Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 385–394
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of interleukin-8 (CXCL8/IL-8) responses by repertaxin, a new inhibitor of the chemokine receptors CXCR1 and CXCR2

Federica Casilli[a,1], Andrea Bianchini[a,1], Isabelle Gloaguen[b], Leda Biordi[c], Edoardo Alesse[c], Claudio Festuccia[c], Barbara Cavalieri[d], Raffaele Strippoli[e], Maria Neve Cervellera[a], Rosa Di Bitondo[a], Elisabetta Ferretti[e], Fabrizio Mainiero[e], Cinzia Bizzarri[a], Francesco Colotta[a], Riccardo Bertini[a,*]

[a]*Dompé S.p.A. Research Center, L'Aquila, Italy*
[b]*Consorzio Biolaq, L'Aquila, Italy*
[c]*Department of Experimental Medicine, University of L'Aquila, Italy*
[d]*Department of Clinical and Biological Sciences, University of Turin, Italy*
[e]*Department of Experimental Medicine and Pathology, University of 'La Sapienza', Rome, Italy*

Received 6 August 2004; accepted 20 October 2004

Abstract

Repertaxin is a new non-competitive allosteric blocker of interleukin-8 (CXCL8/IL-8) receptors (CXCR1/R2), which by locking CXCR1/R2 in an inactive conformation prevents receptor signaling and human polymorphonuclear leukocyte (PMN) chemotaxis. Given the unique mode of action of repertaxin it was important to examine the ability of repertaxin to inhibit a wide range of biological activities induced by CXCL8 in human leukocytes. Our results show that repertaxin potently and selectively blocked PMN adhesion to fibrinogen and CD11b up-regulation induced by CXCL8. Reduction of CXCL8-mediated PMN adhesion by repertaxin was paralleled by inhibition of PMN activation including secondary and tertiary granule release and pro-inflammatory cytokine production, whereas PMN phagocytosis of *Escherichia coli* bacteria was unaffected. Repertaxin also selectively blocked CXCL8-induced T lymphocyte and natural killer (NK) cell migration. These data suggest that repertaxin is a potent and specific inhibitor of a wide range of CXCL8-mediated activities related to leukocyte recruitment and functional activation in inflammatory sites.

Keywords: Interleukin-8; Polymorphonuclear leukocytes; Adhesion; Degranulation; Phagocytosis; Repertaxin

1. Introduction

The recruitment of leukocytes into sites of tissue damage is a normal physiological response designed to fight infection, remove damaged cells and stimulate healing. However, the uncontrolled recruitment of leukocytes often exacerbates tissue damage, slows healing and in some cases may lead to host death.

Among chemotactic factors generated at the site of inflammation, interleukin-8 (CXCL8/IL-8) is a key mediator of human polymorphonuclear leukocyte (PMN) recruitment and activation [1,2]. CXCL8 is also involved in T lymphocyte and natural killer (NK) cell recruitment [3,4]. CXCL8 belongs to the C–X–C branch of chemokine family, which also includes growth-related oncogene (CXCL1/GRO), neutrophil-activating peptide-2 (CXCL7/NAP-2), epithelial neutrophil-activating peptide (CXCL5/ENA-78) and granulocyte chemotactic protein-2 (CXCL6/GCP-2) [5,6]. Interleukin-8 receptor 1 (CXCR1)

Abbreviations: PMN, human polymorphonuclear leukocytes; CXCL8/IL-8, interleukin-8; CXCR1, interleukin-8 receptor 1; CXCR2, interleukin-8 receptor 2; GPCR, G protein-coupled receptor; NK, natural killer; LPS, bacterial endotoxin; C5a, fifth component of complement; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; CXCL12/SDF-1, stromal cell-derived factor-1; CXCL6/GCP-2, granulocyte chemotactic protein-2; PBS, phosphate buffered saline; FBS, fetal bovine serum; MMP-9, matrix metalloproteinase 9; IL-1β, interleukin-1β; EDTA, ethylenediaminetetracetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

* Corresponding author. Tel.: +39 0862 338375; fax: +39 0862 338219.
E-mail address: bertini@dompe.it (R. Bertini).
[1] They contributed equally to this work.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.10.007

and interleukin-8 receptor 2 (CXCR2) are G protein-coupled receptors (GPCRs) known to couple intracellular signal-transduction pathways involving the activation of *Bordetella pertussis* toxin-sensitive GTP-binding proteins, activation of phospholipase C, formation of second messengers inositol 1,4,5-triphoshate and subsequent increase of intracellular calcium concentration. Phosphatidylinositol 3-kinase γ and proline-rich tyrosine kinase 2 (Pyk2) activation have also been shown to increase in response to CXCL8 [7,8].

CXCL8 regulates recruitment and activation of PMNs at inflammatory sites by inducing a coordinated set of responses. CXCL8 up-regulates adhesion molecule expression in PMNs and induces the directional migration of PMNs from the bloodstream to the site of inflammation [1,5]. Moreover, CXCL8 induces functional activities of PMNs including cytokine expression [9] and release of tissue damage mediators including proteases from cytoplasmic granules [1,2].

CXCL8 is supposed to play a pivotal role in the pathology of several inflammatory diseases including psoriasis [10,11], ulcerative colitis [12], cystic fibrosis [11] and ischemia/reperfusion injury [11], suggesting that CXCL8 and its receptors may represent attractive therapeutical targets. All small molecule inhibitors of CXCL8 described so far are still in the preclinical phase [11,13].

We have recently identified and characterized repertaxin, a new small-molecule inhibitor of CXCR1 and CXCR2 [14]. Structural and biochemical data are consistent with a non-competitive allosteric mode of interaction between CXCL8 receptors and repertaxin, which, by locking CXCR1 and CXCR2 in an inactive conformation, prevents the activated receptor-induced intracellular signal transduction cascade and cell response. This mechanism of action is unprecedented in the pharmacological modulation of GPCR. Repertaxin is a potent and specific inhibitor of CXCL8-induced PMN chemotaxis in vitro and protects organs against reperfusion injury in vivo [14].

Given the unique mode of action of repertaxin, it was important to examine the ability of repertaxin to inhibit a whole range of biological activities induced by CXCL8 in human leukocytes. The results reported hereafter show that repertaxin potently and selectively blocked PMN adhesion to fibrinogen and CD11b up-regulation induced by CXCL8. In addition, repertaxin blocked CXCL8-mediated PMN activation including secondary and tertiary granules release and pro-inflammatory cytokine production. By contrast, repertaxin did not affect PMN phagocytosis of opsonized *Escherichia coli* bacteria. Finally, repertaxin, tested in the same range of concentrations affecting PMN activities, selectively blocked CXCL8-induced T lymphocyte and NK cell migration. These data suggest that repertaxin is a potent and specific inhibitor of a wide range of CXCL8-induced activities related to leukocyte recruitment and functional activation in inflammatory sites.

