A role for Syk-kinase in the control of the binding cycle of the β_2 integrins (CD11/CD18) in human polymorphonuclear neutrophils

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Abstract: A fine control of β_2 integrin (CD11/CD18)-mediated firm adhesion of human neutrophils to the endothelial cell monolayer is required to allow ordered emigration. To elucidate the molecular mechanisms that control this process, intracellular protein tyrosine signaling subsequent to β_2 integrin-mediated ligand binding was studied by immunoprecipitation and Western blotting techniques. The 72-kDa Syk-kinase, which was tyrosine-phosphorylated upon adhesion, was found to coprecipitate with CD18, the β-subunit of the β_2 integrins. Moreover, inhibition of Syk-kinase by piceatannol enhanced adhesion and spreading but diminished N-formyl-Met-Leu-Phe-induced chemotactic migration. The enhancement of adhesiveness was associated with integrin clustering, which results in increased integrin avidity. In contrast, piceatannol had no effect on the surface expression or on the affinity of β_2 integrins. Altogether, this suggests that Syk-kinase controls alternation of β_2 integrin-mediated ligand binding with integrin detachment. *J. Leukoc. Biol.* 74: 260–269; 2003.

Key Words: *MAC-1 · adhesion · migration*

INTRODUCTION

The ordered extravasation of human polymorphonuclear neutrophils (PMN) plays a critical role in host defense and inflammation. It is a multistep process that includes margination, initial capturing of free-flowing leukocytes, leukocyte rolling along the vessel wall, firm adhesion, transendothelial diapedesis, and chemotactic migration to sites of inflammation where PMN finally exert their defense functions [1]. Leukocyte adhesion molecules of the β_2-integrin family (CD11/CD18) play an important role in this recruitment process by binding to specific ligands and allowing firm adhesion of PMN to, e.g., endothelial cells or other substrates [2]. The β_2 integrins are heterodimeric molecules, which consist of an α-subunit and a noncovalently bound β-subunit, which span the plasma membrane once [3]. Among the integrin family, which is classified according to the associated β-subunit, the β_1 (CD29), β_2 (CD18), and β_3 (CD61) integrins are expressed on the cell surface of human PMN [2, 4, 5]. Members of the β_2-integrin family represent the most abundant integrins on PMN, which are designated by the different α-subunits as lymphocyte-function-associated antigen 1 (LFA-1; CD11a/CD18), Mac-1 (CD11b/CD18), and gp150/95 (CD11c/CD18) [2]. There are currently no data to show the expression of the fourth β_2 integrin (CD11d/CD18) on human PMN [6].

The β_2 integrins mediate PMN adhesion by binding to specific ligands: LFA-1 is critically involved in PMN emigration by binding to the intercellular adhesion molecules 1 and 2 (ICAM-1, -2) on endothelial cells [7, 8], allowing firm adhesion, shape change, spreading, and subsequent emigration of PMN. Mac-1 is also known as a receptor for ICAM-1 [9], but several reports suggest a subordinate role of Mac-1 for PMN adhesion to endothelial cells as compared with LFA-1 [10, 11]. Mac-1 serves as the receptor for complement factor C3bi, fibrinogen, fibrin, and collagens [12–14]. gp150/95 binds C3bi and fibrinogen as well [15, 16], but the physiological impact of these interactions seems less important as a result of the low surface expression on PMN when compared with the high abundance of Mac-1 [17]. Thus, the β_2 integrins mediate a variety of extracellular cell–cell and cell–substrate interactions of PMN during the inflammatory response.

Although much progress has been made in understanding the adhesive functions of the β_2 integrins, the question of how controlled detachment of the β_2 integrins is achieved is still incompletely understood. A fine control of the β_2 integrin-mediated adhesion and de-adhesion is required to allow shape change and spreading as well as locomotion, e.g., migration of PMN on the endothelial cell monolayer to the intercellular junction between neighboring endothelial cells where PMN eventually transmigrate into the extravascular space: During this step, strong adhesion has to alternate with integrin detachment to allow locomotion of the PMN on its endothelial substrate while resisting blood flow. As integrins transduce signals into the cell [18] and thereby contribute to the activation of

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various PMN functions [19] and to the induction of PMN apoptosis [20, 21], these molecules seem to control PMN in inflammation by integrating adhesion and signaling at the molecular level. The first evidence that β_2 integrins by themselves are involved in the initiation of cellular responses by exerting an intracellular signaling capacity upon ligand binding was obtained by the finding that tumor necrosis factor α (TNF-α)-induced superoxide anion production in human PMN depends on β_2 integrins [22]. Subsequently, activation of different signaling components has been reported upon β_2 integrin-mediated adhesion including tyrosine phosphorylation of the adaptor protein c-Cbl, Syk-kinase, and the Src kinases Fgr and Lyn, respectively [23–26].

The present study was undertaken to investigate the hypothesis that β_2 integrin-mediated signal-transduction events, which are elicited upon ligand binding of the β_2 integrins, may contribute to the control of the binding cycle of the β_2 integrins in human PMN. To elucidate these intracellular events that control firm β_2 integrin-mediated adhesion and de-adhesion, intracellular protein tyrosine phosphorylation following ligand interactions of the β_2 integrins was studied by Western blotting and immunoprecipitation techniques. Furthermore, the cell-surface expression and the affinity state of the β_2 integrins were investigated by flow cytometry, and the subcellular localization of the β_2 integrins was analyzed by confocal microscopy. Allowing PMN adhesion to immobilized fibrinogen, a native ligand of the β_2 integrins Mac-1 and gp150/95, induced the ligand interactions of the β_2 integrins. The role of Syk-kinase for β_2 integrin-mediated signaling was analyzed using the Syk-kinase inhibitor piceatannol. Studying β_2 integrin-mediated adhesion and spreading of PMN on immobilized fibrinogen as well as chemotactic migration in response to the chemoattractant formyl-Met-Leu-Phe (fMLP) elucidated the physiological relevance of Syk-kinase-dependent integrin signaling.

MATERIALS AND METHODS

Isolation of human PMN

Human blood was collected from healthy donors by venipuncture using a heparinized (10 units/ml) syringe. Erythrocyte sedimentation was performed in the presence of 40% (v/v) autologous plasma. The leukocyte-rich plasma was layered onto a discontinuous Percoll gradient as described [27] and centrifuged at 600 *g* for 20 min. The PMN-containing band was collected and washed in Dulbecco's phosphate-buffered saline (PBS). Cells were resuspended in HEPES buffer (20 mM HEPES and 0.9% NaCl) supplemented with 0.1% (w/v) glucose. PMN viability was >97%, as judged by a trypan blue exclusion test. Purity was >99%, as analyzed by microscopy using Hemacolor™ staining (Merck, Darmstadt, Germany).

