Angiopoietin chemotactic activities on neutrophils are regulated by PI-3K activation

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Abstract: Angiopoietins (Ang1 and Ang2) modulate blood vessel integrity during the angiogenic process through the activation of tyrosine kinase receptor (Tie2). We recently detected Tie2 expression on neutrophils and reported that angiopoietins induce acute proinflammatory events including neutrophil β_2-integrin activation and their adhesion onto endothelial cells. Herein, we investigated the effect of angiopoietins on neutrophil migration and their capacity to modulate CXCL8/IL-8 chemotactic properties. Using a Boyden chamber assay, we observed that Ang1 and Ang2 (up to 10 nM; 60 min) increased the migration of neutrophils, and the maximal effect was achieved at 1 nM (72% and 114% increase, respectively) as compared with untreated cells. Angiopoietins induce a rapid and transient Akt phosphorylation, and pretreatment of neutrophils with PI-3K inhibitors, wortmannin (100 nM) and LY294002 (500 nM), reduced Ang1-mediated neutrophil migration by 100% and 78% and Ang2 chemotactic activity by 100% and 71%, respectively. Treatment of neutrophils with CXCL8/IL-8 (up to 50 nM; 60 min) increased basal neutrophil migration by 257% at its optimal concentration (10 nM), and pretreatment of neutrophils with corresponding PI-3K inhibitors reduced CXCL8/IL-8 (1 nM) chemotactic effect. Pretreatment of neutrophils with Ang1 or Ang2 (10 nM; 15 min) potentiated neutrophil migration induced by CXCL8/IL-8 (1 or 10 nM; 60 min) by 263% and 238% and by 177% and 164%, respectively. Finally, both angiopoietins showed a synergistic effect on the induction of Akt phosphorylation mediated by CXCL8/IL-8. In summary, our data demonstrate that angiopoietins increase neutrophil migration through PI-3K activation and can enhance proinflammatory activities of other cytokines. *J. Leukoc. Biol.* 81: 1093–1101; 2007.

Key Words: *cytokines · polymorphonuclear cells · migration · Tie2 receptor*

INTRODUCTION

Angiogenesis is characterized by the formation of new blood vessels from a pre-existing vasculature. This process is tightly regulated and requires the coordinated action of numerous growth factors, including vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2) are structurally related endothelial growth factors, having similar binding affinity for tyrosine kinase receptor (Tie2). Ang1 has been shown to act as a Tie2 agonist, promoting endothelial cell (EC) survival, migration, and proliferation [1, 2]. Conversely, Ang2 has been characterized initially as an endogenous Tie2 antagonist, thereby counteracting Ang1 activities [1, 3]. However, recent evidences suggest that Ang2, under certain circumstances, has a more complex role and may act as a Tie2 agonist. This hypothesis is supported by the capacity of Ang2 to induce Tie2 phosphorylation, EC chemotaxis, and in vitro capillary-like tube formation [4–6]. Furthermore, we reported that Ang1 and Ang2, upon endothelial Tie2 activation, can induce platelet-activating factor (PAF) synthesis and transient P-selectin translocation, which support neutrophil adhesion onto activated EC [7, 8]. Recent studies revealed that angiopoietins can also modulate the responsiveness of EC toward proinflammatory cytokines. For instance, Ang2 was shown to serve as a priming factor for TNF-α at low concentrations [9]. Moreover, Ang1 and Ang2 were shown to potentiate VEGF-mediated angiogenesis in a mouse cornea model [10].

Postnatal angiogenesis is associated with numerous inflammatory conditions such as atherosclerosis, rhumatoïd arthritis, retinopathy, and tumor growth [11]. It has been reported that inflammation precedes and accompanies pathological angiogenesis, as evidenced by increased vascular permeability and recruitment of inflammatory cells at angiogenic sites [12, 13].

Among inflammatory cells, neutrophils are the first ones recruited to the site of inflammation and have been suggested to participate in pathological angiogenesis [14, 15]. Neutrophils produce several proangiogenic cytokines, including VEGF, TNF-α, and ILs (IL-1, IL-6, and IL-8), as well as matrix-degrading enzymes, which together, contribute to trigger and support angiogenic activities. Recruitment of neutrophils is a multistep process implying neutrophil tethering, rolling, and firm adhesion to EC, followed by the migration of

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neutrophils across the vasculature toward the subendothelial chemoattractant source.

Recently, we detected Tie2 expression on the cell surface of polymorphonuclear cells, which has been confirmed later by independent groups [7, 16, 17]. Stimulation of neutrophils with Ang1 or Ang2 induces PAF synthesis and β_2-integrin-dependent neutrophil adhesion to extracellular matrix (ECM) [7, 8]. Taken together, these observations suggest that the angiopoietin/Tie2 system can modulate leukocyte trafficking. In agreement, recent studies showed that angiopoietins can induce neutrophil and eosinophil chemotaxis, an important component of the acute inflammatory response [16, 17]. However, little is known about the cellular pathways involved in angiopoietin-mediated neutrophil migration. In the present study, we thus wanted to define the cellular pathways involved in angiopoietin-mediated neutrophil migration. In addition, as angiopoietins can modulate the effect of proinflammatory cytokines in EC, we were led to investigate if angiopoietins could modulate cytokine activities in neutrophils.

MATERIALS AND METHODS

Neutrophil purification

Venous blood was obtained from healthy donors free from medication for at least 10 days prior to the experiments. Neutrophils were isolated as described previously [7]. Briefly, upon a 2% dextran sedimentation, neutrophils were isolated under sterile conditions using the Ficoll-Paque gradient, and contaminating erythrocytes were removed by hypotonic shock. Unless otherwise noted, neutrophils were resuspended in RPMI containing 25 mM HEPES and 1% of antibiotics. Ninety-five percent of the isolated cells were polymorphonuclear cells, as determined with a Coulter counter, and viability was found to be greater than 98%, as assessed by trypan blue dye exclusion assay. To characterize the ratio of neutrophils and eosinophils in our polymorphonuclear population, cells were centrifuged, spread onto microscope slides, and stained with Diff-Quick solution. Only the populations of polymorphonuclear cells consisting of at least 95% of neutrophils were used in our study.

