinct effects of the three chemoattractants on eosinophil function could be a result of a difference in receptor expression. However, this is not likely because the CCR3 (eotaxin receptor) and CD88 (C5a receptor) expression levels are comparable on eosinophils (7, 37). For the CXCL8 receptor we cannot speculate about expression levels because the identity of the CXCL8 receptor on eosinophils is not well defined. We and others have shown that there is no expression of CXCR1 and CXCR2, the known CXCL8 receptors, on eosinophils in suspension (21, 38). Further research should identify the CXCL8 receptor on eosinophils. All the experiments in this study were performed at a concentration of 10<sup>-8</sup> M of the chemoattractants. This is the optimal concentration in chemotaxis as well as chemokine assays for C5a (39, 40) and also in chemokine assays on activated HUVEC for CCL11 (data not shown). In the Boyden chamber chemotaxis assay we never observed an increase in migration of eosinophils toward CCL11 (tested at 10<sup>-7</sup>, 10<sup>-8</sup>, 10<sup>-9</sup> M, data not shown) suggesting that the lack of migration induced by CCL11 in this assay is not due to a concentration effect. In a fluorescent ICAM-1 bead assay it was also found that CCL11 as well as C5a induced an optimum in  $\beta_2$  integrin-dependent binding at 10<sup>-8</sup> M (data not shown). In the latter assay CXCL8 was not able to induce adhesion at any concentration, which reflects the transient response of CXCL8 on activation of  $\beta_2$  integrins on eosinophils under flow conditions.

CXCL8, CCL11, and C5a all have been detected in tissues of patients with chronic inflammatory diseases, e.g., in the bronchoalveolar lavage fluid of allergic asthma patients (41–43). A role for CXCL8 in the recruitment of eosinophils has been implicated in animal models (44) although the effect of CXCL8 on eosinophil



**FIGURE 8.** Effect of the inhibition of PLC on the C5a-induced transition from rolling to arrest of eosinophils on TNF- $\alpha$ -activated HUVEC under flow conditions (2 dyn/cm<sup>2</sup>). Eosinophils were preincubated with blocking Abs against  $\alpha_4$  (HP2/1) and  $\beta_2$  (IB4) integrins and with the control compound U73343 (10 mM, left) or the PLC inhibitor U73122 (10  $\mu$ M, right) (15 min, 37°C). Cells were perfused over activated HUVEC for 3 min and subsequently C5a (10<sup>-8</sup> M) was perfused over the cells. The percentage of rolling cells was determined before (□), during (▨), and 1 min after (■) stimulation with C5a. Average percentage of rolling cells per field is depicted from four experiments. Statistically significant effects of the different treatments against the situation before chemoattractant were determined by a paired Student *t* test (\*, *p* < 0.05).

recruitment was found to be indirect. In contrast, CCL11 has been shown to directly mediate tissue eosinophilia in animal models (45). C5a, which is thought to play an important role in innate immunity, induced emigration of eosinophils from the skin of guinea pigs within 1 h after intradermal injection of C5a (44). Based on these and our findings we speculate that CXCL8 has a mild, CCL11 has a strong effect and C5a has a dominant effect on eosinophil adhesion and migration processes.

If the difference in potency exist as mentioned, differences in chemoattractant-induced intracellular signaling should also be present. Initial studies have shown that only CCL11 and C5a induce changes in intracellular free Ca<sup>2+</sup> concentration in cells in suspension, whereas CXCL8 induces this response only in adhered eosinophils (21). We set out experiments to study signaling induced by these chemoattractants in eosinophils in more detail. We first focused on inside-out signals that activate integrins on the eosinophil surface.  $\alpha_4$  and  $\beta_2$  integrins on the cell surface of eosinophils bind to VCAM-1 and ICAM-1 on activated endothelium, respectively. We investigated how chemoattractant-induced arrest of eosinophils under flow conditions was dependent on the  $\alpha_4$  and  $\beta_2$  integrins using an in vitro flow chamber. CXCL8- and CCL11-induced arrest of eosinophils to activated endothelial cells was for >75% dependent on  $\alpha_4$  and  $\beta_2$  integrins. Interestingly, the C5a-induced response was only partially inhibited in the presence of blocking Abs directed against  $\alpha_4$  and  $\beta_2$  integrins. This result was not anticipated, but can be explained with different not mutually exclusive hypotheses: 1) C5a induces activation of another yet to be defined adhesion molecule and/or 2) C5a induces the activation of a functional high activation epitope of integrins that is not completely inhibited by the used Abs IB4 (anti- $\beta_2$  integrin) and HP2/1 (anti- $\alpha_4$  integrin). It is known that IB4 binds to an epitope on the  $\beta_2$  integrin chain whereas CBRM1/5 binds to a high activation epitope on the  $\alpha_M$  integrin chain. Theoretically, IB4 might not be able to block the high activation conformation of the  $\alpha_M\beta_2$  integrin. We found that C5a but not CCL11 and CXCL8 induced the high activation epitope of  $\alpha_M\beta_2$ . Therefore, the second hypothesis seems more likely, but these data do not rule out the possibility of additional adhesion molecules playing a role in C5a-induced arrest of eosinophils.

In this study we clearly show that inside-out signals induced by C5a are different compared with those induced by CCL11 or CXCL8. The receptors for these chemoattractants signal in part via PLC in different cell types. The  $\beta$ -isoform of this enzyme may be activated by the  $\beta\gamma$ -subunit from G proteins which leads to hydrolysis of phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol-3,4,5-trisphosphate. The latter releases Ca<sup>2+</sup> from internal stores into the cytosol. Ca<sup>2+</sup> and DAG are known to activate CalDAG-GEF (guanine nucleotide exchange factor), which is highly expressed in leukocytes and activate Rap-1 (46). Rap-1 has been shown to regulate inside-out signaling of  $\beta_2$  and  $\alpha_4$  integrins (47). We showed that the difference in potency of C5a>CCL11>CXCL8 in activating adhesion and migration processes is mirrored by the effect of these chemoattractants on Rap-1 stimulation. We hypothesize that PLC via Rap-1 affects integrin function. Indeed, in neutrophils and platelets it has been shown that PLC inhibition resulted in decreased fMLP- and thrombin-induced homotypic aggregation, respectively (33). Furthermore, recently Katagiri et al. (48) showed that LFA-1 activation was mediated by Rap-1 and dependent on PLC $\gamma$  function. We show in this study that inhibition of PLC leads to 1) the down-regulation of the C5a-induced high activation epitope of  $\alpha_M\beta_2$  recognized by CBRM1/5 and 2) an inhibition of Rap activity after C5a stimulation. Furthermore, blocking  $\alpha_4$  and  $\beta_2$  integrins and PLC simultaneously resulted in a clear inhibition of the C5a-induced arrest of eosinophils on the activated endothelium. This suggests that C5a activates  $\alpha_4$  and  $\beta_2$  integrins and the  $\alpha_M\beta_2$  high activation epitope, the latter being PLC dependent. However, we cannot exclude that PLC inhibits additional adhesion molecules on the eosinophil surface.

In summary, we showed that different cell-derived chemokines (CXCL8 vs CCL11) and end-target chemoattractants (C5a), which all make use of serpentine receptors coupled to G $\alpha_i$  protein-linked signaling pathways, result in differentially regulated inside-out signaling. The difference in potency of C5a>CCL11>CXCL8 might contribute to the direction in the migration process of the eosinophil to chronic inflammatory sites.

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## Disclosures

The authors have no financial conflict of interest.

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