2. Materials and methods

2.1. Reagents and chemicals

Human recombinant CXCL8, CXCL6 and stromal cell-derived factor-1 (CXCL12/SDF-1) were purchased from PeproTech. Diff-Quik was from Harleco. RPMI-1640 and Trizol reagent were from Life Technologies. Fetal bovine serum (FBS) was from Hyclone. Dextran and Ficoll–Hipaque were from Amersham Biosciences. Bacterial endotoxin (LPS) was from Difco. Lysing solution for flow cytometric analysis, fluorescein-conjugated goat anti-mouse and anti-CD11b antibodies were from Becton Dickinson. Anti-CD66b antibody was from Serotec. Repertaxin (R(−)-2-(4-isobuthylphenyl)propionyl methansulfonamide) salified with L-lysine was routinely dissolved at the indicated final concentrations in saline (Bieffe Medital). All other chemicals, fifth component of complement (C5a), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), fibrinogen and cell culture reagents were from Sigma.

2.2. Cells

Human PMNs, NK cells and T lymphocytes were obtained from buffy-coats of heparinized human peripheral blood from healthy volunteers through the courtesy of Centro Trasfusionale, Ospedale S. Salvatore (L'Aquila, Italy). PMNs were prepared to 99% purity by centrifugation through a Ficoll–Hipaque gradient, as previously described [7]. Briefly, after Ficoll–Hipaque centrifugation, pellet was washed in saline and then resuspended in 5 ml PBS + 5 ml 5% dextran solution for 25 min at room temperature. Then, the supernatant (containing PMNs) was carefully layered onto discontinuous gradient (Histopaque-1119/Ficoll–Hipaque). PMNs were collected after a centrifugation at 700 × *g* for 20 min. Cellular viability was >95% in all experiments, as measured by trypan blue dye exclusion.

Human T lymphocytes were prepared to 97% purity using Human T Cell Enrichment Columns isolation kit (R&D Systems). Briefly, mononuclear cells, obtained by centrifugation on Ficoll–Hipaque followed by hypotonic lysis of contaminating red blood cells, were resuspended at a concentration of 15×10^7 cells/ml and loaded into the column. After an incubation at room temperature for 10 min, cells were eluted from the column according to the manufacturer's instructions, washed two times in saline and resuspended at 5×10^6 cells/ml in RPMI 1640 + 1% BSA.

Highly enriched (99%) population of fresh human NK cells was obtained as follows. First, human mononuclear cells were isolated from buffy-coats by Lymphoprep (Nycomed AS) gradient centrifugation as previously described [15]. Then, NK cells were purified by negative selection using a NK cell isolation kit (Miltenyi Biotech). Briefly, mononuclear cells were labeled with a cocktail of biotin-conjugated monoclonal antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and Glycoforin A, followed by a labeling with

anti-biotin antibodies conjugated to magnetic micro-beads and then passed through a MACS column in the magnetic field of a MACS separator. Finally, the magnetic labeled non-NK cells (such as T and B lymphocytes and monocytes) are retained into the column and only NK cells are eluted. The purity of the obtained NK cells was evaluated by immunofluorescence and FACS analysis.

2.3. PMN adhesion

Twelve-well plates were coated with purified human fibrinogen (0.5 mg/ml in PBS, for 90 min at room temperature) and then used for the adhesion assay [16]. PMNs (1.6×10^6 cells/ml), resuspended in Hank's balanced salt solution (HBSS) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 2 mM $MgCl_2$, were pretreated with vehicle or repertaxin for 20 min at room temperature. Subsequently, 500 μl of these cell suspensions were added (in triplicate) to wells containing vehicle, CXCL8 (20 nM), CXCL6 (10 nM), C5a (100 nM) or fMLP (100 nM) and incubated for 20 min at 37°. Unbound cells were removed by repeated washes with HBSS containing 10 mM Hepes and 2 mM $MgCl_2$, and adherent cells were fixed and stained with Diff-Quik. Twelve microscope fields (magnitude 200×) were counted for each sample.

2.4. CD11b and CD66b membrane expression

Expression of CD11b and CD66b on human PMNs was evaluated by using a FACSCalibur flow cytometer (Becton Dickinson) as previously described [17,18]. Briefly, human whole blood samples (100 μl) were preincubated for 20 min with different concentrations of repertaxin or vehicle and then stimulated with CXCL8 (1–10 nM) or CXCL6 (30 nM), for 10 min at room temperature or for 30 min at 37° for CD11b and CD66b expression, respectively. The induction was stopped by placing samples at 4°. Blood samples were then incubated with monoclonal anti-CD11b (10 μg/ml) or monoclonal anti-CD66b (1 μg/ml) antibodies for additional 30 min. Then, samples were washed twice with cold buffer [PBS containing 0.2% bovine serum albumin (BSA)] and incubated with goat anti-mouse-fluorescein isothiocyanate (FITC) immunoglobulin G (IgG) (20 μg/ml) for 30 min at 4° in the dark. Samples were then washed with cold buffer and red cells eliminated by incubation with a FACS lysing solution for 8 min at room temperature. Following a final washing step with cold buffer, leukocytes were fixed with 1% paraformaldehyde in PBS and processed by flow cytometry.

In each sample, a total of 10^4 PMNs were acquired by drawing a gate into the PMN region of leukocytes in the forward scatter/side scatter (FSC/SSC) plot. CD11b and CD66b expression was evaluated as geometric mean fluorescence using the FL-1 fluorescence channel. All samples were tested in triplicate.

2.5. MMP-9 activity

PMNs were resuspended (4×10^6 cells/ml) in RPMI 1640 + 0.05% BSA, plated into 24-well plates (500 μl) containing vehicle or repertaxin (1 μM) and incubated for 20 min at 37°. Then, PMNs were stimulated with CXCL8 (1–10 nM) for 1 h at 37°. Following incubation, culture supernatants were collected, centrifuged at top speed in an Eppendorf Microcentrifuge for 5 min at 4° to remove cell debris and stored at −80° until assayed. Matrix metalloproteinase 9 (MMP-9) activity was analyzed by zymography performed using sodium-dodecyl sulfate (SDS)-polyacrylamide gel copolymerized with 0.1 mg/ml gelatine, as previously reported [19]. Mix of pro-MMP-9 and pro-MMP-2 present in unconcentrated conditioned media obtained from untreated or 12-*O*-tetradecanoylphorbol-13-acetate (100 nM TPA)-treated MDA-MB231 human breast cancer cell line was used as standards. Each lane was loaded with 20 μl of cell-free supernatants. Gels were washed three times for 15 min in 10% Triton X-100 under shaking at room temperature, then incubated at 37° overnight in collagenase buffer (50 mM Tris–HCl pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$). Gels were fixed and stained for 30 min at room temperature with 0.1% Comassie blue in a mixture of acetic acid:methanol:water (1:4:5) and destained in the same solution without dye. Proteolytic activities were identified for the appearance of clear bands.