Cell-surface expression of Tie2

Neutrophils (10^7 cells/ml) were rinsed and resuspended in RPMI, and bovine IgG (150 μg/ml) was added for 30 min to prevent nonspecific binding via FcRs. Neutrophils were centrifuged, rinsed, resuspended in RPMI (10^6 cells/ml), and incubated with mouse monoclonal Tie2 IgG_1 (1–10 μg/ml, R&D Systems, Minneapolis, MN, USA) or with mouse IgG_1 isotype control (1–10 μg/ml, R&D Systems) for 60 min at 4°C. Upon additional rinses, neutrophils were incubated with goat antimouse, FITC-conjugated, secondary IgG (1:100). Cells were rinsed and fixed with 0.5% paraformaldehyde. Flow cytometric analysis (10,000 events) was performed using a FACScan (Becton Dickinson, San Jose, CA, USA).

Neutrophil migration

In vitro chemotaxis was performed in a 48-well microchamber (Neuro Probe Inc., Gaithersburg, MD, USA) using a 3-μm polycarbonate membrane filter as described previously [18, 19]. The bottom wells were loaded with RPMI, with or without agonists (final volume, 25 μl), the polycarbonate membrane was placed over the wells, and the top layer of the chamber was added over the membrane. Neutrophils (50 μl from a RPMI suspension; 10^6 cells/ml) were added into the upper wells. The chamber was incubated at 37°C for 60 min in a humidified incubator in the presence of 5% CO_2. Upon the incubation period, the top of the chamber was removed, and the upper side of the membrane was wiped carefully with the rubber scraper provided by the manufacturer. The polycarbonate membrane was fixed in methanol, colored with the Diff-Quick staining kit, mounted on a glass slide, and examined with a light microscope under 400× magnification. The number of cells in five random fields was counted, and the results were expressed as relative neutrophil migration (number of cells from tested group/number of cells from corresponding control vehicles). Treatment with the chemoattractant CXCL8/IL-8 (25 nM) was used as a positive control.

In another set of experiments, cells were pretreated for 15 min with a blocking goat polyclonal antihuman Tie2 (anti-hTie2) IgG (R&D Systems) or isotypic goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) before cell migration toward angiopoietins (1 nM). In some experiments, cells were pretreated for 15 min with PI-3K inhibitors (wortmannin, 10 and 100 nM [20]; LY294002, 0.5 or 5 μM [21]), p38 MAPK (SB203580, 10 μM [22]), p42/44 MAPK (PD98059, 50 μM [23]), or angiopoietins (0.1–10 nM) prior to neutrophil migration toward agonists. In studies implying the use of inhibitors, the latter was also added into the bottom wells.

Akt Western blot analyses

Neutrophils were resuspended at a concentration of 4×10^7 cells/ml, and 100 μl cell suspension was stimulated with control vehicle, angiopoietins, CXCL8/IL-8, and angiopoietins plus CXCL8/IL-8 at room temperature. Neutrophils were lysed in 4× Laemmli's buffer and boiled for 10 min. An equal volume of samples was loaded in a 7.5% SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, and membranes were probed with a rabbit polyclonal phospho-Akt (Ser^{473}) IgG (1:750, Cell Signaling Technology Inc., Beverly, MA, USA). PVDF membranes were subsequently stripped in glycine stripping buffer, and total Akt protein expression was determined with a rabbit polyclonal anti-Akt IgG (1:1000). Immunoreactive bands were visualized by using LumiGlo™ (Cell Signaling Technology Inc.), digitized using a two-dimensional gel scanner, and quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are mean ± SEM. Statistical comparisons were made by one-way ANOVA, followed by a Tukey *t*-test for multiple comparisons. Data were considered significantly different if values of $P < 0.05$ were observed.

RESULTS

Expression of Tie2 in neutrophils

We recently reported by RT-PCR, confocal microscopy, and immunocytochemistry that Tie2 is expressed at the cell surface of neutrophils, and others demonstrated by RT-PCR and by flow cytometric analysis that Tie2 is expressed on eosinophils [7, 16, 17]. To confirm that the detection of Tie2 on neutrophils in our initial study was not a result of a marginal cross-contamination by eosinophils, we performed a FACS analysis directed against Tie2 expression in a population of purified polymorphonuclear cells containing at least 95% of neutrophils (**Fig. 1**).

Ang1 and Ang2 mediate neutrophil migration

Using a modified Boyden chamber assay, we tested the capacity of angiopoietins to modulate neutrophil migration. Ang1 and Ang2 increased concentration-dependently the migration of neutrophils in a bell-shaped manner. The maximal effect mediated by Ang1 and Ang2 was achieved at 1 nM (72% and 114% increase, respectively) as compared with PBS-treated cells (**Fig. 2A**), whereas at 5 and 10 nM, the effect of both angiopoietins resumed to basal level. To assess if the migration of neutrophils mediated by Ang1 and Ang2 were Tie2-dependent, we pretreated the neutrophils with different concentrations of anti-hTie2 IgG (5 and 10 μg/ml; 15 min) capable of blocking the angiopoietin/Tie2 interaction. Such pretreatment prevented neutrophil migration induced by Ang1 or Ang2 (1 nM), whereas a pretreatment with equivalent concentrations of

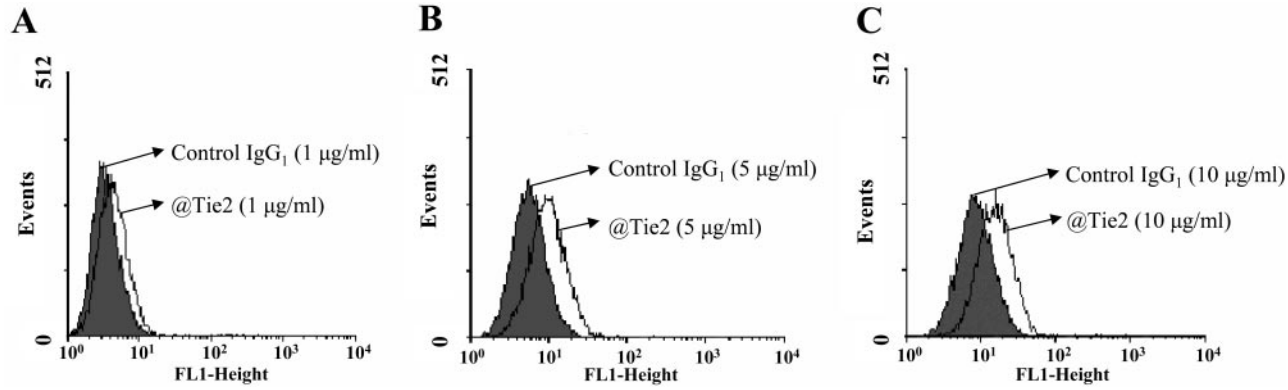

Fig. 1. Detection of Tie2 expression on neutrophil cell surface by FACScan analysis. Neutrophils were incubated with mouse monoclonal Tie2 IgG_1 (@Tie2; 1, 5, or 10 μg/ml) or with control mouse isotypic IgG_1 (Control IgG_1; 1, 5, or 10 μg/ml). Neutrophils were then incubated with a secondary, goat antimouse, FITC-conjugated IgG (1:100). Flow cytometric analysis (10,000 events) was performed using a FACScan. Representative analysis of three independent experiments.