Antibodies

The monoclonal antibody (mAb) directed against phosphotyrosine residues was obtained from Upstate Biotechnology [Lake Placid, NY; clone 4G10; immunoglobulin G ($IgG)_{2b}$]. The anti-Syk mAb 4D10 (IgG_{2a}) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-c-Cbl mAb (clone 17) was obtained from Transduction Laboratories (Lexington, KY). The mAb IB4 (mouse anti-human CD18, IgG2a) was isolated from hybridoma supernatants (American Type Culture Collection, Manassas, VA; 10164-HB) by protein A-Sepharose. Purity was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the saturating concentration was determined by flow cytometry. The anti-CD18 mAb 7E4 (IgG_1) was obtained from Immunotech (Marseille, France). The anti-CD18 mAb 6.5E was provided courtesy of Dr. Martyn K. Robinson (Celltech, Slough, UK) [28]. The anti-CD18 mAb CBRM1/5, which binds to an activation-specific neoepitope of Mac-1, was a generous gift of Dr. Timothy A. Springer (Harvard University, Boston, MA) [29]. The fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG as well as the RPE-conjugated anti-human CD11b mAb (clone 2LPM19c) were obtained from Dakopatts (Glostrup, Denmark). The nonbonding, isotype-matched control mAb of the IgG_1 subclass and the peroxidase-conjugated goat anti-mouse IgG were purchased from Sigma (Deisenhofen, Germany). The isotype-matched control mAb of the IgG_{2a} and IgG_{2b} subclasses were obtained from Calbiochem (La Jolla, CA).

Integrin engagement

Engagement of β_2 integrins was induced by adhesion of PMN to immobilized fibrinogen as described previously [24]. Briefly, 500 µl aliquots of PMN (5×10^6/ml) in HEPES buffer were seeded onto Petri dishes (2 cm diameter) coated with human fibrinogen at a final concentration of 250 µg/ml at 4°C overnight, followed by two extensive washes. Adhesion was induced at 37°C in the presence of 1.2 mM Ca^{2+} and 1 mM Mg^{2+} alone or by additional treatment with 1 mM Mn^{2+}. In the absence of divalent cations, only minimal adhesion was observed (data not shown). After aspiration of the supernatant, PMN stimulation was terminated by addition of 90 µl 1× Laemmli buffer [2% (w/v) SDS, 6% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and a trace amount of bromphenol blue in 200 mM Tris-HCl, pH 6.8], supplemented with 10 mM sodium orthovanadate. For negative control, PMN were kept in suspension under the experimental conditions used for adherent cells. The stimulation of suspended PMN was terminated by addition of 3 vol of 3× Laemmli buffer, and samples were subjected to SDS-PAGE.

Immunoprecipitation

PMN (2.5×10^7) were lysed for 10 min on ice with 500 µl modified radio immunoprecipitation assay (RIPA) buffer [50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, 100 mM sodium fluoride, 5 mM diisopropylfluorophosphate (DFP), 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM tetrasodium pyrophosphate, 10 mM p-nitrophenyl phosphate, 10 µg/ml antipain, 2 µg/ml aprotinin, 2 µg/ml chymostatin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, pH 7.5]. Cell lysates were precleared by centrifugation (12,000 *g*, 4°C, 10 min). The supernatant was subjected to 10 µg primary mAb coupled to 75 µl protein A-Sepharose for 1 h at 4°C under gentle rotation. After washing twice with lysis buffer, immunoprecipitates were eluted by boiling samples in 90 µl 1× Laemmli buffer for 6 min at 100°C and were subjected to SDS-PAGE.

Immunodepletion

PMN (4×10^6) were lysed for 10 min on ice with 80 µl immunodepletion buffer [50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.05% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40 (NP-40), 100 mM sodium fluoride, 5 mM DFP, 2 mM PMSF, 10 µg/ml antipain, 2 µg/ml aprotinin, 2 µg/ml chymostatin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, pH 7.5]. Cell lysates were precleared by centrifugation (12,000 *g*, 4°C, 10 min). The supernatant was subjected to 10 µg primary anti-Syk mAb (or the isotype-matched mAb for negative control) coupled to 75 µl protein A-Sepharose for 1 h at 4°C under gentle rotation. The supernatant was collected by centrifugation, and after addition of 3 vol of 3× Laemmli buffer, the samples were immediately heated for 6 min at 100°C and subjected to SDS-PAGE.

SDS-PAGE and immunoblotting

Total cell lysates, immunoprecipitates or immunodepletates, were subjected to SDS-PAGE on gels containing 10% (w/v) acrylamide under reducing conditions [30]. Separated proteins were transferred to nitrocellulose filters using a semidry technique at 150 mA for 1.5 h. All blots were tested for loading of equal amounts of protein in each lane by Ponceau S staining. Prior to incubation for 1 h with a final concentration of ~1 µg/ml primary mAb in Tris-buffered saline (TBS) supplemented with 0.1% bovine serum albumin

(BSA), filters were blocked by treatment with 3% ovalbumin in TBS for 1 h. After three washes in TBS containing 0.05% Tween-20, filters were incubated for 1 h with the peroxidase-conjugated goat anti-mouse IgG (final dilution, 1:1000) in TBS supplemented with 0.1% BSA and subsequently washed as described above. Detection was performed by chemiluminescence using an enhanced chemiluminescence kit (Amersham Life Sciences, Braunschweig, Germany) and subsequent autoluminography by exposure to X-ray films (XOMAT-AR, Kodak, Germany).

Adhesion assay

PMN (2.5×10^4/100 μl) were seeded onto 96-well microtiter plates coated with human fibrinogen as described above for integrin engagement. After 30 min, unattached PMN were rinsed away by washing wells twice with PBS. Computer micrographs of adherent cells were generated using a Nikon microscope and a 10× objective. Adherent cells in the visual field (~0.74 mm^2) were counted off-line. The obtained results were confirmed by measuring adhesion of the identical samples using the crystal violet assay. For this assay, PMN were fixed with 1% glutaraldehyde in PBS and stained with 0.1% crystal violet. Plates were photometrically measured at 570 nm after lysis in 0.5% Triton X-100 overnight at room temperature. Experiments were done in triplicates. Blanks were measured in the absence of cells to determine background extinction.