2.6. RT-PCR analysis

PMNs were resuspended (4×10^6 cells/ml) in RPMI 1640 supplemented with 2% heat-inactivated fetal bovine serum, incubated for 2 h at 37° and then pretreated with vehicle or repertaxin (1 μM) for additional 30 min. Next, CXCL8 (10 nM) was added and the samples were incubated for 4 h at 37°. Cells were then collected, washed and stored at −80° for total RNA extraction. Total RNA was extracted from PMNs with Trizol reagent according to the manufacturer's instruction. Semiquantitative RT-PCR was carried out on DNase Amp Grade (GIBCO-BRL)-treated RNA using MuLV RT (50 units) in 100 mM Tris–HCl pH 8.3, 500 mM KCl, 5 mM $MgCl_2$, 0.5 μM each dNTP, 1 unit of RNasin, 500 pmol of random hexamer primers. Two microliters of cDNA were amplified in a 50 μl reaction volume containing 0.5 μM of sense and antisense primers, 2.5 units of Taq DNA polymerase (Applied Biosystems), 200 μM each dNTP and 1.5 mM $MgCl_2$. A co-amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for semiquantitative analysis was performed. Samples from PCR reaction were taken from 15 to 35 cycles, in order to monitor the linear exponential phase of amplification for optimal quantitative of the template levels. Primers sequences used for PCR were: interleukin-1β (IL-1β) Fw 5′-TCCCCAGCCCTTTTGTTGA-3′; IL-1β Rev 5′-TTAGAACCAAATGTGGCCGTG-3′; GAPDH Fw 5′-CACCATGGAGAAAGGCCGGGG-3′;

GAPDH Rev 5′-GACGGACACATTGGGGGTAG-3′. After amplification, 20 μl of the PCR reaction mixture was analyzed by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

2.7. Real-time PCR analysis

Quantitative analysis of IL-1β mRNA expression was performed by the ABI Prism 7700 Sequence Detection System (Applied Biosystems) employing Syber green Assay Reagents. Oligonucleotide primers for IL-1β were the same used for qualitative PCR (see Section 2.6). Oligonucleotide primers for the endogenous control GAPDH sequences were as follow: Fw 5′-AGCAATGCCTCCTGCACCACCAAC-3′, Rev 5′-CCGGAGGGGCCATCCACAGTCTTCT-3′. A 25 μl reaction mixture containing 2.5 μl of cDNA template, 12.5 μl TaqMan Universal PCR master mix (Applied Biosystems) and 1.25 μl primer mixture was amplified using the following thermal cycler parameters: incubation at 50 °C for 2 min and denaturing at 95 °C for 10 min, then 40 cycles of the amplification step (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). For each amplification run, standard curve was generated using six serial dilutions of cDNA mix expressing both genes analyzed. All amplification reactions were performed in triplicate, and the averages of the threshold cycles (Cts) were used to interpolate standard curves and to calculate the transcript amount in samples using SDS version 1.7a software (ABI). To obtain the target gene expression rate, the amounts of IL-1β mRNA were normalized with that of GAPDH. Results are expressed in arbitrary units as fold increases.

2.8. IL-1β production

PMNs (5×10^6 cells/ml), resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, were incubated at 37° for 2 h. Next, PMNs were pretreated with vehicle or repertaxin (1 μM) for 30 min at 37°. Then, LPS (50 ng/ml) or LPS + CXCL8 (10 nM) were added, and 300 μl of these cell suspensions were transferred into 96 propylene wells-plate and incubated for 8 h at 37°. After incubation, cell supernatants were collected, centrifuged and stored at −80° for IL-1β analysis. IL-1β production was quantified in cell-free supernatants by ELISA kit (Amersham Biosciences) according to the manufacturer's instructions.

2.9. PMN phagocytosis

PMN phagocytic activity was evaluated using the commercial kit Phagotest (Becton Dickinson). Briefly, 100 μl of human whole blood was pretreated with vehicle, repertaxin (1 μM or 10 μM) or ethylenediaminetetracetic acid (EDTA) (1 mM) for 20 min at room temperature. Subsequently, the samples were put at 4° and after 10 min 20 μl of *E. coli*–FITC opsonized were added. To induce PMN phagocytosis the samples were incubated for 15 min at 37°, while the basal PMN phagocytosis was evaluated in samples maintained at 4° during the incubation time. Then, samples were processed for flow cytometric evaluation according to the manufacturer's instructions. A total of 10^4 PMNs were acquired by drawing a gate into the PMN region of leukocytes in the FSC/SSC plot, and phagocytosis was evaluated as geometric mean fluorescence using the FL-1 fluorescence channel. All samples were tested in triplicate.

2.10. T lymphocyte and NK cell migration

Human T lymphocyte migration was evaluated using a 48-well micro-chemotaxis chamber as previously described [20]. Thirty microliters of control medium (RPMI 1640 + 1% BSA) or chemoattractant solution were seeded in the lower compartment of the chemotaxis chamber. Fifty microliters of cell suspension (5×10^6/ml), preincubated at 37° for 30 min with vehicle or different concentrations of repertaxin, were seeded in triplicates in the upper compartment of the chemotactic chamber. The two compartments of the chemotactic chamber were separated by a 5-μm polycarbonate filter (Poretics) previously treated with collagen IV (10 μg/ml) for 1 h at 37°. Thirty microliters of control medium (RPMI 1640 + 1% BSA) or chemoattractant solution were seeded in the lower compartment. The chamber was incubated at 37° in air with 5% CO_2 for 3 h. At the end of incubation, filters were removed, fixed, stained with Diff-Quik and five oil immersion fields at high magnification (1000×) were counted for each migration well after sample coding.

Cell migration of highly purified fresh human NK cells was measured using a Transwell migration chamber (diameter, 24 mm; pore size, 5 μm; Costar, Cambridge, MA) as previously described [21]. Briefly, 600 μl of control medium (RPMI 1640 + 0.1% BSA) or chemoattractant solution were seeded in the lower compartment of the chemotaxis chamber. One hundred microliters of cell suspension (5×10^6/ml), preincubated at 37° for 30 min with vehicle or repertaxin, were seeded in duplicates in the upper compartment of the chemotactic chamber. After 60 min at 37° in air with 5% CO_2, the number of migrated cells was counted using an inverted microscope with 100× magnification.

3. Results

3.1. Inhibition of CXCL8-induced PMN adhesion

Pretreatment of human PMNs with repertaxin significantly reduced PMN adhesion on fibrinogen-coated wells stimulated by an optimal concentration (20 nM) [16] of

