Chapter 5

Src kinases regulate PKB activation and modulate cytokine and chemoattractant controlled neutrophil functioning

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Abstract
Tyrosine phosphorylation is thought to be critical in the regulation of neutrophil functioning and members of the Src-family of tyrosine kinases have recently been shown to be regulated in activated granulocytes. We have utilized a specific pharmacological inhibitor of Src-kinases, pyrazolopyrimidine 1 (PP1), to evaluate the role of Src-kinases in cytokine/chemoattractant induced regulation of neutrophil function. PP1 inhibits PKB phosphorylation, but not STAT5 phosphorylation or the activation of MAP kinases by either fMLP or GM-CSF. Pre-treatment of neutrophils with PP1 and also with the PI3K inhibitor LY294002 resulted in a strong inhibition of fMLP induced superoxide production and cytokine-mediated survival, but not fMLP induced migration. Interestingly, the kinetics of inhibition of actin polymerization and the respiratory burst are very similar. While initiation of both processes was not effected, sustained activation was inhibited by PP1. Taken together, our results demonstrate a critical role for Src-kinases in regulating neutrophil cytotoxic effector functioning through PI3K-PKB.
Introduction
Neutrophils are critical effector cells in the killing and removal of microorganisms through the regulation of specific effector functions (1). The recruitment of neutrophils from peripheral blood to the inflammatory locus is mediated by several processes, consisting of rolling, and later firm adhesion to the vascular endothelium, followed by transmigration through the endothelium and migration to the specific site (2). At the inflammatory locus, the killing of microorganisms is mediated by different cytotoxic effector mechanisms including phagocytosis, release of cytotoxic proteins, and production of toxic oxygen metabolites initiated by a membrane bound NADPH-oxidase (3). Furthermore, these leukocytes are also involved in maintaining inflammatory reactions by the release of cytokines and bioactive lipids.

A consequence of activation of neutrophils is the ability to cause tissue damage during inflammatory reactions and, therefore, the activity of neutrophils must be tightly controlled. For this reason the activation of neutrophils occurs in a multistep process. Resting neutrophils in the peripheral blood are poorly responsive to many activators including naturally occurring formyl peptides such as fMet-Leu-Phe (fMLP). However, when these cells are exposed to several pre-activating or "priming" agents such as cytokines, effector functions such as the respiratory burst, phagocytosis and degranulation are greatly enhanced (4,5). To understand the mechanism by which specific agents activate or prime granulocytes it is important to define components of signaling pathways responsible for the activation of effector functions in granulocytes.

Recently several intracellular signal transduction cascades have been found to be activated in human neutrophils in response to cytokines such as GM-CSF, as well as G-protein coupled receptor (GPCR) agonists such as PAF and fMLP. One family of proteins that has been demonstrated to be activated by many priming agents are the mitogen activated protein kinases or MAP kinases (6-10). There are three distinct groups of MAP kinases: extracellular signal-related protein kinases (ERKs) activated by a large variety of agonist, stress-activated protein kinases (JNK/SAPKs) and the p38 MAPK family. Another signal transduction pathway that appears to play a critical role in both priming and activation of granulocyte effector functions involves the lipid kinase phosphatidylinositol 3-kinase (PI3K). Utilizing specific inhibitors of PI3K, wortmannin and LY294002, ourselves and others have recently demonstrated that this kinase plays a critical role in several neutrophil effector functions including the respiratory burst and migration (7,11-13). Despite the fact that in neutrophils cytokine and G-protein coupled receptors can activate the signaling cascades mentioned above, little is known about the specific mechanism by which these receptors initiate these cascades. During the last few years increasing evidence shows that tyrosine kinases are involved in transducing signals from both cytokine and G-protein coupled receptors to downstream signal cascades (14). In granulocytes, it has been shown that tyrosine kinases are involved in cytokine and G-
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protein coupled receptor agonist signaling (15-18). In neutrophils several protein tyrosine kinases have been identified, including Janus Kinases (JAKs) and the Src kinase family. The JAKs, which are activated by cytokine receptors but not by G-protein coupled receptors, phosphorylate the STAT transcription factors (19-21). This pathway is important for linking many cytokine receptors to gene regulation but is not involved in activation of cytotoxic mechanisms. Of the family of Src kinases, several members like Lyn, Hck and Fgr are expressed in neutrophils (22,23). These Src kinase members have also been shown to be activated by several cytokine and G-protein coupled receptors (18,24,25). Data from cell lines and knock-out mice show that Src kinases are involved in many cellular processes including differentiation, adhesion/spreading, migration, apoptosis, gene transcription and cell cycle (26,27).