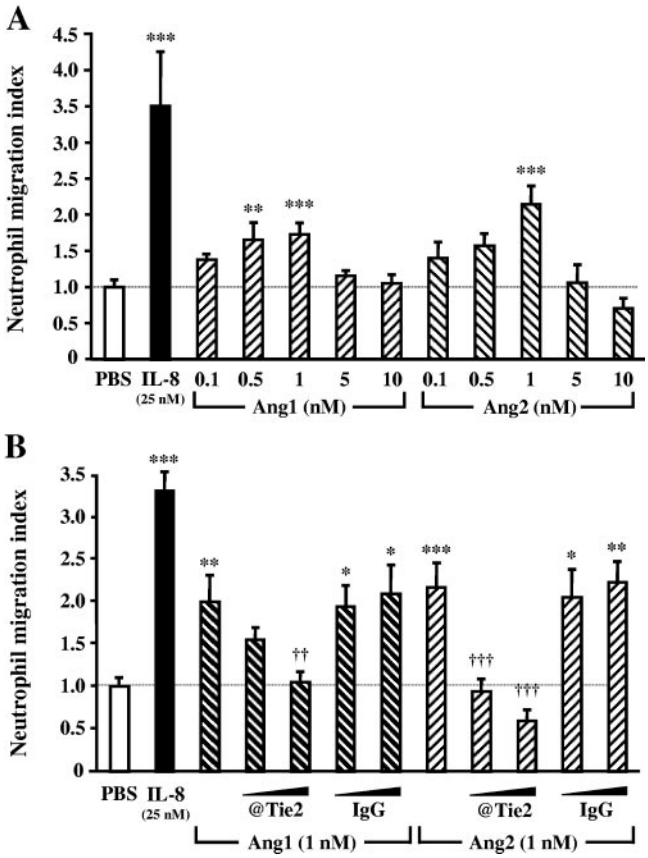

Fig. 2. Ang1 and Ang2 mediate neutrophil migration through Tie2 activation. Neutrophils were added to the upper wells of a modified Boyden chamber apparatus, and the lower wells were filled with RPMI ± PBS, Ang1, or Ang2 (0.1–10 nM; A). In another series of experiments, neutrophils were pretreated with a blocking goat anti-hTie2 IgG (@Tie2; 5 or 10 μg/ml) or control goat isotypic IgG (IgG; 5 or 10 μg/ml) for 15 min prior to the addition of neutrophils in the upper wells of a modified Boyden chamber apparatus in which the lower wells were filled with RPMI ± PBS, Ang1, or Ang2 (1 nM; B). Treatment with CXCL8/IL-8 (25 nM; lower wells) was used as a positive control. Migrating cells were fixed, stained, and counted under light microscopy at 400× original magnification. Values are means ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with PBS. ††, $P < 0.01$; †††, $P < 0.001$, as compared with corresponding agonist.

control isotypic IgG had no such effect (Fig. 2B). In all studies, treatments with CXCL8/IL-8 (10 or 25 nM) were included as a positive control, increasing neutrophil migration by over 200% as compared with PBS-treated cells.

Role of the PI-3K pathway in angiopoietin-mediated neutrophil migration

Previous studies reported that angiopoietins possess the capacity to activate PI-3K/Akt, p38, and p42/44 MAPK pathways [4, 8, 24–26]. Thus, we wanted to address the signaling pathway(s) involved in neutrophil migration mediated by angiopoietins. Neutrophils were pretreated with inhibitors of PI-3K (wortmannin and LY294002), p38 MAPK (SB203580), and p42/44 MAPK (PD98059) pathways. Pretreatment of neutrophils with wortmannin (10 and 100 nM) for 15 min prior to their addition in the upper chamber did not affect basal migration mediated under PBS condition but prevented by over 90% the migration of neutrophils mediated by Ang1 and Ang2 (1 nM; 60 min; **Fig. 3A**). The contribution of the PI-3K pathway was confirmed by using a second independent inhibitor LY294002. Pretreatment of neutrophils with LY294002 (0.5 and 5 μM) reduced basal PBS-mediated migration by 40% and 53%, respectively. Pretreatment of neutrophils with LY294002 (0.5 and 5 μM) decreased by 72% and 78% the migration of neutrophil-mediated by Ang1 (1 nM) and by 80% and 71% under Ang2 (1 nM) stimulation, respectively, as compared with PBS-treated cells in the presence of LY294002 (0.5 and 5 μM; Fig. 3A). In another set of experiments, neutrophils were pretreated with PD98059 (50 μM) or SB203580 (10 μM) as defined above, and both did not affect neutrophil migration induced by Ang1 or Ang2 (1 nM; data not shown).

As the migration of neutrophils induced by angiopoietins was sensitive to PI-3K inhibitors, we therefore tested the capacity of angiopoietins to increase Akt phosphorylation in neutrophils. Treatment of neutrophils with Ang1 and Ang2 (1 nM) induced a rapid and transient Akt phosphorylation, which was maximal within 2 min (3.0- and 2.8-fold of increase, respectively), as compared with control, PBS-treated cells, and returning to basal level within 30 min (Fig. 3, B and C).

