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c-Abl and Src-family kinases cross-talk in regulation of myeloid cell migration

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Abstract

Cytoskeleton dynamics are regulated by Src-family tyrosine kinases (SFKs) and c-Abl. We found that the SFK members Hck and c-Fgr regulate tyrosine phosphorylation of c-Abl and c-Abl associates with β1 integrin-bound Hck or c-Fgr in murine macrophages. Studies with selective inhibitors and cells from SFK-deficient mice showed that c-Abl and SFK regulate migration and activation of the small GTPases Cdc42 and Rac in macrophages. Additionally, human neutrophil chemotactic activity was reduced by c-Abl inhibitors, and neutrophils from chronic myeloid leukaemia patients displayed an increased chemotactic ability. Hence, Src-family kinase and c-Abl cross-talk in the regulation of myeloid cell migration.

Keywords

Macrophage; Neutrophil; Tyrosine kinase; Chemotaxis; Signal transduction

1. Introduction

Src-family kinases (SFKs) are implicated in signal transduction by different surface receptors in haematopoietic cells [1]. In myeloid leukocytes, these kinases regulate different cell responses [1,2] and genetic inactivation of Hck, c-Fgr and Lyn, the SFKs predominantly expressed in these cells, results in defective integrin-dependent spreading, polarization and migration [2–5]. Similarly to classical immune receptors, signaling by adhesive receptors (integrins or the endothelial selectin counter-receptor PSGL-1) in phagocytic cells involves, besides SFKs and Syk, the immunoreceptor tyrosine activation motif (ITAM)-containing adaptors DAP12 and FcRγ [6–8]. Because signal transduction by the T cell receptor (TCR) via a SFK/ITAM-containing adaptor/Zap70 module requires c-Abl [9,10], we addressed whether c-Abl is implicated in signal transduction by myeloid leukocyte non-immune receptors. Here we report that c-Abl associates to integrin-bound c-Fgr or Hck and its

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phosphorylation is regulated by these SFKs. Additionally, we show that inhibition of c-Abl activity results in a marked reduction of mouse macrophage and human neutrophil migration and polarization. These findings suggest that cross-talk between SFKs and c-Abl plays a critical role in regulation of myeloid cell migration.

2. Materials and methods

2.1. Cell isolation and culture

Bone marrow-derived macrophages (BMDMs) were isolated from femurs and tibias of 6- to 8-week-old wild-type, *hck*−/−*fgr*−/− double and *hck*−/− and *fgr*−/− single knockout mice (see [4]), backcrossed 4 generations into the wild-type C57BL/6J strain [4]. Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Biowhittaker, Walkersville, MD) supplemented with 15% FCS, 10% L-cell conditioned medium (LCM) as a source of CSF-1, 100 U/ml penicillin, and 100 μg/ml streptomycin (BMDMs complete medium), and cultured at 37 °C/5% CO_2 in 75 cm^2 flasks. After 24 h, the non-adherent cells were removed, counted and plated on bacteriologic (non-tissue culture treated) plastic dishes in the above medium. Human PMNs were prepared from buffy coats of healthy volunteers or from peripheral blood of first diagnosis Chronic Myeloid Leukemia (CML) patients. The murine wild-type (pSrl) pro-B lymphocyte cell line BaF3 was cultivated at 37 °C/5% CO_2 in RPMI 1640 medium containing 10% foetal bovine serum and 10% WEHI-3B-conditioned medium as a source of IL-3 [11]. BaF3 cells expressing Bcr/Abl (BaF3/p210wt) or Bcr-Abl with the T315 mutation (Baf3/T315) were cultured in RPMI 1640 medium with 10% foetal bovine serum, without any source of IL-3.

2.2. Antibodies and reagents

Antibodies were obtained from the following sources: anti-phosphotyrosine mAb (4G10), UBI (Lake Placid, NY), anti-SFKs (reactive with Src, Yes, Fyn and c-Fgr) and anti-Abl, Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-β1 and anti-β2 integrin, Chemicon International (California, USA).