The development of a pharmacological Src family-selective tyrosine kinase inhibitor, PP1, has allowed the investigation of the role of this tyrosine kinase family (28). In this study we have utilized this inhibitor to evaluate the role of Src-kinases in cytokine and chemoattractant signaling regulating neutrophil functions. Our results demonstrate that Src kinases are involved in PKB but not in MAPK activation in neutrophils. Furthermore, we show that Src kinases play a role in fMLP-induced superoxide production, migration, actin polymerization and cytokine mediated survival.
Materials and Methods

Reagents and Antibodies
N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma (St. Louis, MO, USA). Recombinant human GM-CSF (2.5 x 10^8 U/mg) was from Genzyme (Boston, MA). Human serum albumin (HSA) and human pasteurised plasma-protein solution (40 g/L) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Heps buffered RPMI 1640 medium with L-Glutamine and Hyclone were purchased from Life Technologies, Breda, The Netherlands. Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Polyclonal anti-phospho-PKB (ser473), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Thr182) and anti-phospho-STAT5 (Tyr694) antibodies were from New England Biolabs (Westburg, Leusden, The Netherlands). ERK-1 (C-16), ERK-2 (C-14), Hck (N-30) and c-Fgr (N-47) polyclonal antisera were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-Lyn was purchased from Transduction Laboratories (Lexington, KY). Anti-PKB antisera has been described previously (29). The inhibitors PP1, PD098059, and LY294002 were from Biomol (Plymouth, PA).

Isolation of human neutrophils
Blood was obtained from healthy volunteers. Mixed granulocytes were isolated from 50-100 ml blood, which was anti-coagulated with 0.32% sodium citrate as described before (7). Blood was diluted 1.4 times with phosphate-buffered saline (PBS) containing 0.32% sodium citrate and 10% human pasteurized plasma-protein solution (40 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Paque for 20 minutes at 2000 rpm. The erythrocytes were lysed in isotonic ice-cold NH₄CL solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 minutes at 37°C in Hepes buffered RPMI 1640 medium Technologies, supplemented with L-glutamine and 0.5% HSA. All preparations contained >97% neutrophils. Before stimulation, neutrophils were resuspended in incubation buffer (20 mM Hepes pH 7.4, 132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM glucose, 1 mM CaCl₂ and 0.5% (v/v) HSA) for 15-30 minutes at 37°C.

Lyn, Hck and Fgr kinase activity
Neutrophils were isolated as described above and incubated at 37°C for 30 minutes. After stimulation with fMLP, cells (5 x 10⁶ cells) were lysed in 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM Na₃VO₄, 1% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and benzamidine. Lysates were pre-cleared for 30 minutes at 4°C with protein G-sepharose and
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subsequently, Lyn, Hck and Fgr kinase was immunoprecipitated with 1 µg of Lyn, Hck or Fgr antibody for 1 hour at 4°C on a rotating-wheel. To immunoprecipitate Lyn monoclonal antibody, protein G-sepharose was added for a further 1 hour at 4°C, while protein A-sepharose was used to immunoprecipitate Hck and Fgr antibodies. After washing twice with lysis-buffer and twice with wash buffer (25 mM Tris pH 7.5, 150 mM NaCl and 0.1 mM Na$_3$VO$_4$), precipitates were incubated in 30 µl kinase buffer (20 mM Hepes pH 7.5, 10 mM MnCl$_2$, 1 µM rATP, and 0.3 µCi [$\gamma$-$^{32}$P]ATP) with increasing concentrations of PP1 for 2 minutes at room temperature. The Hck and Fgr kinase reactions contained 10 µg of enolase. Reaction was stopped by the addition of 5x Laemmli sample buffer and boiled for 5 minutes. Samples were separated by electrophoresis on 15% SDS-polyacrylamide gels. Kinase activity was detected by autoradiography.

MAP kinase activity in vitro

MAP kinase activity was measured as described before (7). In short, neutrophils were isolated as described above and incubated at 37°C for 30 minutes. After pre-treatment with PP1 or PD98059 and stimulation with fMLP or GM-CSF, cells (5 × 10$^6$ cells) were lysed in 50 mM Tris-Cl pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and benzamidine. Lysates were pre-cleared for 30 minutes at 4°C with protein A-sepharose and subsequently, MAP kinase was immunoprecipitated with 1 µg of both ERK-1/ERK-2 or p38 polyclonal antisera for 1 hour at 4°C on a rotating-wheel. Protein A-sepharose was then added for a further 1 hour at 4°C. After washing twice with lysis-buffer, samples were washed twice with kinase buffer (30 mM Tris-Cl pH 8.0, 20 mM MgCl$_2$, 20 mM MnCl$_2$, 10 µM rATP, 10 µg/ml myelin basic protein and 0.3 µCi [$\gamma$-$^{32}$P]ATP) without ATP and MBP. Precipitates were then incubated in 25 µl kinase buffer for 20 minutes at room temperature. Reaction was stopped by the addition of 5x Laemmli sample buffer. Samples were separated by electrophoresis on 15% SDS-polyacrylamide gels. MBP phosphorylation was detected by autoradiography.