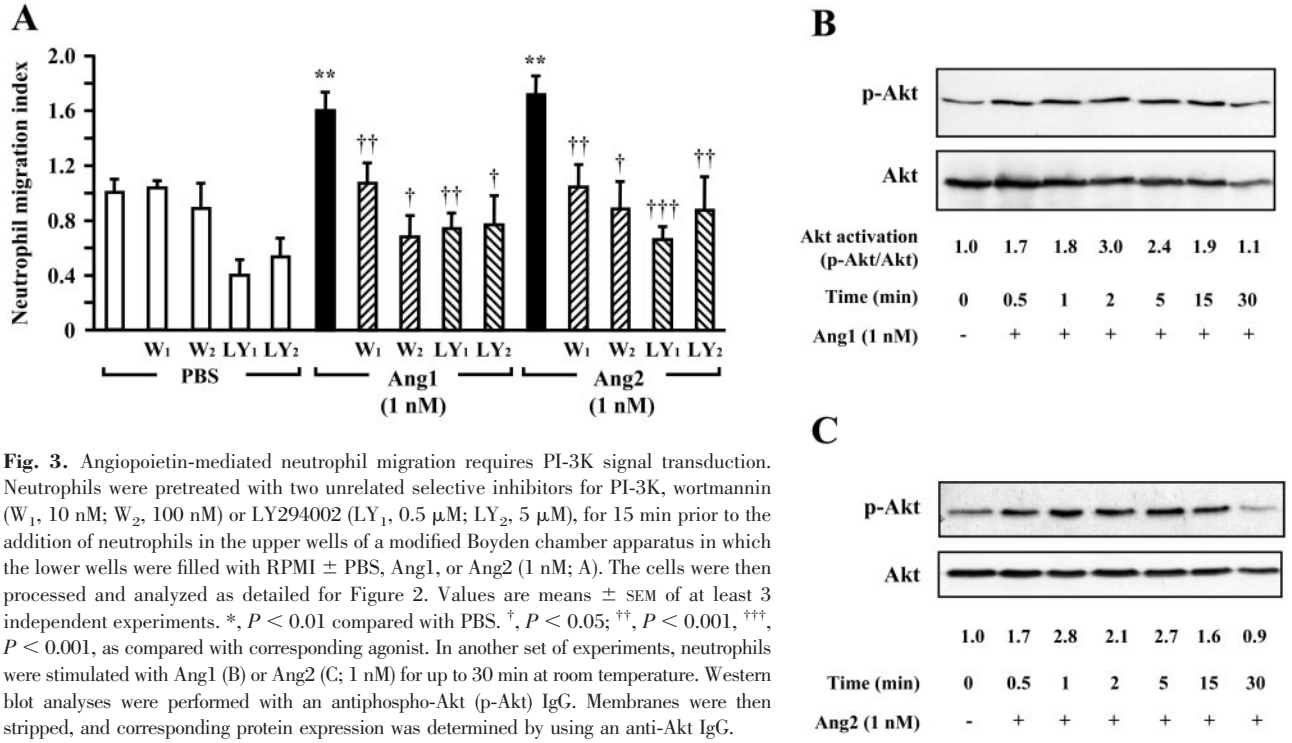

Fig. 3. Angiopoietin-mediated neutrophil migration requires PI-3K signal transduction. Neutrophils were pretreated with two unrelated selective inhibitors for PI-3K, wortmannin (W_1, 10 nM; W_2, 100 nM) or LY294002 (LY_1, 0.5 μM; LY_2, 5 μM), for 15 min prior to the addition of neutrophils in the upper wells of a modified Boyden chamber apparatus in which the lower wells were filled with RPMI ± PBS, Ang1, or Ang2 (1 nM; A). The cells were then processed and analyzed as detailed for Figure 2. Values are means ± SEM of at least 3 independent experiments. *, $P < 0.01$ compared with PBS. †, $P < 0.05$; ††, $P < 0.001$, †††, $P < 0.001$, as compared with corresponding agonist. In another set of experiments, neutrophils were stimulated with Ang1 (B) or Ang2 (C; 1 nM) for up to 30 min at room temperature. Western blot analyses were performed with an antiphospho-Akt (p-Akt) IgG. Membranes were then stripped, and corresponding protein expression was determined by using an anti-Akt IgG.

Enhancing activity of angiopoietins on CXCL8/IL-8-mediated neutrophil migration

A recent report revealed that angiopoietins can modulate EC responsiveness to proinflammatory cytokines [9]. Different studies provided evidence that PI-3K activation contributes to CXCL8/IL-8 chemotactic properties [22, 27]. Our data suggest as well that PI-3K activation contributes to angiopoietin chemotactic activity on neutrophils; thus, we wanted to assess if angiopoietins can modulate CXCL8/IL-8-mediated neutrophil migration. In a first series of experiments, we performed a CXCL8/IL-8 concentration-dependent assay (0.001–50 nM) on neutrophil migration and assessed the extent of PI-3K contribution. CXCL8/IL-8 induced a significant neutrophil migration at 1, 10, and 50 nM, and a maximal effect was achieved at 10 nM (257% increase as compared with PBS-treated cells; **Fig. 4A**). Pretreatment of neutrophils with wortmannin (10 and 100 nM) or LY294002 (0.5 and 5 μM) for 15 min prevented neutrophil migration induced by CXCL8/IL-8 (1 nM), whereas at a higher concentration of CXCL8/IL-8 (10 nM), wortmannin (10 and 100 nM) or LY294002 (0.5 and 5 μM) provided a partial inhibitory effect (52–59% inhibiton) on neutrophil migration (Fig. 4B). In another set of experiments, neutrophils were pretreated with PD98059 (50 μM) or SB203580 (10 μM), which did not affect neutrophil migration induced by CXCL8/IL-8 (10 nM; data not shown).

As the migration of neutrophils induced by CXCL8/IL-8 was sensitive to PI-3K inhibitors, we therefore tested the capacity of CXCL8/IL-8 to increase Akt phosphorylation in neutrophils, which were treated with CXCL8/IL-8 (0.001–50 nM) for 2 min [22, 28], and we observed Akt phosphorylation at 10 and 50 nM—11.9- and 15.6-fold of increase, respectively—as compared with control, PBS-treated cells (Fig. 4C). Pretreatment of neutrophils with increasing concentrations of PI-3K inhibitors (wortmannin, 10 and 100 nM; LY294002, 0.5 and 5 μM) for 15 min prior to stimulation with CXCL8/IL-8 (10 nM; 2 min) reduced Akt phosphorylation, and maximal inhibition reached 80% and 99% under wortmannin and LY294002 pretreatment, respectively (Fig. 4D).