Analysis of cell morphology

PMN were subjected to morphological analysis 30 min after the onset of adhesion in the presence of immobilized fibrinogen following the experimental procedure described for the adhesion assay. PMN were analyzed before the removal of unattached cells on a Nikon microscope using an HMC 40/0.6 objective.

"Under-agarose" assay

Tissue-culture dishes (Becton Dickinson, Plymouth, UK; 8.5 cm diameter) were filled with an agarose solution containing 0.4% agarose and 0.04% BSA in PBS supplemented with 1.2 mM Ca^{2+} and 1 mM Mg^{2+}. After solidification, two 1.2-mm diameter holes were cut into the gel 2.4 mm apart from each other using a template. After thermoequilibration at 37°C, one hole was filled with 10 μl PMN suspension (5×10^4/sample); the second hole was filled with 10 μl 50 nM fMLP as chemoattractant. PMN were allowed to migrate in response to the stimulus through the agarose gel for 2 h at 37°C. The results were analyzed by light microscopy using a Nikon microscope with an HMC 10/0.25 objective. In the absence of a stimulus, no site-directed migration of PMN was observed (data not shown).

"Boyden"-chamber assay

PMN (1×10^6/sample) were allowed to transmigrate in a Boyden-chamber for 1 h at 37°C through Transwell filters (6.5 mm diameter, 3 μm pore size; Corning Costar, Cambridge, MA) in response to 10 nM fMLP. Transmigrated PMN were harvested from the lower chamber in the presence of 5 mM EDTA and counted under the microscope. The assay was done in duplicates.

Cell-surface expression of CD antigens

PMN (5×10^5/100 μl) were incubated with a saturating concentration of 10 μg/ml primary mAb for 1 h on ice and washed twice. After incubation for 1 h with the secondary FITC-conjugated rabbit anti-mouse IgG (final dilution of 1:20) on ice and in the dark, samples were subjected to flow cytometry (FACscan, Becton Dickinson). In each sample, 10^4 cells were counted and analyzed off-line using CellQuest™ software.

Analysis of integrin clustering

Prior to the staining procedure, PMN were fixed with 3% paraformaldehyde, washed twice, and treated with 1% BSA in PBS for 30 min at room temperature. Subsequently, PMN were incubated with a final concentration of 10 μg/ml RPE-conjugated anti-CD11b mAb 2LPM19c for 1 h at room temperature and in the dark. After washing twice, PMN were subjected to confocal microscopy (LSM 410/Axiovert 135 M, Zeiss, Oberkochen, Germany).

Reagents

Antipain, aprotinin, BSA, chymostatin, crystal violet, DFP, deoxycholic acid, human fibrinogen, fMLP, leupeptin, NP-40, ovalbumin, pepstatin, Percoll, p-nitrophenyl phosphate, PMSF, Ponceau S, protein A-Sepharose, sodium fluoride, SDS, sodium orthovanadate, tetrasodium pyrophosphate, Triton X-100, TNF-α, and Tween-20 were obtained from Sigma. Piceatannol was obtained from Calbiochem. Buffers were obtained from Biochrom (Berlin, Germany). Electrophoresis calibration standards for molecular mass determination were purchased from Pharmacia (Freiburg, Germany).

Statistical analysis

Data shown represent mean ± SD where applicable. Statistical significance was determined using Student's *t*-test; $P < 0.05$ was considered statistically significant.

RESULTS

To study the question whether β_2 integrins (CD11/CD18) by themselves may control their binding cycle, i.e., alternation of ligand binding and detachment, by evoking signal-transduction events in human PMN, intracellular protein tyrosine phosphorylation subsequent to β_2 integrin-mediated adhesion was studied by Western blotting and immunoprecipitation techniques. Extracellular ligand interactions of β_2 integrins were allowed in the presence of 1.2 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 mM Mn^{2+} by incubating PMN on immobilized fibrinogen, a native ligand of the β_2 integrins Mac-1 (CD11b/CD18) and gp150/95 (CD11c/CD18). When compared with suspended PMN, adhesion of PMN immobilized fibrinogen-induced intracellular protein tyrosine phosphorylation within 10 min after the onset of adhesion (**Fig. 1**): The proteins that became tyrosine-phosphorylated showed apparent molecular masses of 120 kDa, 78 kDa, 72 kDa, 68 kDa, and 56 kDa, respectively. Western blotting using an anti-Syk-kinase mAb revealed that Syk-kinase showed the same molecular mass as the 72-kDa protein, which became tyrosine-phosphorylated upon adhesion to immobilized fibrinogen. Immunoprecipitation of Syk-kinase using an anti-Syk-kinase mAb revealed that the 72-kDa protein was identical to Syk-kinase as demonstrated by blotting the Syk-kinase immunoprecipitates for phosphotyrosine residues or Syk-kinase, respectively. This was confirmed by the analysis of phosphotyrosine immunoprecipitates in a Western blot for Syk-kinase. Moreover, immunodepletion of Syk-kinase from whole-cell lysates resulted in a loss of this protein in the cell lysate, further confirming the specificity of the observed effect. Together, this shows that β_2 integrin-mediated firm adhesion induces tyrosine phosphorylation of Syk-kinase in human PMN.

To find out whether Syk-kinase may be associated with the β-subunit of the β_2 integrins, CD18 was immunoprecipitated using the specific anti-CD18 mAb 7E4 or 6.5E, respectively (**Fig. 2A**). The analysis of the precipitates in a Western blot for Syk-kinase showed that Syk-kinase coprecipitated with CD18 in suspended and in adherent PMN, suggesting that Syk-kinase is constitutively associated with the β-subunit of the β_2 integrins. However, adherent PMN showed a slightly higher degree of Syk-CD18 association when compared with suspended cells, suggesting that adhesion may promote association between CD18 and Syk. For control, association of CD18 with c-Cbl was