Western blotting

Neutrophils (10$^6$ cells) were pre-incubated with several concentrations of inhibitor for 20 minutes followed by stimulation for the indicated time points, washed in ice-cold PBS and immediately lysed in Laemmli sample buffer. Total cell lysates were boiled for 5 min at 95°C and analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P. The blots for hybridization with phospho-specific antibodies were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 3% BSA for 1 hour followed by incubation with phospho-specific antibody (1/1000) in hybridization buffer with
1% BSA for 2 hours at room temperature. Second antibody was incubated in hybridization buffer for 1 hour. The western blots for hybridization with all other antibodies were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 5% non-fat milk for 1 hour followed by incubation with antibody (1/1000) in hybridization buffer with 0.5% non-fat milk for 2 hours at room temperature. Second antibody was incubated in hybridization buffer containing 0.5% non-fat milk for 1 hour. Detection of all western blots was performed by enhanced chemiluminescence (ECL; Amersham, UK).

**F-actin measurement**

Fluorescent F-actin staining was performed as described previously (30). In short, neutrophils (2.5 x 10^6 cells/ml) were stimulated for the indicated time points and subsequently fixed and permeabilized with ice-cold 3% formaldehyde in PBS, containing 100 µg/ml lysophosphatidylcholine for 10 minutes at room temperature. F-actin was stained with 30 U/ml NBD-phalloidin for 30 minutes at room temperature. The intracellular fluorescence was determined by FACS-analysis (FACSVantage, Becton Dickinson, San Jose, CA), by measuring a total cell count of 5000 cells per sample.

**Measurement of neutrophil migration**

Neutrophil migration was measured using a modification of the method according to Boyden as described before (7); using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). Chemoattractants or incubation buffer (30 µl) were added to the lower compartments. Two filters were placed between lower and upper compartments. The lower filter had a pore width of 0.45 µM (Millipore, Bedford, MA), while the upper filter (cellulose nitrate) had a pore width of 8 µM (thickness 150 µM; Sartorius, Gottingen, Germany). Before use the filters were soaked in incubation buffer. Neutrophils were placed in the upper compartment (25 µl of 2 x 10^6 cells/ml). The chambers were subsequently incubated for 1.5 hours at 37°C. The upper filters were removed, fixed in butanol/ethanol (20/80% v/v) for 10 minutes and stained with Weigert solution (composition: 1% v/v haematoxylin in ethanol mixed with a 70 mM acidic FeCl₃ solution at 1:1 ratio). The filters were dehydrated with ethanol, made transparent with xylene and fixed upside down. All migratory responses were quantified with an image analysis system (QuantiMet 570C, Leica Cambridge Ltd.) using QuantiMet 570 Control Software (QUIC version 2.02) together with custom made software. An automated microscope, Leitz DMRXE (Leica, Weitzlar, Germany) was used to step through the filters in the Z direction with 16 intervals of 10 µM. Neutrophils were counted at each level and the total migration to each level was calculated. The results are expressed as migration index, which is calculated by the cumulative migration of all intervals (µm) divided by the total number of cells multiplied by the amount of migrated cells. The mean of four randomly chosen points on each filterspot was calculated.
Measurement of NADPH-oxidase activation
Superoxide was measured by cytochrome c reduction according to a modified method described by Pick and Mizel (31). In short, neutrophils (4 x 10^6 cells/ml) were pre-incubated for 5 min at 37°C in incubation buffer. Subsequently, neutrophils were pre-incubated with inhibitors and/or cytokines for the indicated periods of time. Hereafter, the cells (200 µl) were transferred to a microtitre plate in a thermostat-controlled microtitre plate reader (340 ATTC; SLT LabInstruments, Austria), mixed with cytochrome c (75 µM) and the incubation was continued for 5 min, the plates shaken every 3 seconds. The cells were then stimulated with fMLP (1 µM) and cytochrome c reduction measured every 12 seconds as an increase in absorbance at 550 nm.
Oxygen consumption was measured as describe before (32). In short, granulocytes were resuspended (3 x 10^6 cells/ml) in the incubation buffer and pre-incubated with GM-CSF (10^{-10}M) for 30 min. After incubation, cells were brought in a stirred and thermostated airtight vessel and inhibitor was added for 5 min. Subsequently, fMLP (1 µM) was added to activate the respiratory burst and oxygen consumption was continually measured with an oxygen probe (Yellow Springs Instrument) for several minutes.