We then assessed the capacity of angiopoietins to modulate CXCL8/IL-8 chemotactic activities on neutrophils. Pretreatment of neutrophils with Ang1 or Ang2 (0.1, 1, and 10 nM) for 15 min prior to the addition of neutrophils in the upper wells with their corresponding Ang1- or Ang2-conditioned media did not modulate basal neutrophil migration toward control PBS (lower wells). However, pretreatment of neutrophils with Ang1 (10 nM) under the same condition potentiated neutrophil migration toward a CXCL8/IL-8 concentration gradient (0.1, 1, or 10 nM) for 60 min. For instance, addition of CXCL8/IL-8 (0.1, 1, or 10 nM) alone in the lower wells increased neutrophil migration by 35%, 101%, and 286%, and the pretreatment of neutrophils with Ang1 (10 nM) prior to the addition of CXCL8/IL-8 (0.1, 1, or 10 nM) in the lower wells raised the migration of neutrophils to 154%, 266%, and 507%, respectively (**Fig. 5A**). Similarly, in another series of experiments, basal migration of neutrophils under CXCL8/IL-8 (0.1, 1, or 10 nM) exposure alone increased by 27%, 63%, and 222%, respectively, and a pretreatment of neutrophils with Ang2 increased those mediated toward CXCL8/IL-8 (0.1, 1, or 10 nM) by 41%, 150%, and 363%, respectively (Fig. 5B).

As angiopoietins and CXCL8/IL-8 increase neutrophil migration through the activation of a PI-3K pathway, we hypothesized that it could be implicated in the priming effect of angiopoietins on CXCL8/IL-8-mediated neutrophil migration. To address this issue, neutrophils were pretreated initially for 15 min with wortmannin (100 nM) or LY294002 (5 μM) prior to the addition of PBS, Ang1, or Ang2 (10 nM) for an additional

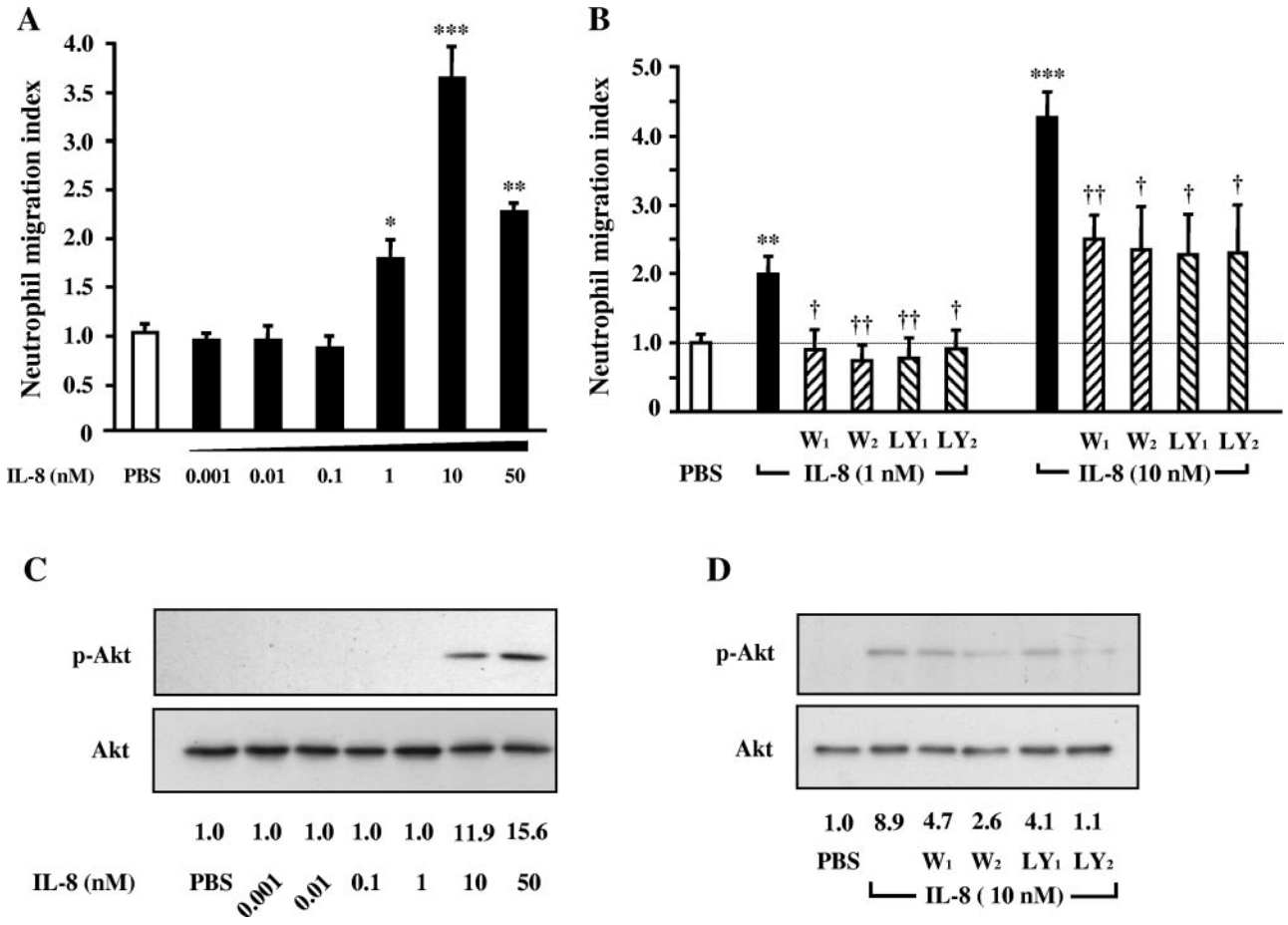

Fig. 4. CXCL8/IL-8-mediated neutrophil migration requires PI-3K/Akt signal transduction. Neutrophils were added to the upper wells of a modified Boyden chamber; the lower wells were filled with RPMI ± PBS or CXCL8/IL-8 (0.001–50 nM; A). In a second set of experiments, neutrophils were pretreated with wortmannin (W_1, 10 nM; W_2, 100 nM) or LY294002 (LY_1, 0.5 μM; LY_2, 5 μM) for 15 min prior to the addition of neutrophils in the upper wells, and the lower wells were filled with RPMI ± PBS or CXCL8/IL-8 (1 or 10 nM; B). The cells were then processed and analyzed as detailed for Figure 2. Values are means SEM of at least 3 independent experiments. *, $P > 0.05$; **, $P < 0.01$; ***, $P > 0.001$, compared with PBS. †, $P < 0.05$; ††, $P < 0.01$, as compared with CSCCL8/IL-8. In another set of experiments, neutrophils were stimulated with PBS or CXCL8/IL-8 (0.001–50 nM) for 2 min (C), and neutrophils were also pretreated with wortmannin (W_1, 10 nM; W_2, 100 nM) or LY294002 (LY_1, 0.5 μM; LY_2, 5 μM) for 15 min prior to stimulation with PBS or CXCL8/IL-8 (10 nM; 2 min; D). Western blot analyses of Akt activation were performed as detailed for Figure 3.