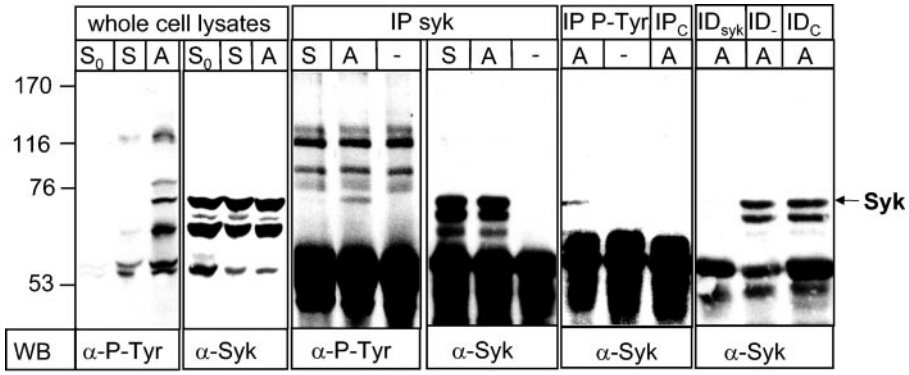

Fig. 1. The 72-kDa protein was identical to Syk-kinase. Human PMN were lysed immediately (S_0) or were incubated for 10 min at 37°C in the presence of 1.2 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 mM Mn^{2+} in suspension (S) or were allowed to adhere to immobilized fibrinogen (A). Western blots (WB) of whole-cell lysates, immunoprecipitates of Syk-kinase (IP syk), phosphotyrosine residues (IP P-Tyr), immunodepleted cell lysates using an anti-Syk-kinase mAb (ID_{syk}), an isotype-matched control mAb (ID_C), and whole-cell lysates without mAb (ID_-). For negative control, immunoprecipitation was performed in the absence of whole-cell lysates with lysis buffer alone (–) or in the presence of whole-cell lysates using an isotype-matched control mAb (IP_C). Blots shown are representative of three independent experiments.

studied, a 120-kDa protein that was previously shown to become tyrosine-phosphorylated upon β_2 integrin-mediated adhesion of human PMN [24]. The Western blot for c-Cbl revealed that c-Cbl was only detectable in the whole-cell lysate analyzed for positive control, but it was absent from the immunoprecipitates of CD18 (Fig. 2B). This shows that Syk-kinase but not c-Cbl is specifically associated with CD18, the β-subunit of the β_2 integrins.

To study the biological relevance of the observed signaling pathway, PMN were treated with different concentrations of piceatannol, an inhibitor of Syk-kinase, before the induction of adhesion in the presence of immobilized fibrinogen and divalent cations. As demonstrated in a Western blot for phosphotyrosine residues (**Fig. 3A**), a final concentration of 10 μM piceatannol inhibited tyrosine phosphorylation of Syk-kinase in adherent PMN to some extent when compared with vehicle-treated, adherent control cells. Tyrosine phosphorylation of Syk-kinase was almost completely absent at a final concentration of 30 μM piceatannol. However, the tyrosine phosphorylation of other proteins also decreased, suggesting that they may be downstream of the Syk in the signaling pathway.

The treatment of PMN with final concentrations of 1, 3, 10, or 30 μM piceatannol significantly promoted adhesion of PMN up to ~660% of the values measured in vehicle-treated control cells (100%) in a dose-dependent manner (Fig. 3B). Also, spreading of PMN on immobilized fibrinogen within 30 min after the onset of adhesion, which was analyzed by light microscopy, was enhanced in the presence of 30 μM piceatannol when compared with vehicle-treated cells (Fig. 3C). Thus, inhibition of Syk-kinase not only enhanced β_2 integrin-mediated adhesion of PMN but also promoted spreading over immobilized fibrinogen, suggesting that Syk-kinase plays a role in the control of β_2 integrin-mediated adhesion of human PMN. In contrast, inhibition of Syk-kinase by piceatannol decreased TNF-α-induced (300 U/ml) adhesion and spreading of PMN to immobilized fibrinogen (data not shown), which may show that Syk has a dual function in regulating integrin adhesiveness.

Next, the role of Syk-kinase in migration was studied in an under-agarose assay. PMN were treated with 30 μM piceatannol or vehicle before fMLP-induced chemotactic migration (**Fig. 4A**). Within 2 h after the onset of the experiment, a substantial migration of PMN was observed in response to 50

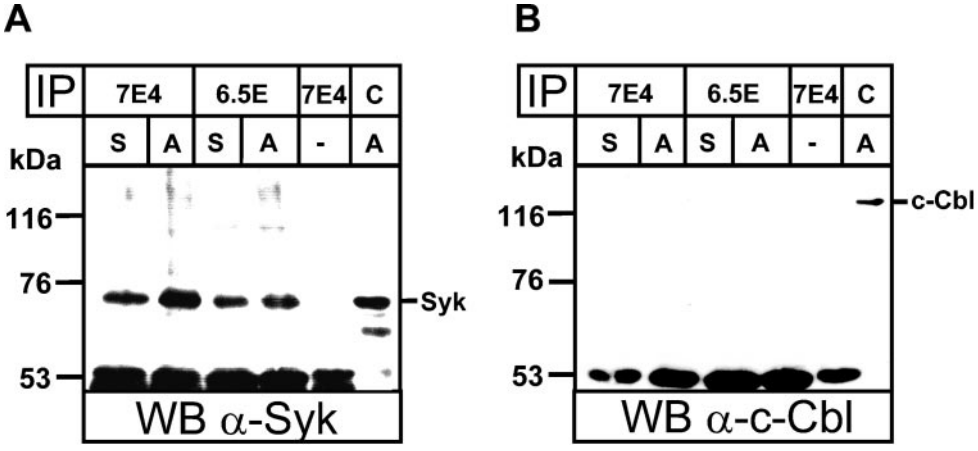

Fig. 2. Syk-kinase but not c-Cbl was associated with CD18. Human PMN were kept in suspension (S) or were allowed to adhere to immobilized fibrinogen (A) for 10 min at 37°C in the presence of 1.2 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 mM Mn^{2+}. Whole-cell lysates (C) or immunoprecipitates (IP) of CD18 using the mAb 7E4 or 6.5E were analyzed in Western blots (WB) using an anti-Syk-kinase mAb (α-Syk; A) or an anti-c-Cbl mAb (α-c-Cbl; B). For negative control, immunoprecipitation was performed in the absence of whole-cell lysates with lysis buffer alone (–). Blots shown are representative of three independent experiments.