Measurement of neutrophil apoptosis
Apoptosis of neutrophils was measured by analyzing Annexin V-FITC binding (Alexis; Kordia bv, The Netherlands). In short, freshly isolated neutrophils (0.5 x 10^6/ml) were resuspended in Hepes buffered RPMI medium supplemented with 8% Hyclone serum. After treatment with inhibitors/cytokines, cells were incubated for the indicated times at 37 °C. At the end of incubation, cells were stored at 4°C until the last incubation time point had been reached. Subsequently, cells were washed with ice-cold PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2). Annexin V-FITC (1/40) was added to the cells and incubated for 10 min at room temperature. After washing, cells were resuspended in binding buffer containing 1 µg/ml propidium iodide. The fluorescence was determined by FACS-analysis (FACSvantage, Becton Dickinson, San Jose, CA), by measuring a total cell count of 10000 cells per sample.
Results

**PP1 inhibits Lyn and Hck kinase activity but not STAT5 phosphorylation in neutrophils**

To analyze the role of Src kinases in neutrophil signaling and effector functions we utilized the recently identified inhibitor pyrazolopyrimidine (PP1) (28). This compound has been described to specifically inhibit Src kinases including Fyn and Lck but not ZAP-70, JAK2 and EGF-R protein kinases. As previously mentioned, several members of the Src kinase family including Lyn, Hck and Fgr are expressed in neutrophils and have been shown to be activated by several cytokine and G-protein coupled receptors (18,22,23). Furthermore, a recent study found that the sensitivity of the various members of the Src kinase family to PP1 was different (33). To characterize the inhibition of Lyn, Hck and Fgr by PP1 we stimulated neutrophils with fMLP and an immunocomplex kinase assay was performed in the presence of different concentrations of PP1. Whereas Lyn and Hck demonstrated kinase activity, no Fgr kinase activity could be found in fMLP stimulated neutrophils (data not shown). As shown in Figure 1A and 1B, increasing concentrations of PP1 resulted in a concentration-dependent decrease in Lyn and Hck kinase activities.

Recently it has been suggested that Src kinases are critically involved in the activation of STAT transcription factors in many cell types (34). c-Src was found to be involved in IL-3 mediated activation of STAT3 and it has been shown that Lyn can enhance STAT5 activation (35,36). To investigate whether Src kinases may be involved in STAT activation in neutrophils, cells were pre-incubated with increasing concentrations of PP1 and subsequently stimulated with GM-CSF. After stimulation, samples were analyzed by performing a western blot utilizing an activation-specific phospho-STAT5 antibody. Figure 1C shows no inhibition of STAT5 phosphorylation by PP1 at a concentration of 50 µM PP1, demonstrating that Src kinases are not involved in GM-CSF induced STAT5 phosphorylation in neutrophils. This observation also establishes that PP1 does not aspecifically inhibit other tyrosine kinases.

**Activation of MAPKs by GM-CSF or fMLP is not inhibited by PP1**

Stimuli such as GM-CSF and fMLP have been described to transiently activate MAPKs in neutrophils (6-10,37). Whereas Src kinases have been proposed to be important regulators of MAPKs in several cell types (38-41), very little is known about the role of Src kinases in neutrophils. To determine whether fMLP and GM-CSF activate MAPKs via Src kinase we performed two different assays to measure the activation state of MAPKs, an *in vitro* kinase assay (Fig. 2A and C) and western blotting using phospho-specific antibodies to ERK1/2 and p38 (Fig. 2B and D respectively). Before stimulation, neutrophils were pre-incubated with increasing concentrations of PP1 for 20 minutes. As shown in Figure 2 neither ERK1/2 nor p38 kinase activities were inhibited by the highest concentration of 50
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µM PP1 in both the in vitro kinase assay as well as the western blots using activation-specific phospho-antibodies against p38 and ERK1/2. However a specific inhibitor of MEK (PD098059), the upstream activator of ERK1/2, completely blocks ERK1/2 kinase activity. Thus in neutrophils neither the GM-CSF nor fMLP receptors require Src kinases in the regulation of ERK1/2 and p38 MAPK activation.

A role for Src kinases in GM-CSF- and fMLP-induced PKB activation

Recent studies have shown that both cytokines and chemoattractants activate PI3K which plays a critical role in regulating a variety of neutrophil effector functions (7,12,13,42-45). A role for tyrosine kinases in activation of the PI3K has also been proposed in neutrophils (17,18,46). To investigate the role of Src kinases in PI3K activation in neutrophils, we used the phosphorylation status of PKB, a downstream target of PI3K (29,47), as a measurement of PI3K activation. Firstly, we analyzed the ability of fMLP and GM-CSF to induce PKB activation in neutrophils using an activation-specific phospho-antibody against serine 473 of PKB. Figure 3A shows a transient phosphorylation of PKB upon either GM-CSF or fMLP stimulation. fMLP induced PKB phosphorylation is much stronger and more rapid than by GM-CSF, similar to the kinetics of MAPK and PI3K activation by these two stimuli (7,18). As shown in Figure 3B, increasing concentrations of PP1 resulted in a decrease in both GM-CSF and fMLP induced PKB phosphorylation with a half-maximal inhibition of approximately 1-5 µM of PP1 and a complete block at 5-20 µM of PP1 respectively. Addition of the specific PI3K inhibitor LY294002 (48), also completely blocks GM-CSF and fMLP induced PKB phosphorylation, indicating that PKB
activation requires PI3K. Furthermore we have found that PP1 does not directly inhibit PKB kinase activity (data not shown). Taken together, our data demonstrate that Src kinases are critical in activation of the PI3K-PKB signaling.