15-min exposure. Then, neutrophils were added to the upper chambers allowing them to migrate toward the CXCL8/IL-8 (10 nM) gradient for 60 min.

In the current set of experiments, pretreatment of neutrophils with wortmannin and LY294002 partially blocked (50% and 51%, respectively) CXCL8/IL-8-mediated neutrophil migration. If the priming effect of angiopoietins on CXCL8/IL-8-mediated neutrophil migration is PI-3K-dependent, thus the inhibition of the angiopoietin priming effect by PI-3K inhibitors should decrease the migration of neutrophils to the level mediated by CXCL8/IL-8 in the presence of PI-3K inhibitors (**Fig. 6A**, dotted line). Pretreatment of neutrophils with wortmannin or LY294002 decreased by 70% and 79% the priming effect of Ang1 and by 88% and 82% the priming effect of Ang2 on CXCL8/IL-8-mediated neutrophil migration (Fig. 6A).

As the activation of the PI-3K pathway is involved in the angiopoietin priming effect on CXCL8/IL-8-mediated neutrophil migration, we then tested the capacity of angiopoietins to potentiate CXCL8/IL-8-mediated Akt phosphorylation. Treatment of neutrophils with CXCL8/IL-8 (0.1 and 1 nM; 2 min) did not mediate Akt phosphorylation, whereas a treatment with CXCL8/IL-8 (10 nM) induced Akt phosphorylation (28-fold increase) as compared with PBS-treated cells (Fig. 6, B and C). A combination of Ang1 (0.1, 1, and 10 nM) to suboptimal CXCL8/IL-8 (1 nM) concentration potentiated Akt phosphorylation by 2.1-, 3.9-, and 7.0-fold, respectively, as compared with CXCL8/IL-8 alone (1 nM; Fig. 6B). Similarly, cotreatment of neutrophils with increasing concentrations of Ang2 (0.1, 1, and 10 nM) plus CXCL8/IL-8 (1 nM) potentiated as well by 1.3-, 5.1-, and 4.8-fold, respectively, the phosphorylation of Akt mediated by CXCL8/IL-8. Cotreatment of neutrophils with Ang1 or Ang2 (0.1, 1, and 10 nM) did not provide a synergistic effect on Akt phosphorylation mediated by the lowest and the highest concentrations of CXCL8/IL-8 (0.1 and 10 nM; Fig. 6, B and C).

DISCUSSION

In the present study, we showed that Ang1 and Ang2 possess a similar agonistic capacity to mediate neutrophil migration

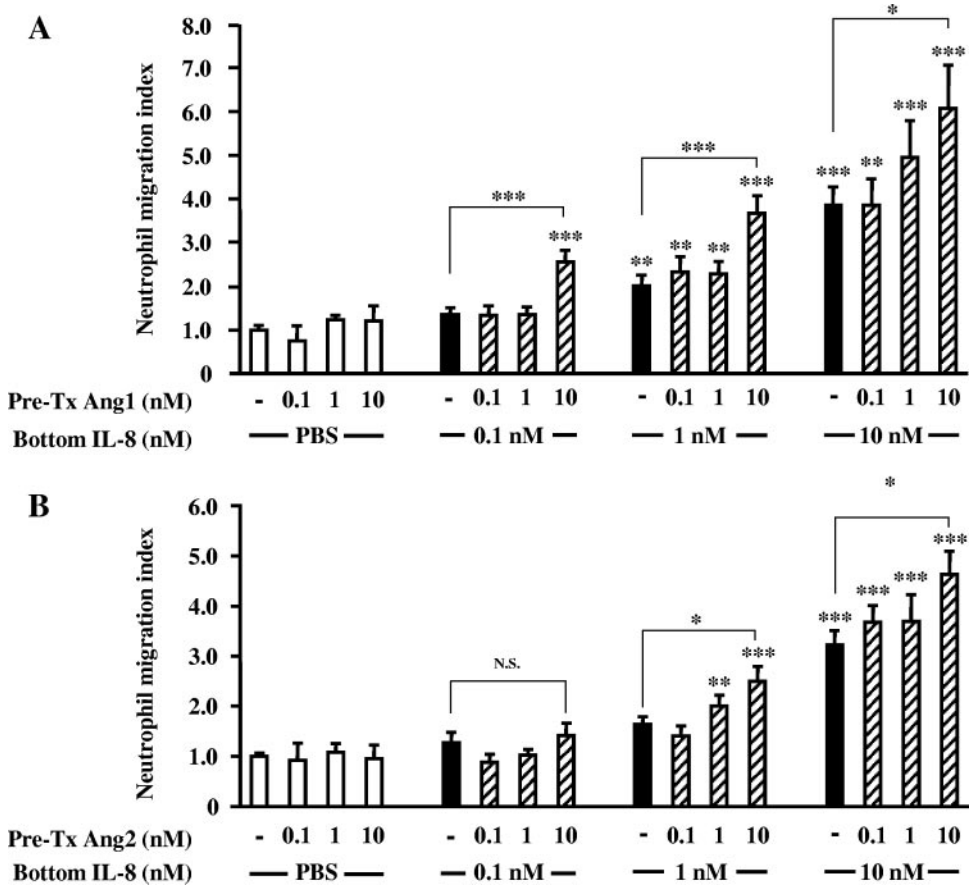

Fig. 5. Angiopoietins enhance CXCL8/IL-8-mediated neutrophil migration. Neutrophils were pretreated (Pre-Tx) with Ang1 (A) or Ang2 (B; 0.1, 1, or 10 nM) for 15 min prior to the addition of neutrophils in the presence of corresponding Ang1- or Ang2-conditioned media in the upper wells of a modified Boyden chamber apparatus in which the lower wells (Bottom IL-8) were filled with RPMI ± PBS or CXCL8/IL-8 (0.1, 1. or 10 nM). Following a 60-min incubation period at 37°C, the cells were then processed and analyzed as detailed for Figure 2. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with PBS or CXCL8/IL-8 (0.1, 1, or 10 nM). N.S., Not significant.

through the activation of the PI-3K pathway. These data are in accordance with our previous findings in which we defined proinflammatory activities of angiopoietins in neutrophils. Furthermore, we have observed that both angiopoietins can potentiate the effect of CXCL8/IL-8 on neutrophil migration.