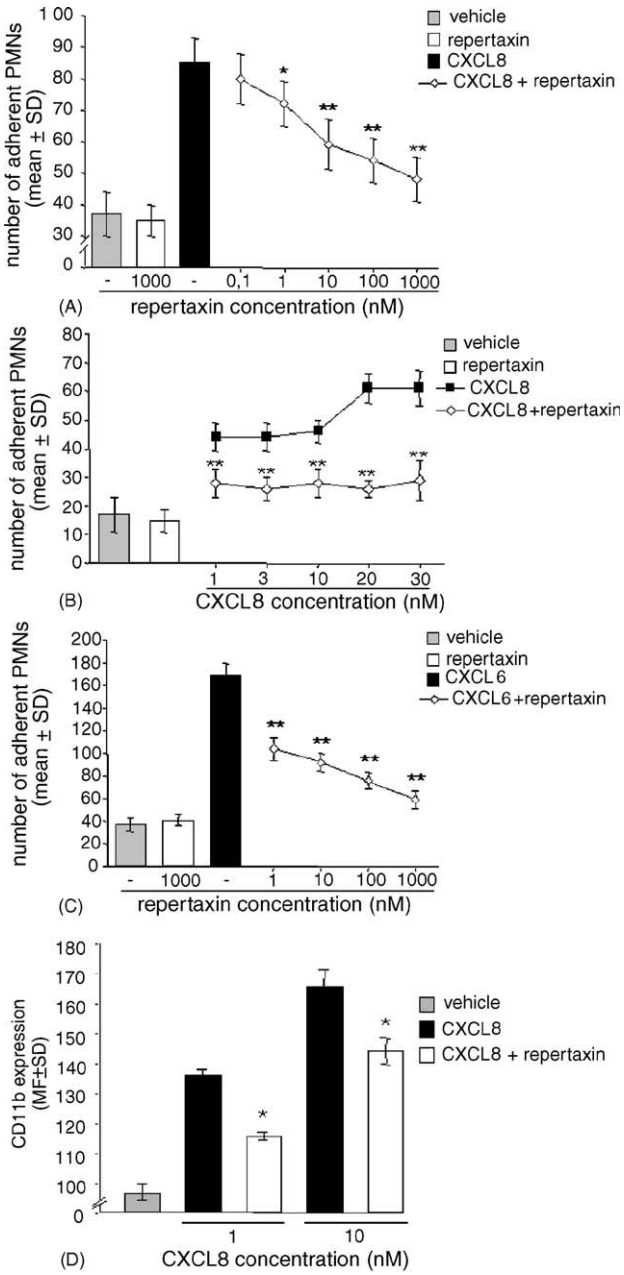

Fig. 1. Effect of repertaxin on PMN adhesion and CD11b up-regulation. (A) PMN adhesion. Human PMNs were pretreated for 20 min at room temperature with vehicle, or increasing concentration of repertaxin. PMNs were then tested for their ability to adhere on fibrinogen-coated wells in response to CXCL8 (20 nM). (B) PMNs were pretreated with vehicle or repertaxin (1 μM). PMN adhesion was stimulated with increasing concentrations of CXCL8 (1–30 nM). (C) Human PMNs were pretreated for 20 min at room temperature with vehicle, or increasing concentration of repertaxin. PMNs were then tested for their ability to adhere on fibrinogen-coated wells in response to CXCL6 (10 nM). PMN adhesion was determined as described in Section 2. Data are expressed as mean values ± S.D. of three independent experiments. (D) CD11b expression. Whole blood samples were pretreated with vehicle or repertaxin (1 μM) for 20 min at room temperature. Both experimental groups were then stimulated for 10 min with CXCL8 (1 nM or 10 nM). CD11b expression was determined by flow cytometry as described in Section 2. Data are expressed as mean fluorescence intensity (MFI) ± S.D. and are from one experiment of five. Statistical analysis was performed by one-way ANOVA test, followed by Dunnett multiple comparison. Statistical threshold was set at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. CXCL8 or CXCL6 group.

CXCL8 (Fig. 1A). Reduction of PMN adhesion by repertaxin was concentration dependent, being statistically significant at 1 nM (30% of inhibition) and maximal at 1 μM (80% of inhibition). The inhibitory effect of repertaxin was independent of CXCL8 concentration (1–30 nM; Fig. 1B). Similarly, repertaxin inhibited PMN adhesion induced by CXCL6, a well-known agonist of CXCR1/2. As shown in Fig. 1C, repertaxin inhibited CXCL6-mediated PMN adhesion in the range of concentrations and with similar efficacy to CXCL8-induced PMN adhesion. In the absence of chemokine stimulation, repertaxin alone was unable to modify PMN spontaneous adhesion. Since CXCL8 is known to induce β2-integrin Mac-1 (CD11b/CD18) membrane up-regulation [1] and fibrinogen is a well-known ligand for β2-integrins of PMNs [22], we investigated the effect of repertaxin on CXCL8-induced CD11b membrane over-expression on human PMNs. As shown in Fig. 1D, repertaxin (1 μM) significantly inhibited CD11b expression induced by CXCL8 (1 nM and 10 nM).

To investigate the specificity of repertaxin-induced inhibition of PMN adhesion, we examined the effect of repertaxin on fMLP- and C5a-induced PMN adhesion. The effect of repertaxin was evaluated in the same range of

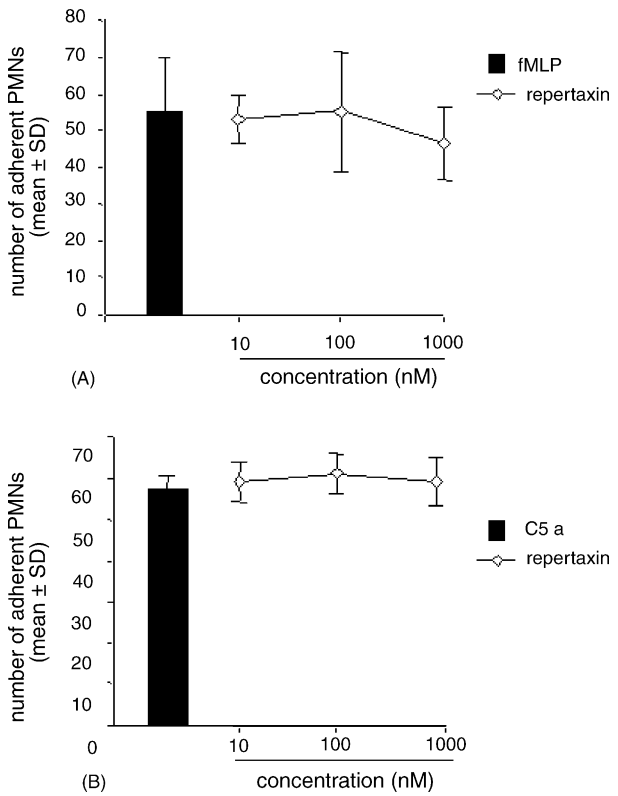

Fig. 2. Effect of repertaxin on fMLP- and C5a-induced PMN adhesion. PMNs were pretreated for 20 min at room temperature with vehicle or increasing concentration of repertaxin. PMNs were then tested for their ability to adhere in response to fMLP (100 nM; panel A) or C5a (100 nM; panel B). PMN adhesion was determined as described in Section 2. Data, subtracted of spontaneous PMN adhesion, are expressed as mean values ± S.D. of three independent experiments. Spontaneous PMN adhesion was 27 ± 4.

concentrations affecting CXCL8-induced PMN adhesion. Results reported in Fig. 2 (panels A and B) show that repertaxin did not affect PMN adhesion induced by fMLP and C5a over a wide range of concentrations. Thus, repertaxin is a potent and specific inhibitor of CD11b-mediated adhesion of PMN induced by CXCR1/2 agonists.