Figure 2: Activation of ERK1/2 and p38 MAPK by fMLP and GM-CSF do not require Src kinases. Freshly isolated neutrophils were pre-incubated with DMSO, PD-98059 (50 µM) or increasing concentrations of PP1 for 20 min and subsequently stimulated with GM-CSF (10^{-10} M) and fMLP (10^{-6} M) for 10 and 2 min respectively. (A and C) After stimulation, 5x10^6 cells were lysed and 1/25th was used for Western blotting with anti-ERK1/2 and anti-p38 MAPK antibodies to confirm as equal amount of protein. The remainder of the sample was used for immunoprecipitation with a mixture of ERK1 and ERK2 (1:1) antibodies or with p38 MAPK antibody. Kinase activity of both ERK1/2 and p38 MAPK was measured for 20 minutes at room temperature using Myelin Basic Protein (MBP) as substrate. Phosphorylation of MBP was detected by autoradiography. (B and D) Neutrophils (10^6 cells) were treated as above and immediately lysed in Laemmli sample buffer. Proteins were analyzed by SDS-PAGE followed by Western blotting with indicated antibodies. Data is representative of three independent experiments.
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Figure 3: Src kinases are critical for fMLP and GM-CSF induced PKB activation. (A) Isolated neutrophils were incubated with GM-CSF (10⁻¹⁰ M) and fMLP (10⁻⁶ M) for the times indicated. After stimulation, cells were immediately lysed in Laemmli sample buffer and samples were analyzed by SDS-PAGE followed by Western blotting with anti-phospho-PKB (ser 473) or with anti-PKB. (B) Neutrophils were pre-treated with DMSO, increasing concentrations of PP1 or 20 µM of LY294002 followed by stimulation with GM-CSF (10⁻¹⁰ M) and fMLP (10⁻⁶ M) for 10 or 2 min respectively. All samples were analyzed as previously described. Data is representative of three independent experiments.

Src-kinase activity is required for activation of the respiratory burst in neutrophils

Stimulation of neutrophils with the chemotactic peptide fMLP induces the rapid formation of microbicidal oxidants, a process termed the respiratory burst and is dependent on prior priming of cells with cytokines, chemoattractants or lipopolysaccharide (1,4,5,7). Previously we have shown a role for PI3K but not ERK in this process (7). Taken this finding together with our result that PKB but not ERK activation is inhibited by PP1, suggests a possible role for Src kinases in fMLP-induced superoxide production. We analyzed the effect of pre-incubation of neutrophils with various concentrations of PP1 on the fMLP induced respiratory burst using GM-CSF as a priming agent.

As shown in Figure 4A, GM-CSF and fMLP individually do not activate the respiratory burst in neutrophils, whereas neutrophils treated with GM-CSF prior to fMLP activation show a large increase in superoxide production which is markedly decreased by both 50 µM PP1 and 20 µM LY294002. Neutrophils incubated with increasing concentrations PP1 show that the maximum decrease of superoxide production is already reached at 5 µM of
PP1 (Fig. 4B). The same decrease in fMLP-induced respiratory burst was observed in cells first primed with GM-CSF, followed by treatment with different concentrations of PP1 before finally being activated with fMLP (Fig. 4C). This demonstrates that indeed Src kinases are involved in superoxide production, but it is not apparent whether Src kinases are critical for GM-CSF mediated neutrophil priming. Interestingly, PMA, which strongly induces the respiratory burst independently of priming, is not inhibited by 50 µM of PP1 (Fig. 4D). This demonstrates that under certain conditions, activation of the respiratory burst can occur independently of Src-kinases.

Figure 4: PP1 inhibits fMLP induced respiratory burst, but not PMA stimulated respiratory burst. (A and B) Neutrophils were pre-incubated with DMSO, increasing amounts of PP1 or 20 µM of LY294002 for 20 min before incubation of GM-CSF (10^{-10} M) for 30 min followed by stimulation with fMLP (10^{-6}). (C) Neutrophils were incubated with GM-CSF (10^{-10} M) for 30 min, before treatment with DMSO or increasing amounts of PP1 for 20 min followed by stimulation with fMLP (10^{-6}). (D) Neutrophils were pre-incubated with DMSO or with 50 µM PP1 for 20 min followed by stimulation with PMA (0.1 µg/ml). Superoxide production was monitored continuously by measurement of cytochrome c reduction. Results are expressed as the OD at a wavelength of 550 nm and are representative of four independent experiments.
Src kinases are not involved in fMLP induced neutrophil migration

To reach the site of infection, chemoattractants guide neutrophils to the inflammatory locus by a process termed chemotaxis. In order to investigate the role of Src kinases in neutrophil migration we utilized a modification of the method described by Boyden with a 48-well microchemotaxis chamber where the stimuli was placed in the lower chamber and migratory activity was measured as described previously (7). To induce migration we used the chemoattractant fMLP, which is able to potently induce neutrophil migration (Fig. 5A).