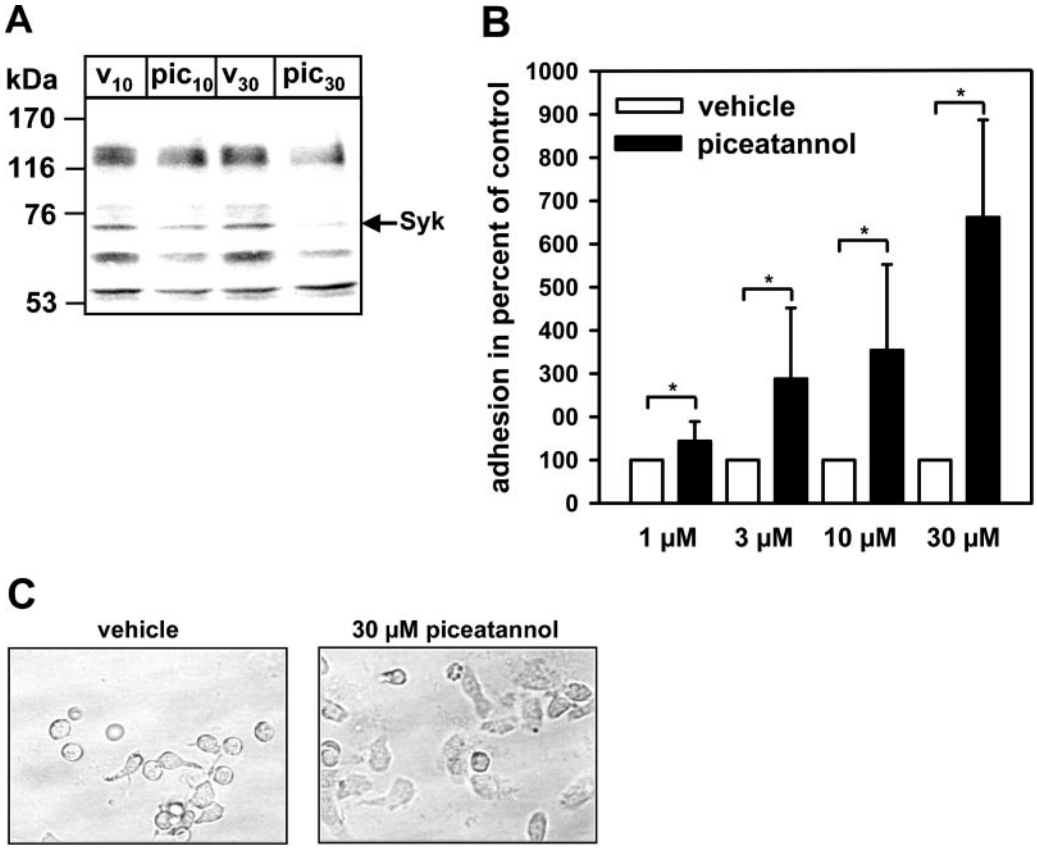

Fig. 3. Inhibition of Syk-kinase enhanced adhesion and spreading. Human PMN were treated with indicated concentrations of piceatannol (pic) or vehicle (v) for 30 min at 37°C before induction of adhesion to immobilized fibrinogen at 37°C in the presence of 1.2 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 mM Mn^{2+}. (A) Antiphosphotyrosine Western blot of whole-cell lysates of vehicle- or piceatannol-treated PMN (10 µM or 30 µM) 10 min after the onset of adhesion. Blot shown is representative of three independent experiments. (B) Adhesion of PMN to immobilized fibrinogen after treatment with piceatannol or vehicle in percent of the vehicle-treated control (100%). The number of adherent PMN was determined 30 min after the onset of adhesion by counting adherent cells under the microscope. Mean ± SD; n= 6; *, $P < 0.05$. Measuring adhesion with the crystal violet assay gave similar results (data not shown). (C) Photomicrographs of PMN 30 min after the onset of adhesion. Data are representative of three independent experiments.

nM fMLP. The fMLP-induced response was almost completely absent in the presence of piceatannol. This demonstrates that the Syk-kinase inhibitor abolished the chemotactic migration of PMN. In additional experiments, the effect of the inhibition of Syk-kinase by piceatannol on migration was studied in a Boyden-chamber using 10 nM fMLP as chemoattractant (Fig. 4B). Within 1 h after the onset of the experiment, 6.4.% of the PMN added (100%) transmigrated spontaneously without further stimulation as measured for control. In contrast, fMLP induced transmigration of 61.1% of PMN. This effect was significantly impaired upon inhibition of Syk-kinase by piceatannol, which decreased fMLP-induced transmigration to 37.5%. The presence of the function blocking anti-CD18 mAb IB4 decreased migration to 28.3 ± 5.0% of total cells added (n=7), demonstrating that PMN migration in the Boyden-chamber assay was partially dependent on CD18 (data not shown). Coapplication of piceatannol and the anti-CD18 mAb IB4 had no further effect and resulted in 26.8 ± 10.9% of migration, demonstrating that there was no additive effect on inhibition of Syk-kinase and CD18 (data not shown). Altogether, these experiments suggest that the inhibition of tyrosine phosphorylation of Syk-kinase by piceatannol decreases chemotactic migration of PMN.

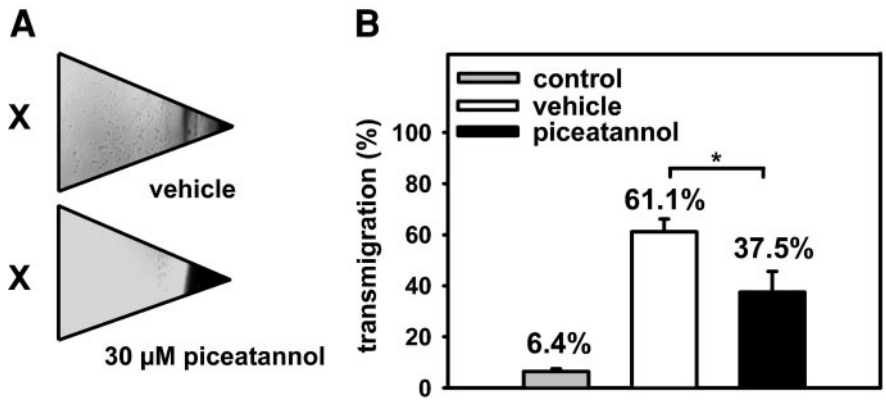

Fig. 4. Inhibition of Syk-kinase profoundly diminished chemotactic migration. (A) PMN (5×10^4/sample) were treated with 30 µM piceatannol or vehicle for 30 min at 37°C, and cells were allowed to migrate from a hole in the agarose gel to a second hole in the distance of 2.4 mm filled with 50 nM fMLP (X) in the presence of 1.2 mM Ca^{2+} and 1 mM Mg^{2+}. The results were analyzed after 2 h by light microscopy. Data are representative of three independent experiments. (B) PMN (1×10^6/sample) were treated with 30 µM piceatannol or vehicle for 30 min at 37°C, and cells were allowed to transmigrate in a Boyden-chamber for 1 h at 37°C through Transwell filters in response to 10 nM fMLP in the presence of 1.2 mM Ca^{2+} and 1 mM Mg^{2+}. Spontaneous transmigration was measured in the absence of a chemotactic stimulus (control). Transmigrated cells were harvested and counted under the microscope. Transmigration was calculated as transmigrated cells in percent of total cell number (100%). N = 3; mean ± SD; *, $P < 0.05$.