3.2. Inhibition of CXCL8-induced PMN activation

Next, we investigated whether repertaxin could exert its inhibitory activity on CXCL8-mediated biological activities other than PMN chemotaxis [14] and adhesion. To this aim, we evaluated the effect of repertaxin on CXCL8-induced CD66b plasma membrane expression, a well-known marker of secondary and tertiary granules release on human PMNs [23]. As shown in Fig. 3A, repertaxin significantly reduced CD66b membrane expression stimulated by CXCL8 (1 nM; 40% and 52% of inhibition in the presence of repertaxin 100 nM and 1 μM, respectively). The effect of repertaxin was independent of CXCL8 concentration (1–10 nM; Fig. 3B and C). As expected, repertaxin (1 μM) also inhibited CXCL6-induced CD66b membrane expression (Fig. 3B). To further demonstrate that the inhibition of CD66b over-expression by repertaxin was paralleled by the reduction of secondary and tertiary granules release, supernatants of CXCL8-stimulated PMN from vehicle or repertaxin-pretreated cells were evaluated in vitro by zymography analysis. MMP-9 is a well-known product of PMN secondary and tertiary granules release [24]. As expected, CXCL8 (1–10 nM) induced the release of MMP-9 and the heterodimer of MMP-9 with lipocalin was also barely visible (Fig. 3D) [25]. PMN pretreatment with repertaxin (1 μM) blocked the release of MMP-9 gelatinase activity induced by CXCL8 (Fig. 3D). In the absence of CXCL8 stimulation, repertaxin alone was unable to modify PMN spontaneous MMP-9 release and CD66b membrane expression.

Moreover, we investigated the effect of repertaxin on CXCL8-primed IL-1β production in response to LPS on human PMNs [9]. First, we evaluated the effect of repertaxin on CXCL8-induced IL-1β mRNA expression. Total RNA was isolated from PMNs, reverse-transcribed into cDNA, and PCR-amplified with specific primers for IL-1β. As shown in Fig. 4A, PMN pretreatment with repertaxin (1 μM) blocked CXCL8-induced IL-1β mRNA expression. Inhibition of CXCL8-stimulated IL-1β mRNA by repertaxin was further confirmed by real-time PCR analysis (Fig. 4B). In addition, repertaxin (1 μM) completely reverted CXCL8 priming for IL-1β production in response to LPS on human PMNs (Fig. 4C). Repertaxin did not affect IL-1β release induced by LPS alone and, as expected [9], CXCL8 alone did not induce IL-1β production.

Finally, we evaluated the effect of repertaxin on PMN phagocytosis. PMN phagocytosis was evaluated in human whole blood by flow cytometric analysis. As shown in Fig. 5 (panels A and C) incubation of whole blood with

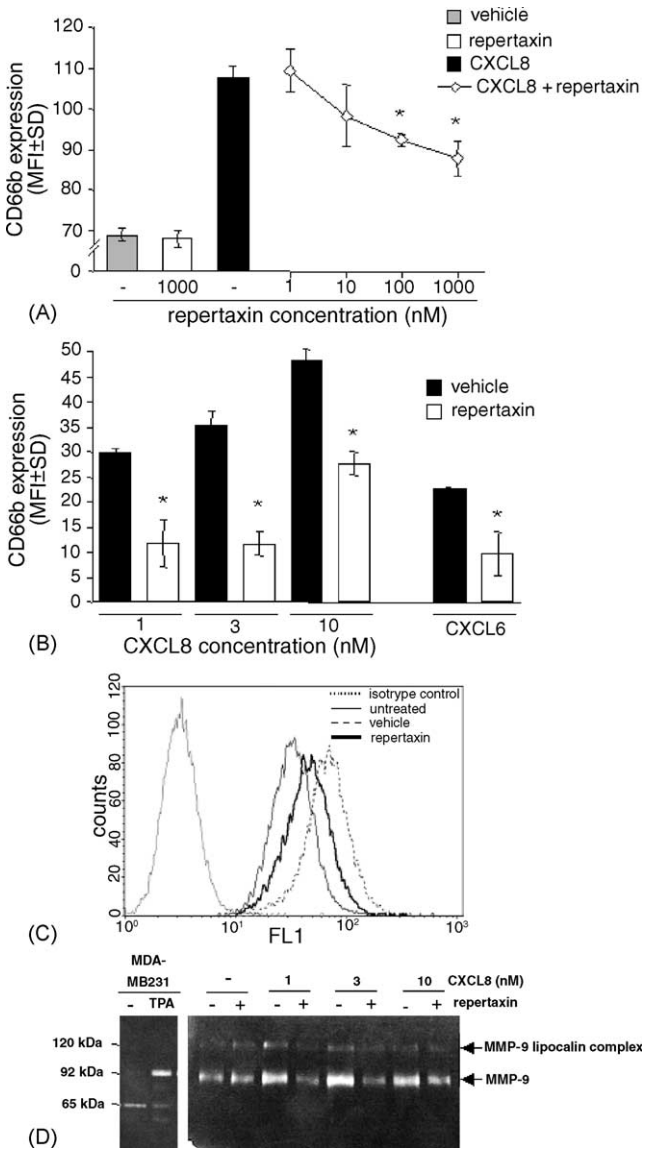

Fig. 3. Effect of repertaxin on CXCL8-induced PMN degranulation. (A) CD66b expression. Whole blood samples were preincubated with vehicle or increasing concentration of repertaxin for 20 min at 37°. PMNs were then stimulated with CXCL8 (1 nM) for 30 min at 37°. CD66b expression was evaluated by flow cytometry as described in Section 2. Data are expressed as mean fluorescence intensity (MFI) ± S.D. of three independent experiments. (B) Whole blood samples were pretreated with vehicle or repertaxin (1 μM) and then stimulated with increasing concentration of CXCL8 (1–10 nM) or CXCL6 (30 nM). Data, subtracted of spontaneous CD66b expression, are expressed as mean fluorescence intensity (MFI) ± S.D. of five independent experiments. Spontaneous CD66b expression was 34 ± 2. Statistical analysis was performed by one-way ANOVA test, followed by Dunnett multiple comparison. $^{*}P < 0.05$ vs. CXCL8 or CXCL6 alone (vehicle pretreated) group. (C) Histograms of CD66b expression on PMNs stimulated with CXCL8 (3 nM) in the presence or absence (vehicle) of repertaxin (1 μM) pretreatment. (D) MMP-9 activity. PMNs were pretreated with vehicle or repertaxin (1 μM) for 20 min at 37° and then stimulated with CXCL8 (1–10 nM). Following incubation for 1 h at 37°, culture supernatants were collected, centrifuged to remove cell debris and analyzed for MMP-9 activity by zymography as described in Section 2. Mix of pro-MMP-9 and pro-MMP-2, present in unconcentrated conditioned media obtained from untreated or TPA (100 nM)-treated MDA-MB231 human breast cancer cell line, were used as standards. Data are from one experiment of three.