To determine the role of Src kinases in neutrophil migration, we pre-incubated neutrophils with various concentrations of PP1 before performing Boyden-chamber migration assays. As is shown in Figure 6B, fMLP induced migration is not blocked by PP1. Similar results were also obtained by incubating the cells with 20 µM of LY294002 (Fig. 5B).

![Figure 5: Src kinases are not involved in fMLP induced neutrophil migration.](image)

**Figure 5: Src kinases are not involved in fMLP induced neutrophil migration.** Neutrophil migration was monitored in microchemotaxis Boyden chambers in response to buffer or fMLP (10^-8 M) as described in the Materials and Methods. Stimulus was placed in the lower compartment and cells were left to migrate for 1.5 hours at 37 °C. Neutrophils were pre-incubated with DMSO (A), increasing amounts of PP1 (B) or 20 µM of LY294002 (B). Results are expressed as migration index ±S.E.M. (n=3)

PP1 and LY294002 prevent GM-CSF mediated neutrophil survival

The life span of circulating neutrophils is relatively short compared to other leukocytes and cannot be dramatically extended (49). However, several stimuli can delay apoptosis including GM-CSF and LPS (50), whereas other stimuli, such as TNF-α, accelerate neutrophil apoptosis (51,52). In several cell lines it has been shown that the PI3K-PKB pathway plays an important role in cell survival (53). Since, we have demonstrated in this

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study that Src kinases regulate PI3K-PKB activity it is reasonable to conclude that Src kinases might also play a role in inhibiting neutrophil apoptosis. To investigate the role of Src kinase and PI3K in apoptosis of neutrophils, we incubated freshly isolated neutrophils for 20 min with various concentrations of PP1 or 20 \( \mu M \) of LY294002 before addition of GM-CSF. To measure apoptosis we utilized Annexin-V, which binds to phosphatidylserine (PS), present on the outer leaflet of the plasma membrane only in those cells which have initiated an apoptotic program. In Figure 6A, dot plots are shown in which the lower left corner represents living cells and the lower right corner represents early apoptotic cells. Few apoptotic cells were observed in freshly isolated neutrophils whereas after 12 hours approximately 50% of the cells were apoptotic. Stimulating cells with GM-CSF results in a decrease of the number of apoptotic cells from 50% to 20%, while PP1 completely blocks this GM-CSF rescue (Fig. 6A). In Figure 6B we analyzed different time points of apoptosis using several concentrations of PP1. Figure 6B shows that neutrophils initiate the apoptotic program after 4-8 hours of isolation and that this process is not blocked but delayed by GM-CSF for approximately 4 hours. This delay is completely blocked with PP1 at a concentration of 20 \( \mu M \), as well as with 20 \( \mu M \) of LY294002. Neutrophils treated with PP1 alone give similar kinetics for apoptosis as neutrophils without treatment (data not shown).

**Figure 6: Src-kinases and PI3K are involved in GM-CSF mediated survival.** Neutrophils were pre-incubated for 20 min with DMSO or increasing concentrations of PP1 before adding GM-CSF. Apoptosis of the cells was measured using Annexin-V in combination with propidium iodide followed by FACS analysis. Cells positive for Annexin-V represent cells in apoptosis. FACS results are presented in dot plots (A) or (B) cells positive for Annexin-V were plotted in a graph. Results of the graph are expressed as percentage apoptosis. (n=3)
PP1 inhibits fMLP induced actin polymerization in neutrophils with the similar kinetics as for respiratory burst

Cytoskeletal rearrangement, which involves changes in levels of filamentous actin (F-actin) is considered to be an essential event for several neutrophil effector functions including the respiratory burst and migration. In neutrophils, chemoattractants like fMLP and PAF induce a rapid increase in relative F-actin content (54,55). To determine whether Src kinases may also be involved in actin polymerization in neutrophils, cells were pre-incubated with different concentrations of PP1 and subsequently stimulated with fMLP. Actin polymerization was determined by measuring F-actin content using NBD-phalloidin followed by FACS analysis as described in Materials and Methods. Cells pre-treated with 20 µM PP1 demonstrate a similar rapid increase of F-actin as control cells, however the decline in F-actin, which normally occurs after several minutes of fMLP stimulation, occurs much more rapidly (Fig. 7A). This suggests a possible role for Src-kinases in regulating the depolymerization reaction.