Ang1 and Ang2 promote neutrophil migration through Tie2 activation

Classically, Tie2 was recognized as being expressed on endothelial and hematopoietic stem cells [29]. However, we recently detected Tie2 expression on neutrophils by RT-PCR, confocal microscopy, and immunocytochemistry and reported the capacity of Ang1 and Ang2 to induce PAF synthesis, β_2-integrin functional up-regulation, and neutrophil adhesion onto ECM and EC [7]. During the same time, Tie2 receptor expression was observed on eosinophils and neutrophils by RT-PCR and flow cytometry [16, 17]. In eosinophils, Feistritzer et al. [16] observed that Ang1 has a chemotactic activity, which is partially driven through PI-3K activation, whereas Ang2 has no or a marginal effect. In neutrophils, Sturn et al. [17] reported that both angiopoietins promote chemotaxis; however, they did not address the contribution of the PI-3K pathway. By FACS analysis, we confirmed our initial data demonstrating Tie2 expression on neutrophils [7], which is in agreement with the observation made by Sturn and colleagues [17].

Using a modified Boyden chamber assay, we demonstrate that angiopoietins increase concentration-dependently the migration of neutrophils in a bell-shaped manner. Maximal agonistic activity is observed at 1 nM, whereas at a higher concentration (10 nM), Ang1 and Ang2 lose their capacity to promote neutrophil migration. This latter observation is in line with previous studies, where we observed a similar bell-shaped response, namely VEGF-A_{165}- and angiopoietin-mediated endothelial P-selectin translocation, angiopoietin-mediated PAF synthesis [7, 8]. This phenomenon can be explained by the fact that the binding of a ligand to a receptor tyrosine kinase induces receptor hetero- and homodimerization, which is essential to trigger receptor autophosphorylation and signal transduction [8, 30]. However, an overabundance of ligands impedes receptor dimerization [31]. In addition, by using an antibody capable of blocking the angiopoietin/Tie2 interaction, we demonstrate that neutrophil migration mediated by both angiopoietins is Tie2-dependent.

The PI-3K activation pathway is essential to angiopoietin-mediated neutrophil migration

In EC, the Ang1/Tie2 interaction activates PI-3K/Akt, p42/44, and p38 MAPK pathways [25, 32–37], and Ang2/Tie2 interaction mediates PI-3K/Akt signal transduction [4]. More recently, we observed that Ang2 is also capable of activating p38 and p42/44 MAPK pathways with a similar kinetic as induced by Ang1 [8]. Furthermore, we reported that both angiopoietins promote a similar endothelial PAF synthesis, and the aforementioned intracellular pathways are contributing to angiopoietin-mediated PAF synthesis [8].

In the current study, we observed that a pretreatment with two PI-3K inhibitors prevented angiopoietin-mediated neutrophil migration, whereas the blockade of p38 and p42/44 MAPK