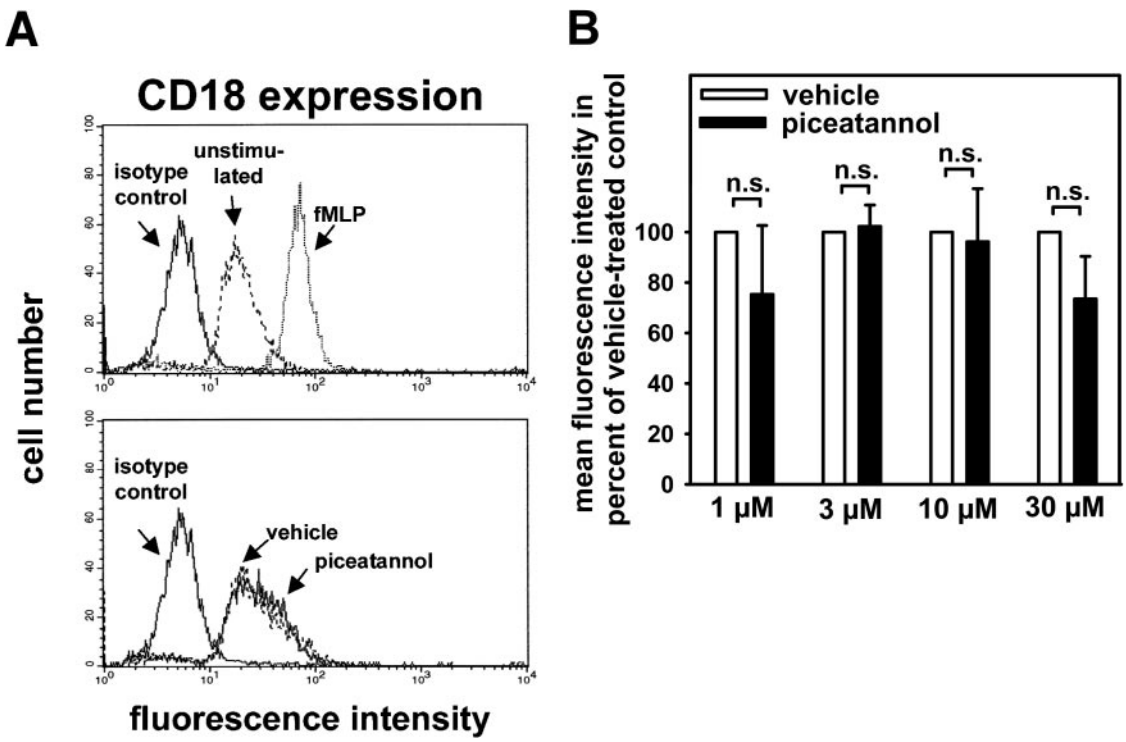

Fig. 5. Inhibition of Syk-kinase had no effect on CD18 expression. Expression of CD18 on the cell surface as measured by flow cytometry. (A) PMN were treated with 10 μg/ml nonbonding, isotype-matched control mAb (isotype control) or with the anti-CD18 mAb 7E4 without stimulation (unstimulated) or after stimulation with 100 nM fMLP for 10 min, respectively. Samples were labeled with the secondary FITC-conjugated rabbit anti-mouse IgG in a final dilution of 1:20 (upper panel). PMN were treated with 30 μM piceatannol or vehicle for 30 min at 37°C and were stained as described above (lower panel). Data are representative of three independent experiments. (B) PMN were incubated for 30 min at 37°C with indicated concentrations of piceatannol or vehicle, respectively. Data represent mean fluorescence intensity in percent of vehicle-treated control (100%) and were stained as described above. N = 4; mean ± SD; n.s., not significant.

To elucidate the mechanism by which inhibition of tyrosine phosphorylation of Syk-kinase enhanced β_2 integrin-mediated adhesion and spreading of PMN and thereby down-regulated their mobility, the expression of CD18 on the cell surface of PMN treated with piceatannol or vehicle was studied by flow cytometry (**Fig. 5A**). For positive control, PMN were stimulated with 100 nM fMLP, which induced a substantial increase of CD18 expression on the cell surface when compared with the expression of CD18 on unstimulated cells. In contrast, treatment of PMN with 30 μM piceatannol had no effect on CD18 expression on the cell surface. This was also true for concentrations of 1, 3, and 10 μM piceatannol (Fig. 5B). Piceatannol also had no effect on fMLP-induced up-regulation of CD18 expression (data not shown). Thus, enhanced adhesion and spreading of PMN after inhibition of tyrosine phosphorylation of Syk-kinase were not a result of an altered expression of β_2 integrins.

Next, the affinity state of β_2 integrins after treatment of PMN with piceatannol was investigated by use of the mAb CBRM1/5, which is directed against activation-specific neoepitopes on β_2 integrins [29]. For positive control, PMN were stimulated with 100 nM fMLP. As expected, fMLP substantially enhanced the number of activated β_2 integrins on PMN when compared with unstimulated cells (**Fig. 6A**). In contrast, the affinity state of the β_2 integrins was not affected by the Syk-kinase inhibitor piceatannol when compared with vehicle-treated cells. This was true for unstimulated cells and for cells that were stimulated with 1, 3, 10, 30, or 100 nM fMLP after pretreatment with 30 μM piceatannol for 30 min (Fig. 6B). This shows that enhanced adhesive interactions of the β_2 integrins upon inhibition of Syk-kinase are not a result of an up-regulation of the affinity state of the β_2 integrins.

Finally, the effect of piceatannol on the clustering of the β_2 integrins was investigated by confocal microscopy. PMN were treated with 30 μM piceatannol or vehicle before incubation of PMN in suspension or induction of adhesion on immobilized fibrinogen for 30 min. Cell-surface expression of CD18 was analyzed using the RPE-conjugated anti-CD11b mAb 2LPM19c (**Fig. 7**). Ligand binding of the β_2 integrins induced the well-known clustering of the β_2 integrins in adherent PMN when compared with suspended PMN, which showed a random surface expression of CD18. When compared with vehicle-treated cells, inhibition of tyrosine phosphorylation of Syk-kinase induced enhanced clustering of the β_2 integrins in suspended and adherent PMN. Thus, inhibition of Syk-kinase may promote PMN adhesiveness by enhancing the clustering of the β_2 integrins which is known to result in increased integrin avidity.