A link between actin polymerization and correct functioning of the NADPH oxidase has recently been proposed (56). Furthermore, we also observe inhibition of both F-actin polymerization and superoxide production by PP1 (Fig. 4 and 7A). Utilizing an oxygen probe we analyzed the detailed kinetics of fMLP induced oxygen consumption (Fig. 7B). Interestingly, similar to actin polymerization, while the overall respiratory burst is reduced dramatically, the initial phase of activation appears to be unaffected. Although indirect this supports a link between sustained actin polymerization and activation of the respiratory burst complex.
Fig. 7: Kinetics of PP1 inhibition of F-actin polymerization and oxygen consumption in neutrophils. (A), Human neutrophils were pre-treated with DMSO or PP1 (20 µM) for 20 min before stimulation with fMLP (1µM). At the indicated time-points cells were fixed and F-actin staining was performed followed by FACS analysis. The results are expressed as the relative F-actin content ±S.E.M. (n=3) (B), Respiratory burst was measured as oxygen consumption by neutrophils as described in Materials and Methods. Cells were stimulated with GM-CSF (10^{-10} M) for 30 min before the addition of DMSO, 50 µM of PP1 or 20 µM of LY294002 for 5 min. Subsequently, fMLP (1µM) was added to activate the respiratory burst and oxygen consumption was continually measured for several min. △ Represents addition of fMLP
Discussion
During the last few years increasing evidence has demonstrated that tyrosine kinases are involved in the coupling between cytokine and G-protein coupled receptor activation of agonist effector functions in granulocytes (57-59). Among the tyrosine kinases, the family of Src kinases are activated upon stimulation of several cytokine and G-protein coupled receptors in neutrophils (18,24) and therefore might play an important role in neutrophil signaling and effector functions. Data obtained utilizing knock-out mice of different Src family members demonstrate that these kinases are involved in degranulation (60), and migration (61) in human neutrophils, whereas other studies have shown a role of Lyn in superoxide production (33) and in regulation of neutrophil survival by cytokines (62). Furthermore a link between Src kinases and PI3K (18) (63) and p38 MAPK (64) has been proposed in human neutrophils. Recently a specific Src family kinase inhibitor called PP1 has been developed (28). By using this inhibitor we have been able to determine the role of Src kinase in regulating human neutrophil signal transduction and effector functions. Recent studies show that PP1 can inhibit several members of the Src kinase family including Fyn, Lck, Lyn, slightly Fgr, but not Hck in vitro (28) (33). In our study we found that Lyn kinase was slightly more sensitive to PP1 than Hck kinase (Fig. 1A and 1B). The finding that Hck is also inhibited is in contrast with a previous study (33). However in this study only 2 µM PP1, was utilized, whereas we found inhibition of Hck kinase activity between 2.5 and 10 µM PP1. Previous studies have shown that neutrophils stimulated with GM-CSF have enhanced phosphorylation of STAT1, STAT3 and STAT5 (65,66). Whereas Src kinases have recently been implicated in cytokine induced STAT activation in various cell types (34-36,67), little is known in neutrophils. In this study we have demonstrated that Src kinases are apparently not involved in GM-CSF induced STAT5 phosphorylation in human neutrophils (Fig. 1C). This also demonstrates that PP1 is not a general tyrosine kinase inhibitor since cytokine-induced tyrosine phosphorylation of STAT is not perturbed. Importantly, responses such as fMLP induced migration and also PMA activated respiratory burst, are not sensitive for the inhibitor PP1, indicating that PP1 is not simply toxic to these cells.

Both the cytokine GM-CSF and G-protein coupled receptor agonist fMLP are able to transiently activate ERK, p38 and PI3K-PKB signaling cascades in neutrophils (6-9,17,18,37,68). Currently little is known about the specific upstream signals by which receptors activate these cascades. In Figure 2 and 3B we demonstrate that PP1 inhibits PKB phosphorylation but not ERK1/2 and p38 MAPK activities upon both fMLP and GM-CSF stimulation. Our observation that PP1 does not inhibit ERK, correlates well with data showing that in neutrophils the tyrosine kinase inhibitors, genistein and erbstatin, and also PP1 do not inhibit activation of the small GTPase p21ras whose activity is critical as a regulator of ERK (69,70). In the case of activation of p38, opposing data has been published showing that fMLP stimulated neutrophils from Hck−/−Fgr−/−Lyn−/− mice are
unable to activate p38 MAPK (64). This contradictory finding is difficult to reconcile with our data but may be due to functional differences between murine and human derived neutrophils. The inability of Hck−/− Fgr−/− Lyn−/− mice to activate p38 could also be through a variety of alternative mechanisms not directly linked to receptor mediated stimulation. Furthermore, the same study also demonstrates a partial inhibition of p38 MAPK phosphorylation with PP1 in human neutrophils. An alternate explanation for this opposing result might be due to the different concentrations of fMLP used in these assays. We have also demonstrated that both fMLP and GM-CSF require Src kinases to activate the PI3K effector kinase PKB. It is likely that activation PI3K is mediated by Src kinases since pre-incubation of cells with either PP1 or the PI3K inhibitor LY294002 inhibited PKB activation. This is supported by data showing that a member of the Src family kinases, Lyn, can bind to the GM-CSF receptor and to the p85 subunit of PI3K in neutrophils (18,71). The observation that Src kinases are involved in fMLP-mediated activation of PKB is supported by data showing that in neutrophils stimulated with agonists of G-protein-coupled receptors, PI3K is activated through a genistein-sensitive target, presumably a protein tyrosine kinase (16,46).