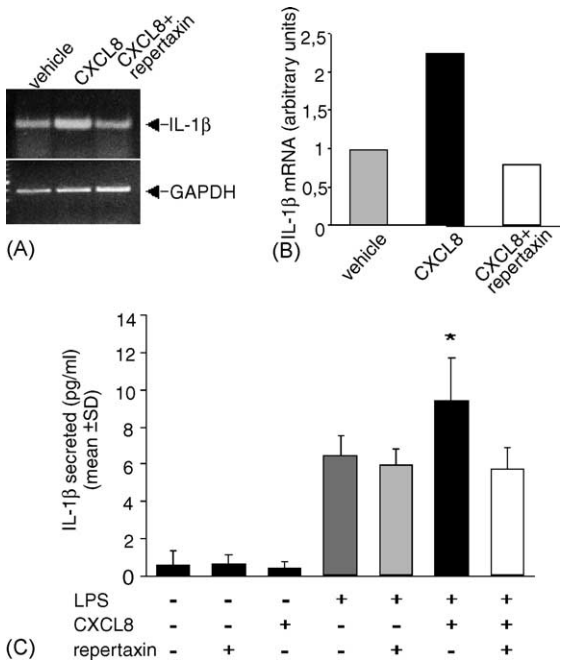

Fig. 4. Effect of repertaxin on CXCL8-primed IL-1β production. (A and B) IL-1β mRNA expression. PMNs were pretreated with vehicle or repertaxin (1 μM) for 30 min at 37° before addition of 10 nM CXCL8. Four hours later, RNA was extracted and IL-1β mRNA was measured by RT-PCR (A) and real-time quantitative PCR (B) analysis as described in Section 2. (C) IL-1β production. PMNs were preincubated with vehicle or repertaxin (1 μM) for 20 min at 37° before addition of LPS (50 ng/ml) or LPS + CXCL8 (10 nM). IL-1β production was measured in cell supernatants 8 h after stimulation as described in Section 2. Data are expressed as mean values ± S.D. of two independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA test, followed by Dunnett multiple comparison: $^{*}P < 0.05$ vs. LPS alone group.

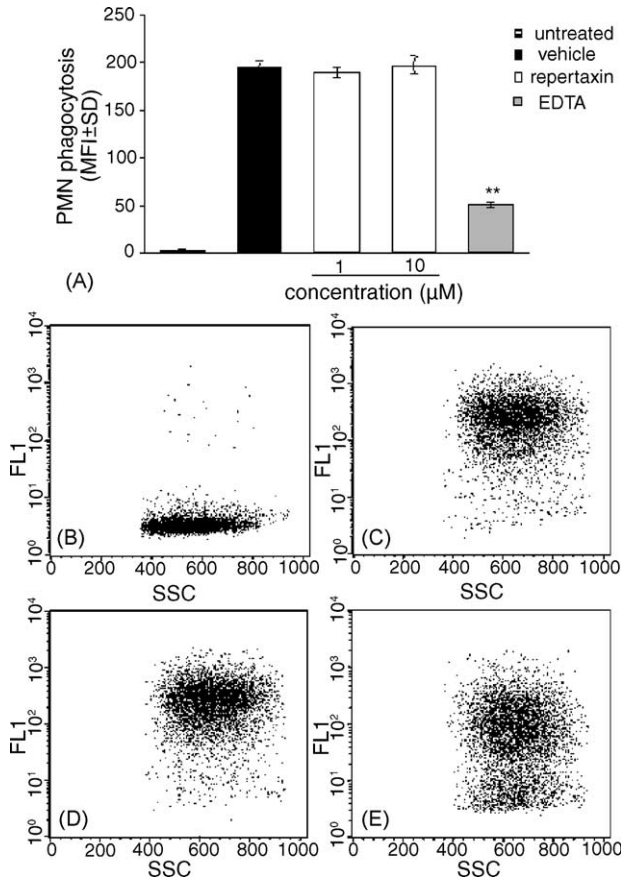

Fig. 5. Effect of repertaxin on PMN phagocytosis. Human whole blood samples were pretreated with vehicle, repertaxin (1 μM or 10 μM) or EDTA (1 mM) for 20 min at room temperature. PMN phagocytosis was induced by addition of fluorescein-labeled opsonized *Escherichia coli* bacteria for 15 min at 37°. PMN phagocytosis was evaluated as described in Section 2. Control (untreated) group was maintained at 4° during the incubation time. Data are expressed as mean fluorescence intensity (MFI) ± S.D. of three independent experiments. (B–E) Representative dot plot analysis of phagocytosis in untreated (panel B) and *Escherichia coli* incubated PMNs in the presence of vehicle (panel C), repertaxin (10 μM; panel D) or EDTA (1 mM; panel E) pretreatment.

fluorescein-labeled opsonized *E. coli* bacteria induced their phagocytosis by PMNs. Pretreatment of whole blood with repertaxin (1 μM and 10 μM) did not affect PMN phagocytosis (Fig. 5; panels A and D). As positive control, inhibition of PMN phagocytosis was obtained by 1 mM EDTA pretreatment (Fig. 5; panels A and E).

3.3. Inhibition of CXCL8-induced T cell chemotaxis

To evaluate whether repertaxin could exert its inhibitory activity on CXCL8-mediated chemotaxis other than PMN chemotaxis, its effect on CXCL8-induced T lymphocyte migration was investigated. The effect of repertaxin was evaluated in the same range of concentrations affecting CXCL8-mediated PMN adhesion (see above; Fig. 1A). Results in Fig. 6A show that repertaxin strongly reduced CXCL8-induced T lymphocyte migration in a concentration-dependent manner. On the other hand, repertaxin (10 nM–1 μM) did not affect CXCL12-induced T lymphocyte migration (Fig. 6B). Finally, we investigated the effect of repertaxin on NK cell migration. As shown in Fig. 6C, preincubation of NK cells with repertaxin (1 μM) blocked NK cell migration in response to CXCL8 (82% of inhibition). On the contrary, repertaxin (1 μM) did not affect CXCL12-induced NK cell migration (Fig. 6C). In the absence of agonist stimulation, repertaxin alone was unable to modify NK cell and T lymphocyte spontaneous migration.

4. Discussion

Repertaxin is the first low molecular weight inhibitor of CXCL8 in clinical studies. This molecule is a potent and specific inhibitor of CXCL8-mediated chemotaxis that binds to CXCR1/2 inducing a conformational constrain in the transmembrane region of the receptor, thus preventing receptor-induced signal transduction [14]. Repertaxin exploits a new concept in the pharmacological inhibition of GPCRs, namely the non-competitive allosteric blocking of receptor activation. Given the unique mode of action of repertaxin it was important to evaluate if beyond chemo-