DISCUSSION

Leukocyte adhesion molecules of the β_2 integrin family, which mediate the firm adhesion of human PMN to the

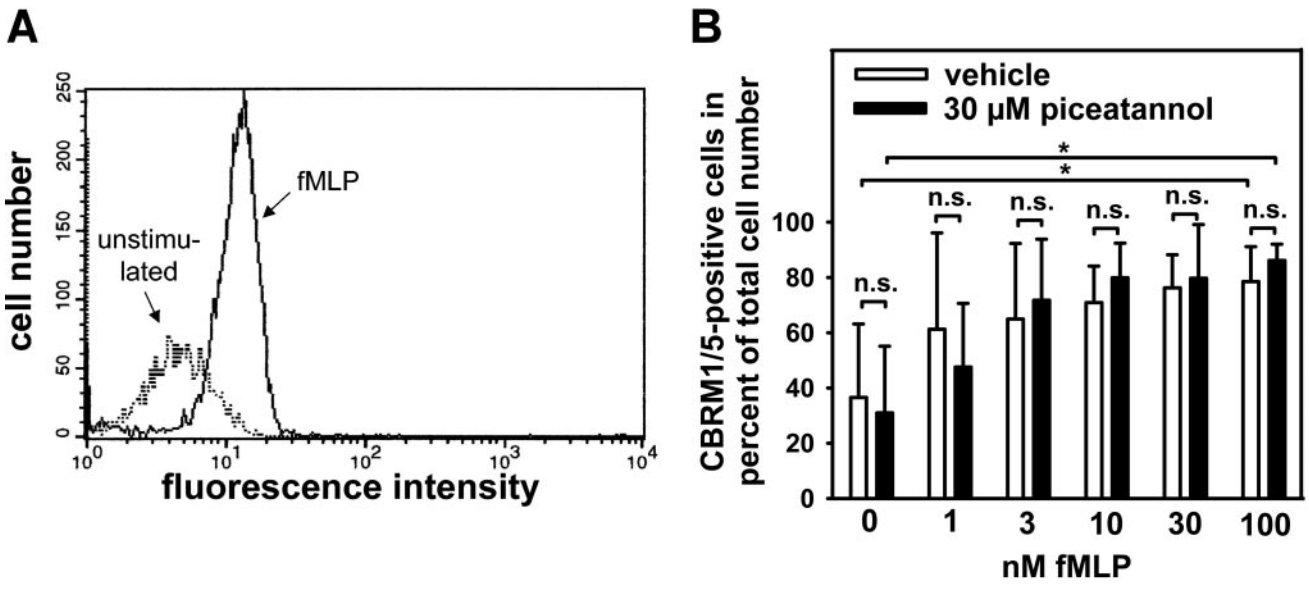

Fig. 6. Inhibition of Syk-kinase had no effect on expression of activation-specific neoepitopes on β_2 integrins. (A) PMN (2×10^6/sample) were left untreated for negative control (unstimulated) or were stimulated for 10 min with 100 nM fMLP. Expression of activation-specific neoepitopes on CD18 was detected by flow cytometry using 10 μg/ml anti-CD18 mAb CBRM1/5 and the secondary FITC-conjugated rabbit anti-mouse IgG in a final dilution of 1:20. Original fluorescence histograms are shown. (B) PMN were treated for 30 min at 37°C with 30 μM piceatannol and were stimulated with 1, 3, 10, 30, or 100 nM fMLP for 10 min. Data represent CBRM1/5-positive cells in percent of total cell number (100%). Mean ± SD; n=4; *, $P < 0.05$, versus unstimulated control; n.s., not significant.

endothelial cell monolayer during the inflammation response, are known to transduce signals into the cell by evoking intracellular protein tyrosine phosphorylation [24–26]. In this study, the 72-kDa protein, which became tyrosine-phosphorylated upon β_2 integrin-mediated adhesion, was identified as Syk-kinase by immunoprecipitation experiments. Tyrosine phosphorylation of the 72-kDa protein was also observed upon antibody cross-linking of CD18 [24], demonstrating that clustering of CD18 causes phosphorylation of Syk. Furthermore, coimmunoprecipitation experiments revealed that the Syk-kinase was associated with CD18, the β-subunit of β_2 integrins. These findings are consistent with other studies, which have shown that ligand binding of β_2 integrins leads to the formation of protein complexes containing the β-subunit of β_2 integrins, Syk-kinase, and the Src-kinases Fgr and Lyn [25, 26]. The present study, however, provides evidence for a direct association of Syk-kinase and the β_2 integrins. Moreover, we found that both molecules are constitutively associated in suspended and adherent cells. Similar results were obtained in the macrophage cell line BAC1, where CD18 and Syk-kinase were found to be constitutively associated [31]. Moreover, another report presented evidence that the binding of Syk-kinase to the cytoplasmic domain of the β-subunit of the β_1, β_2, and β_3 integrins is mediated by a phosphotyrosine-independent interaction via the tandem N-terminal Src homology 2 domains of Syk [32]. However, these results are in contrast to the above-mentioned study [25], where an association of Syk-kinase and CD18 was only found in TNF-α-stimulated, adherent PMN but not in PMN that were exposed to immobilized fibrinogen in the absence of TNF-α. This discrepancy may be a result of the fact that different detergents were used for the immunoprecipitation experiments, which may affect the stability of the protein complexes: In the present study, the immunoprecipitation was performed with modified RIPA buffer with 0.1% SDS and 0.5% deoxycholate in the absence of Triton X-100, whereas 0.2% Triton X-100 was used in the above-mentioned study [25]. In the present study, we observed that adherent PMN showed a slightly higher degree of Syk-CD18 association when compared with suspended PMN, suggesting that ligand binding may promote this interaction. Furthermore, we showed that the 120-kDa adaptor protein c-Cbl, which is tyrosine-phosphorylated upon β_2 integrin engagement [24], was not associated with the β-subunit of β_2 integrins, confirming the specificity of the observed effect. However, the fact that c-Cbl regulates Syk-kinase negatively [33] and acts as a substrate of Src-kinase [34, 35] suggests a signaling pathway in which the Syk-kinase connects the β_2 integrins and Src-kinases via c-Cbl. Although it is known that tyrosine phosphorylation via Src kinases initially activates Syk-kinase, the majority of Syk-phosphorylation is generated by transphosphorylation, a mechanism described for Syk-kinase-mediated Fc receptor signaling and designated as an activation loop phosphorylation chain reaction [36]. Moreover, this step was previously reported to be essential for the propagation of Syk-kinase-mediated, downstream signaling events [37]. However, the activation loop chain reaction requires that the Syk molecules come into a critical vicinity to each other. As the present study provides evidence that Syk is associated with CD18, clustering of β_2 integrins, which is a consequence of β_2 integrin-mediated adhesion [23], fulfills this requirement. This suggests that ligand binding of the β_2 integrins and subse-