The observation that Src kinases are involved in PI3K-PKB activation together with previous data showing that PI3K is involved in several neutrophil effector functions, also predicts a role for Src kinases in these processes (42). Indeed, we observed a decrease of fMLP-induced respiratory burst with PP1 incubated either before or after GM-CSF priming, but we did not observe inhibition of the PMA induced response. This demonstrates that Src kinases are involved in fMLP activation of the respiratory burst but that these kinases are not essential for the assembly of the NADPH oxidase per se. Furthermore, we found a similar inhibition of fMLP induced respiratory burst with LY249002 (Fig. 4A), suggesting that Src kinases may act through PI3K-PKB to regulate fMLP induced respiratory burst.

Again supporting a functional link between Src kinases and PI3K-PKB signaling, we demonstrated that both PP1 and LY294002 inhibit GM-CSF mediated survival (Fig. 6). This data is supported by a study demonstrating a role of Lyn in GM-CSF delayed apoptosis (62) and by a recent study showing that the PI3K inhibitor LY294002 blocked GM-CSF dependent PKB and BAD phosphorylation in neutrophils (72). It has been shown in several cell types that PKB can phosphorylate the pro-apoptotic Bcl-2 family member BAD, which in turn prevents its association with anti-apoptotic Bcl-2 family members by association with 14-3-3 proteins, resulting in prolonged survival (73,74). Thus it is tempting to speculate that Src-kinases act through PKB to inhibit pro-apoptotic Bcl-2 family members resulting in a delay in neutrophil apoptosis.

The effector functions, migration and superoxide production, involve cytoskeletal rearrangement requiring actin polymerization. Previous studies have shown that fMLP induces actin polymerization in neutrophils in suspension (54,75). Here we demonstrate that while the rapid induction of F-actin formation is not inhibited by PP1, the decline in
F-actin content is more rapid in PP1 treated neutrophils (Fig. 7A). This suggests that Src kinases may play a role in the stabilization and duration of actin polymerization. This finding might be considered in contrast with our data that Src kinases and PI3K do not affect fMLP induced migration, which also involves cytoskeletal rearrangement. The role of PI3K in regulating migration however is still controversial. Whereas some studies, including utilization of PI3Kγ knockout mice, show that PI3Ks play an important role in neutrophil migration (12,13,45,76), other studies demonstrate that PI3K inhibitors do not inhibit fMLP induced migration (77,78). Furthermore, the small GTPase p21Rac, which is an upstream regulator of actin polymerization, superoxide production and migration (79,80), does not appear to require Src-kinases or PI3Ks in neutrophils (76,81). This feature might be cell type specific since in several cell lines p21Rac is regulated by PI3K. Thus it might be that Src-kinases and PI3K/PKB act in parallel with p21Rac regulating actin depolymerization and superoxide production in neutrophils. The kinetics of PP1 inhibition of actin polymerization (Fig. 7A) are very similar to that of inhibition of the respiratory burst, see also Figure 4 and 7B. In both cases, PP1 does not block the initiation of the response, but rather the maintenance of the signal. One model might be that Rac is involved in initiating of actin polymerization and superoxide production, whereas Src kinases and PI3K/PKB are necessary in maintaining these responses. A recent report has shown that neutrophil NADPH oxidase could be deactivated by actin depolymerization agents in a cell-free system, suggesting that actin filaments, which grow during the activation of NADPH oxidase, prolong the lifetime of the oxidase (56). This might indicate a mechanism by which Src kinases act via PI3K/PKB to regulate fMLP-induced respiratory burst through maintenance of actin polymerization.

The difference in sensitivity of the respiratory burst and GM-CSF delayed apoptosis and also between fMLP and GM-CSF activation of PKB might suggest that different Src kinases are involved in these processes. Therefore we tested the sensitivity of Lyn and Hck kinases to PP1. However the difference between the sensitivity of Lyn and Hck to PP1 is too small to draw any definitive conclusions. Furthermore the sensitivity of Lyn and Hck to PP1 is probably different whether PP1 is added to an in vitro kinase assay compared to adding PP1 to cells, as has been suggested by others (28). This difference may be attributed in part to permeability of PP1 and its distribution within the cell.

The finding that Src kinases are involved in PKB phosphorylation but not in MAPK activation together with the comparison of data of the Src kinase inhibitor PP1 with those of PI3K inhibitor LY294002, suggests that Src kinases and PI3K-PKB act in the same pathway that is responsible for GM-CSF induced survival, prolonging the fMLP induced respiratory burst and regulating F-actin polymerization. Thus the regulation of Src kinase family members by both cytokine and chemoattractant receptors provides a critical upstream control point modulating human neutrophil function.
References


Src kinases regulate PKB activation and modulate neutrophil functioning


78. Harakawa, N., Sasada, M., Maeda, A., Asagoe, K., Nohgawa, M., Takano, K., Matsuda, Y., Yamamoto, K., Okuma, M. Random migration of polymorphonuclear leukocytes induced by GM-CSF involving a signal transduction pathway different from that of fMLP.