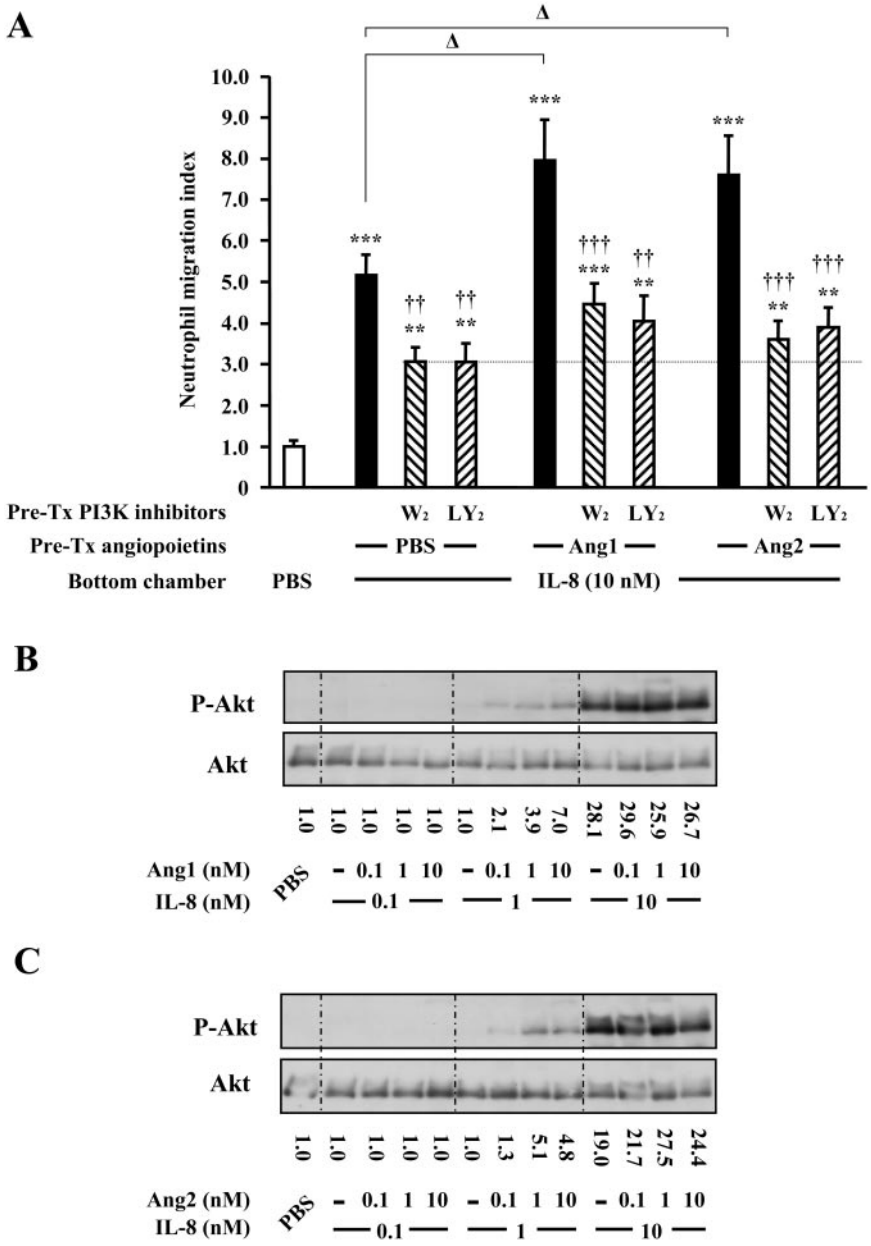

Fig. 6. Angiopoietins potentiate CXCL8/IL-8-mediated neutrophil migration through PI-3K/Akt activation pathway. Neutrophils were pretreated initially with wortmannin (W_2, 100 nM) or LY294002 (LY_2, 5 μM) for 15 min, which was followed by the addition of PBS, Ang1, or Ang2 (10 nM) for an additional 15 min of pretreatment. Neutrophils were then added to the upper wells of a modified Boyden chamber apparatus, and the lower wells were filled with RPMI ± PBS or CXCL8/IL-8 (10 nM; A). Following a 60-min incubation period at 37°C, the cells were then processed and analyzed as detailed for Figure 2. **, $P < 0.01$; ***, $P > 0.001$, compared with PBS; ††, $P < 0.01$; †††, $P > 0.001$, compared with CXCL8/IL-8 (10 nM) ± PBS, Ang1, or Ang2; Δ, $P < 0.05$, compared with CXCL8/IL-8 (10 nM) + PBS. In another set of experiments, neutrophils were stimulated with PBS, CXCL8/IL-8 (0.1, 1, or 10 nM) ± Ang1 (0.1, 1, or 10 nM; B), or Ang2 (0.1, 1, or 10 nM; C) for 2 min at room temperature. Western blot analyses of Akt activation were performed as detailed for Figure 3.

activity had no such effect. Our data are in line with previous studies reporting that PI-3K activation is essential for Ang1- and Ang2-mediated EC survival, migration, and sprouting [4, 5, 26, 38, 39], whereas the activation of p38 and p42/44 MAPKs by angiopoietins modulates cell survival [25]. In addition, our data demonstrate that in neutrophils, PI-3K activation is essential to Ang1 and Ang2 chemotactic properties, whereas in eosinophils, Ang1 chemotactic activity is predominant and partially dependent on PI-3K activation [16].

As Akt is a target of PI-3K signaling upon stimulation of neutrophils with various proinflammatory cytokines [40], we assessed the capacity of angiopoietins to induce Akt phosphorylation. We observed that Ang1 and Ang2 can induce a rapid and transient phosphorylation of Akt in neutrophils. Our data are in agreement with the previous study, in which a similar Akt kinetic activation was observed upon stimulation of neutrophils with chemoattractants including CXCL8/IL-8 and fMLP [41].

Ang1 and Ang2 potentiate CXCL8/IL-8-mediated neutrophil migration

Stimulation of EC by proinflammatory mediators can induce the release of Ang2 and CXCL8/IL-8 from Weibel-Palade bodies, which promotes neutrophil migration and their adhesion onto EC [7, 41, 42]. As we observed that PI-3K activation contributes to angiopoietin chemotactic activity on neutrophils and that different studies provided evidence that PI-3K activation contributes to CXCL8/IL-8 chemotactic properties [22, 27], we thus hypothesized that angiopoietins might modulate CXCL8/IL-8-mediated neutrophil migration.

Herein, we show that CXCL8/IL-8 increases migration of neutrophils in a bell-shaped manner, which is in agreement with previous reports [43–45]. Our data are in line with previous studies indicating that PI-3K pathway activation contributes to CXCL8/IL-8-mediated neutrophil migration [22, 27]. We observed that a pretreatment with PI-3K inhibitors pre-

vented neutrophil migration induced by CXCL8/IL-8 (1 nM). However, identical pretreatment reduced by ~50% the migration of neutrophils mediated by CXCL8/IL-8 (10 nM), despite the fact that those PI-3K inhibitors were capable of preventing CXCL8/IL-8 (10 nM)-mediated Akt phosphorylation. The difference between the effects of PI-3K inhibition on neutrophil migration induced by CXCL8/IL-8 at 1 nM versus 10 nM might be explained by the stimulation of additional pathway(s) independently from PI-3K/Akt activation. However, based on our current data, we can discard the contribution of p38 and p42/44 MAPK pathways, as their inhibition had no effect on CXCL8/IL-8 (10 nM) chemotactic activity.

Our data showed that a pretreatment of neutrophils with Ang1 or Ang2, even at the highest concentration, did not promote their migration in the absence of chemotactic mediators but potentiated a neutrophil migration response toward CXCL8/IL-8 (Fig. 5, A and B). These data suggest that neutrophil activation by Ang1 and Ang2 is priming their migration toward chemotactic mediators such as CXCL8/IL-8. By Western blot analyses, we also observed the capacity of Ang1 and Ang2 to potentiate Akt phosphorylation mediated by a suboptimal CXCL8/IL-8 concentration, which per se, is insufficient to increase the basal level of Akt phosphorylation. Finally, by using selective PI-3K inihibitors, we observed that the potentiating activity of Ang1 and Ang2 on CXCL8/IL-8-mediated neutrophil migration is driven through PI-3K activation.

In a recent study, we observed under an in vitro condition that Ang1 and Ang2 can promote a rapid and transient endothelial P-selectin translocation and neutrophil adhesion onto activated EC. Under in vivo condition, Roviezzo et al. [6] reported that a s.c. injection of Ang2 in a mouse air pouch model promoted transmigration of circulating leukocytes, whereas Ang1 did not induce such effect. Taken together, these data show that under in vitro conditions, both angiopoietins can share common, proinflammatory activities. However, under in vivo conditions, Ang1 and Ang2 may have a different capacity to support a prolonged adhesion of leukocytes onto EC and subsequently, their transmigration.

In summary, we observed that both angiopoietins promote neutrophil chemotaxis through the activation of a PI-3K pathway. In addition, both angiopoietins have the capacity to potentiate CXCL8/IL-8 chemotactic activity on neutrophils.

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