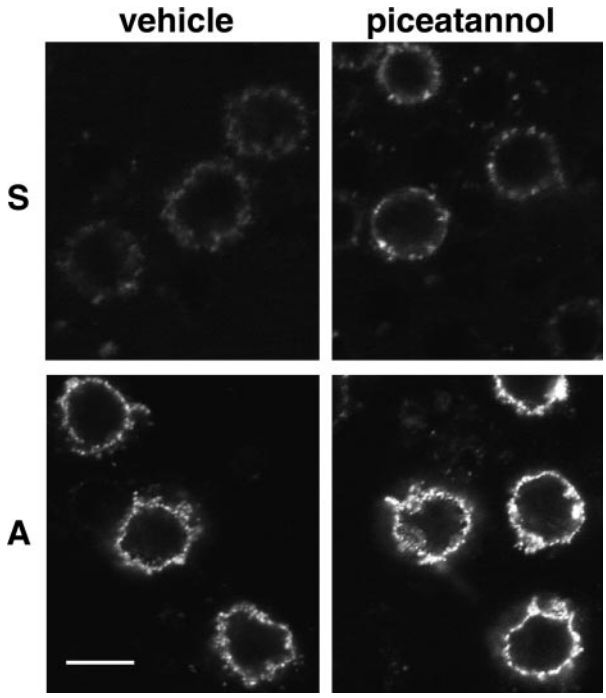

Fig. 7. Inhibition of Syk-kinase induced clustering of the β_2 integrins. PMN were treated for 30 min at 37°C with 30 μM piceatannol or vehicle and were kept in suspension (S) or were allowed to adhere to immobilized fibrinogen (A) for 30 min at 37°C in the presence of 1.2 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 mM Mn^{2+}. PMN were stained with 10 μg/ml PE-conjugated anti-CD11b mAb 2LPM19c. Samples were analyzed by confocal microscopy. Original bar = 10 μM. Data are representative of three independent experiments.

quent integrin clustering may suffice to initiate the Syk-mediated signaling pathway. To elucidate the functional role of β_2 integrin-mediated activation of Syk-kinase, we used the Syk-kinase-specific inhibitor piceatannol [38, 39]. However, piceatannol has been found to inhibit the activity of other kinases, i.e., focal adhesion kinase and Scr-kinase [40], but this was only true for concentrations that were far beyond the concentration of 30 μM, which was used in our study. We found that the concentration of 30 μM piceatannol inhibited tyrosine phosphorylation of Syk-kinase almost completely. However, tyrosine phosphorylation of other proteins also decreased in the presence of piceatannol. This is also true for Syk-deficient neutrophils, which show decreased phosphorylation of c-Cbl, Pyk2, and Vav1 [41], suggesting that these proteins are downstream of Syk in the signaling pathway.

By means of adhesion assays, we found that the inhibition of Syk-kinase by piceatannol induced a profound increase of PMN adhesion and spreading on immobilized fibrinogen when adhesion was induced by divalent cations in the absence of soluble mediators. In contrast, TNF-α-stimulated adhesion and spreading of PMN on immobilized fibrinogen were substantially decreased upon inhibition of Syk-kinase with piceatannol. This is in accordance with a previous study where Syk-deficient BAC1 cells failed to spread on ICAM-1 in response to various proinflammatory mediators [31]. Decreased TNF-α-induced adhesion was also observed in Syk-deficient neutrophils exposed to immobilized fibrinogen [41]. Thus, Syk-kinase seems to exert the opposite effect upon costimulation by β_2 integrin-mediated adhesion and soluble mediators when compared with the role of Syk-kinase upon induction of β_2 integrin-mediated adhesion in the absence of soluble mediators. This would suggest a dual function of Syk-kinase, but the molecular mechanism underlying this effect is still elusive. We found that inhibition of Syk-kinase inhibited migration of PMN in response to 10 and 50 nM fMLP. In a recent report, Mocsai et al. [42] observed that migration of Syk-deficient PMN was only inhibited in response to 300 nM fMLP but not at higher concentrations. The authors concluded that fMLP-induced migration was not affected in Syk-deficient PMN. This conclusion is probably not correct, as high concentrations of fMLP (>100 nM) induce profound chemokinesis. Thus, the results by Mocsai et al. [42] probably show that chemokinesis is independent of Syk. Moreover, they used filters with a pore size of 8 μm, which is comparably large for detecting chemotactic migration of PMN. Finally, the chemotactic migration of PMN in this assay system only partially depends on CD18. Altogether, this may suggest the experimental design used by Mocsai et al. [42] is probably not suitable to measure chemotaxis of PMN.

However, we found that Syk-kinase negatively regulates clustering of β_2 integrins, which is known to control the avidity of the β_2 integrins [43]. This finding is consistent with data obtained from K562 cells, which show that LFA-1-mediated cell adhesion to ICAM-1 is predominantly regulated by receptor clustering and that affinity alterations do not necessarily coincide with strong ICAM-1 binding [44]. Thus, avidity regulation of integrins seems to represent the pivotal mechanism for the control of leukocyte adhesion. Other studies propose specialized membrane regions, called rafts, serving as platforms for signaling molecules such as Src family members, G-proteins, phosphatidylinositol-3 kinase, and adaptor proteins [45, 46]. Clustering of membrane rafts was suggested to regulate LFA-1-mediated adhesion in T cells [47]. It is interesting that clustering and not only ligand binding of β_2 integrins by itself are known to initiate the signaling process in human PMN [23]. Together, this supports the concept that clustering of β_2 integrins by immobilized ligands may critically approach signaling molecules, e.g., constitutively β_2 integrin-associated Syk-kinase, which induces signaling events that control adhesion-dependent cell functions. Integrin clusters have been found to be highly motile in stationary fibroblasts [48]. In contrast, the integrin clusters were stationary and only moved in the tail of the migrating cell, suggesting that the dynamics of integrin clustering are important for the control of cell motility [48]. Our study suggests that Syk-kinase promotes the dynamic turnover of integrin clusters of human PMN in the absence of soluble mediators and therefore may be critical to ensure the migratory capacity of PMN, which represent an important function for their role in host defense.

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