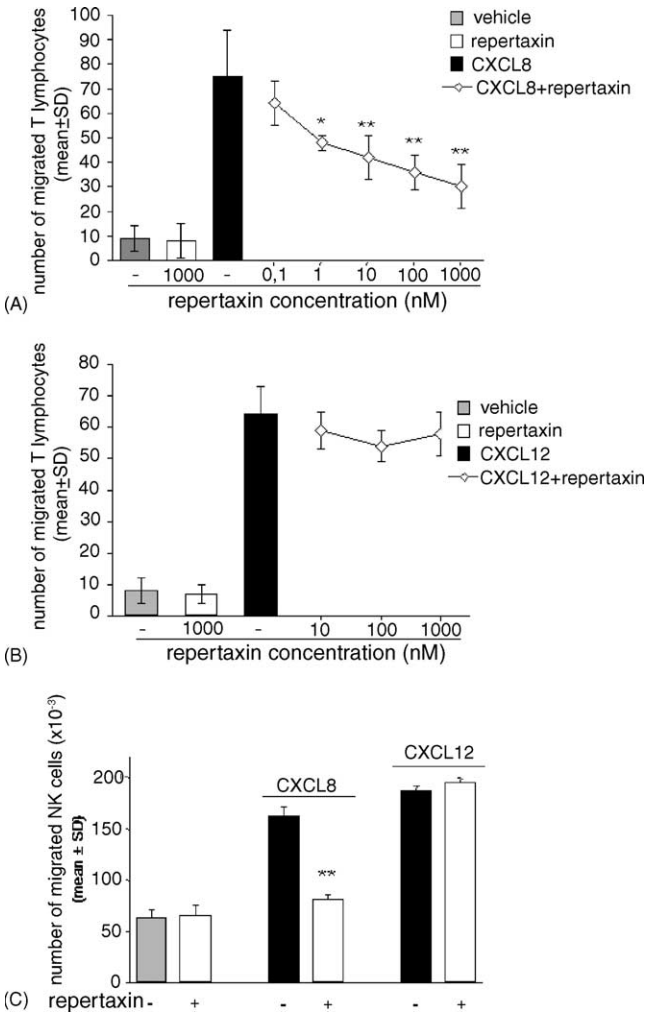

Fig. 6. Effect of repertaxin on T lymphocyte and NK cell migration. (A and B) T lymphocyte migration. Human T lymphocytes were pretreated for 20 min at room temperature with vehicle or increasing concentration of repertaxin. PMNs were then tested for their ability to migrate in response to CXCL8 (1 nM; panel A) or CXCL12 (10 nM; panel B). (C) NK cell migration. Human NK cells were pretreated for 20 min at room temperature with vehicle or repertaxin (1 μM) and then tested for their ability to migrate in response to CXCL8 (10 nM) or CXCL12 (10 nM). T lymphocyte and NK cell migration was determined as described in Section 2. Data are expressed as mean values ± S.D. of three independent experiments. Statistical analysis was performed by one-way ANOVA test, followed by Dunnett multiple comparison. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. CXCL8 or CXCL12 group.

taxis also other biological activities induced by CXCL8 in leukocytes could be inhibited by repertaxin. Our data show that PMN adhesion, release of granule content and pro-inflammatory cytokine production as well as T lymphocyte and NK cell migration induced by CXCL8 are efficiently inhibited by repertaxin.

CXCL8 modulates a wide range of activities in PMNs, including up-regulation of adhesion molecules, rearrangement of cytoskeleton and actin filaments, thus inducing directional movements, and functional activation, such as cytokine production and release of enzymes from primary and secondary granules [1,2]. The coordinated action of CXCL8 in up-regulating both PMN recruitment and release of tissue damaging mediators suggests that CXCL8 may represent a key mediator of acute inflammatory conditions characterized by an acute infiltration of PMNs and tissue damage. In ischemia reperfusion injury, a pathological condition characterized by an early and massive infiltration of PMNs followed by tissue damage of the infiltrated tissue, high molecular weight inhibitors of CXCL8 binding to cellular receptors were efficacious in preventing both PMN recruitment and tissue damage in several organs [26–28]. The finding that several biological activities induced by CXCL8 are inhibited by repertaxin suggests that this molecule might have the potential to block PMN recruitment and activation in inflammation. Accordingly, repertaxin was found to be efficacious in animal models of PMN recruitment and of ischemia reperfusion injury [14]. On the basis of these data, the first clinical indication in which repertaxin is being investigated is the prevention of post-ischemia reperfusion injury in organ transplantation, a clinical condition known as Delayed Graft Function.

Inhibition of CXCL8-induced T lymphocyte and NK cell chemotaxis by repertaxin suggests that the potential therapeutic use of this compound might be extended to the treatment of chronic inflammatory diseases. Indeed, CXCL8 is supposed to be, at least in part, responsible for T lymphocyte recruitment and activation in chronic inflammatory diseases [11]. Moreover, increasing evidences indicate that NK cells are important players in the pathogenesis of different inflammatory and immunological diseases. In particular, it has been demonstrated that NK cells are present in dermis of psoriasis plaques where they secrete CXCL8 and Th1 cytokines such as interferon-γ [29], and thus recruiting additional effector cells, such as PMNs and T lymphocytes, and affecting the progression and evolution of the inflammatory response.

CXCL8 receptors CXCR1 and CXCR2 belong to the GPCR superfamily. Multiple intracellular signal transduction pathways are activated by CXCL8 receptors. G-protein activation is responsible for second messengers induction including phospholipase Cβ and phosphatidylinositol 3-kinase γ activation [8,30–32]. The ability of repertaxin to inhibit multiple biological responses induced by CXCL8 is in keeping with the molecular mechanism of action of this molecule. Indeed, we have previously shown that repertaxin acts as a non-competitive allosteric blocker of CXCR1/2, thus preventing G-protein activation induced by CXCL8 but not by fMLP [14].

The effect of repertaxin on leukocyte function and activation was specific. Indeed, repertaxin blocked PMN activities induced by CXCL8 and CXCL6, two chemokines known to act through the activation of CXCR1/2. On the contrary, we found that G-protein activation and, accordingly, PMN adhesion and T cell chemotaxis induced by other stimuli, namely C5a, fMLP and CXCL12, were not affected by repertaxin. Beyond chemotactic factors, repertaxin does not affect receptor activation induced by

several other agonists of GPCRs, including biogenic amines, whereas other chemokine receptor antagonists have been shown to cross react with biogenic amines [33–35]. Along the same line, we found that phagocytosis of opsonized *E. coli* bacteria was not affected by repertaxin. These data suggest that effector functions of PMNs related to infection control should not be affected by repertaxin. The specificity of action of repertaxin reflects in the high tolerability shown by this molecule in normal volunteers in phase I studies.

In summary, we found that repertaxin, a new small molecule inhibitor of CXCL8 receptor activation, potently and selectively inhibits a wide range of biological activities induced by CXCL8 on human PMNs including adhesion, degranulation and cytokine expression. Repertaxin inhibits PMN adhesion and chemotaxis in the same range of concentrations. Similarly, repertaxin strongly inhibited CXCL8-induced T lymphocyte and NK cell chemotaxis in the same range of concentrations affecting PMN activities. Taken together, these data suggest that beside the proposed clinical indication of repertaxin in the prevention of Delayed Graft Function, the potential therapeutic use of this compound might conceivably be extended to the treatment of chronic inflammatory diseases.

References

[1] Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines – CXC and CC chemokines. Adv Immunol 1994;55:97–179.

[2] Baggiolini M, Loetscher P. Chemokines in inflammation and immunity. Immunol Today 2000;21:418–20.

[3] Taub DD, Anver M, Oppenheim JJ, Longo DL, Murphy WJ. T lymphocyte recruitment by interleukin-8 (IL-8). IL-8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both in vitro and in vivo. J Clin Invest 1996;97:1931–41.

[4] Sebok K, Woodside D, Al-Aoukaty A, Ho AD, Gluck S, Maghazachi AA. IL-8 induces the locomotion of human IL-2-activated natural killer cells. J Immunol 1993;50:1524–34.

[5] Mantovani A. The chemokine system: redundancy for robust out-puts. Immunol Today 1999;20:254–7.

[6] Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity 2000;12:121–7.

[7] Di Cioccio V, Strippoli R, Bizzarri C, Troiani G, Cervellera MN, Gloaguen I, et al. Key role of proline-rich tyrosine kinase 2 in interleukin-8 (CXCL8/IL-8)-mediated human neutrophil chemotaxis. Immunology 2004;111:407–15.

[8] Hirsh E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, et al. Central role for G protein-coupled phosphoinositide 3-kinase γ in inflammation. Science 2000;207:1049–53.

[9] Martinez FO, Sironi M, Vecchi A, Colotta F, Mantovani A, Locati M. IL-8/CXCL8 induces a specific transcriptional profile in human PMN: synergism with LPS for IL-1 production. Eur J Immunol 2004;34:2286–92.

[10] Nickoloff BJ, Karabin GD, Barker JNWN, Griffiths CEM, Sarma V, Mitra RS, et al. Cellular localization of interleukin-8 and its inducer, tumor necrosis factor-α in psoriasis. Am J Pathol 1991;138:129–40.

[11] Bizzarri C, Allegretti M, Di Bitondo R, Cervellera MN, Colotta F, Bertini R. Pharmacological inhibition of interleukin-8 (CXCL8) as a new approach for the prevention and treatment of several human diseases. Curr Med Chem – Anti-Inflammatory Anti-Allergy Agents 2003;2:67–79.

[12] Mahida YR, Ceska M, Effenberg F, Kurlak L, Lindley I, Hawkey CJ. Enhanced synthesis of neutrophil-activating peptide-1/interleukin-8 in active ulcerative colitis. Clin Sci 1992;82:273–5.

[13] Horuk R, Ng HP. Chemokine receptor antagonists. Med Res Rev 2000;20:155–68.

[14] Bertini R, Allegretti M, Bizzarri C, Moriconi A, Locati M, Zampella G, et al. A new class of non-competitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. Proc Natl Acad Sci USA 2004;101:11791–6.

[15] Mainiero F, Gismondi A, Milella M, Morrone S, Palmieri G, Piccoli M, et al. Long term activation of natural killer cells results in modulation of β1 integrin expression and function. J Immunol 1994;152:446–54.

[16] Takami M, Yerry V, Petruzzelli L. Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1. J Immunol 2002;168:4559–66.

[17] Bryan SA, Jose PJ, Topping JR, Wilhelm R, Soderberg C, Kertersz D, et al. Responses of leukocytes to chemokines in whole blood and their antagonism by novel CC-chemokine receptor 3 antagonists. Am J Respir Crit Care Med 2002;165:1602–9.

[18] Wittmann S, Rothe G, Schmitz G, Fröhlich D. Cytokine upregulation of surface antigens correlates to the priming of the neutrophil oxidative burst response. Cytometry 2004;57A:53–62.

[19] Mackay AR, Ballin M, Pelina MD, Farina AR, Nason AM, Hartzler JL, et al. Effect of phorbol ester and cytokines on matrix metalloproteinase and tissue inhibitor of metalloproteinase expression in tumor and normal cell lines. Invasion Metast 1992;12:168–84.

[20] Xu L, Kelvin DJ, Ye GQ, Taub DD, Ben-Baruch A, Oppenheim JJ, et al. Modulation of IL-8 receptor expression on purified human T lymphocytes is associated with changed chemotactic responses to IL-8. J Leukoc Biol 1995;57:335–42.

[21] Gismondi A, Jacobelli J, Strippoli R, Mainiero F, Soriani A, Cifaldi L, et al. Pyk-2 integrates chemokine and integrin receptor signaling to control NK transendothelial migration. J Immunol 2003;170:3065–73.

[22] Berton G, Yan SR, Fumagalli L, Lowell CA. Neutrophil activation by adhesion: mechanisms and pathophysiological implications. Int J Clin Lab Res 1996;26:160–77.

[23] Mollinedo F, Martín-Martín B, Calafat J, Nabokina SM, Lazo PA. Role of vesicle-associated membrane protein-2, through Q-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor/R-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor interaction, in the exocytosis of specific and tertiary granules of human neutrophils. J Immunol 2003;170:1034–42.

[24] Borregaard N, Cowland JB. Granules of human neutrophilic polymorphonuclear leukocyte. Blood 1997;89:3503–21.

[25] Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, et al. Gelatinase B function as regulator and effector in leukocyte biology. J Leukoc Biol 2001;69:851–9.

[26] Sekido N, Mukaida N, Harada A, Nakanishi I, Watanabe Y, Matsushima K. Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. Nature 1993;365:654–7.

[27] Matsumoto T, Ikeda K, Mukaida N, Harada A, Matsumoto Y, Yamashita J, et al. Prevention of cerebral edema and infarct in cerebral reperfusion injury by an antibody to interleukin-8. Lab Invest 1997;77:119–25.

[28] Morita K, Miura M, Paolone DR, Engeman TM, Kapoor A, Remick DG, et al. Early chemokine cascades in murine cardiac grafts regulate T cell recruitment and progression of acute allograft rejection. J Immunol 2001;167:2979–84.

[29] Cameron AL, Kirby B, Fei W, Griffiths CE. Natural killer and natural killer-T cells in psoriasis. Arch Dermatol Res 2002;294:363–9.

[30] Ben-Baruch A, Michiel DF, Oppenheim JJ. Signals and receptors involved in recruitment of inflammatory cells. J Biol Chem 1995;270:11703–6.

[31] Smith RJ, Sam LM, Leach KL, Justen JM. Postreceptor events associated with human neutrophil activation by interleukin-8. J Leukoc Biol 1992;52:17–26.

[32] Naccache PH, Levasseur S, Lachance G, Chakravarti S, Bourgoin SG, McColl SR. Stimulation of human neutrophils by chemotactic factors is associated with the activation of phosphatidylinositol 3-kinase γ. J Biol Chem 2000;275:23636–41.

[33] Tagat JR, McCombie SW, Steensma RW, Lin S, Nazareno DV, Baroudy B, et al. Piperazine-based CCR5 antagonists as HIV-1 inhibitors. I: 2(*S*)-methyl piperazine as a key pharmacophore element. Bioorg Med Chem Lett 2001;11:2143–6.

[34] Mirzadegan T, Diehl F, Ebi B, Bhakta S, Polsky I, McCarley D, et al. Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. J Biol Chem 2000; 275:25562–71.

[35] Hesselgesser J, Ng HP, Liang M, Zheng W, May K, Bauman JG, et al. Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. J Biol Chem 1998;273:15687–92.