2.3. Cell migration assay

In vitro wound-healing assays were performed as described [4,5] in the presence of 100 ng/ml lysophosphatidic acid (LPA) in medium supplemented or not with imatinib mesylate (STI; 10 μM; Novartis Pharma), bosutinib (SKI; 10 μM; Wyeth) or PP2 (10 μM). The number of cells migrating into the open space was counted microscopically [5]. Neutrophil migration assays were performed as previously described [12] using FCS-coated 3 μm transwells.

2.4. Protein immunoprecipitation and immunoblotting

BMDM were lysed in RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 1% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 1 mM DTT, 100 μM Na_2VO_4, 10 μM phenilarsine oxide (PAO) and one tablet of Complete™ Mini EDTA-free protease inhibitor cocktail from Roche Molecular Biochemicals for each 10 ml lysis buffer). After clarification in a microfuge, lysates were incubated with 2 μg of Abs bound to 10 μl of protein A or protein G immobilized to Trysacryl (Sigma) for each sample

and precipitated proteins washed three times with RIPA buffer. Immunoprecipitated proteins or proteins in whole-cell lysates were electrophoresed on SDS–polyacrylamide gel (SDS–PAGE) and transferred to nitrocellulose for 90 min at 70 V. Filters were then analyzed with Odyssey Infrared Imaging System (Li-cor Biosciences, Nebraska, USA) using specific antibodies. For analysis of tyrosine phosphorylated proteins with phospho-specific Abs, cells were lysed with sample buffer: 25 mM Tris, pH 6.8, 50 mM β-mercaptoethanol, 1% SDS and 5% glycerol.

2.5. Cdc42 and Rac activity assay

Cdc42 and Rac activity was assayed using EZ-Detect Rac activation kit according to the manufacturer instructions (Pierce Chemical; Thermo Fisher Scientific; Rockford, IL). Proteins were separated by SDS–PAGE and, after blotting, membranes were probed with anti-Cdc42 and anti-Rac Abs.

3. Results

3.1. c-Abl binds to and is phosphorylated by SFKs in mononuclear phagocytes

A bi-directional cross-talk between SFKs and c-Abl, or the oncoprotein Bcr-Abl, has been demonstrated in several studies [9,13–18]. Examining c-Abl tyrosine phosphorylation in BMDM we found the constitutive, phosphorylation of c-Abl was much more robust in wild-type compared to *hck*−/−*fgr*−/− cells (Fig. 1A). Because SFKs interact with integrins [19], we asked whether integrin-bound SFKs associate with c-Abl. We found that c-Fgr and/or Hck associate with the β1 (Fig. 1B and C) or the β2 (not shown) integrin chain in wild-type BMDM lysates. Comparing β1 integrin-associated c-Abl in wild-type, *fgr*−/−, *hck*−/− or *hck*−/−*fgr*−/− BMDM lysates (Fig. 1B and C), we found that only in the absence of both c-Fgr and Hck expression c-Abl association with the β1 integrin chain was undetectable, thus suggesting that c-Abl interacts with either integrin-bound c-Fgr or Hck. A tripartite complex between c-Fgr or Hck and c-Abl was also detectable in anti-β2 immunoprecipitates (not shown).

3.2. c-Abl regulates mononuclear phagocyte migration

Because c-Fgr and Hck associate with c-Abl and regulate its extent of tyrosine phosphorylation (Fig. 1) we addressed whether c-Abl is implicated in a SFK-dependent signaling network regulating macrophage migration [2]. Wild-type BMDM repair a wound generated by scratching a confluent cell monolayer with a pipette tip in a few hours (Fig. 2A and B), and the chemoattractant LPA hastens it (data not shown). The c-Abl specific inhibitor imatinib mesylate (STI) [20] or the Src specific inhibitor PP2 inhibited BMDM migration in this assay (Fig. 2C–E). To exclude that, in our assays, STI affected also SFKs we used an Ab reacting with a phosphorylated tyrosine residue (Y416) of the kinase domain of Src and other SFK members (Fig. 2F). While treatment with PP2 of wild-type BMDM or deficiency of Hck and c-Fgr expression markedly reduced the reactivity of the anti-phospho Y416 antibody, STI did not affect the intensity of the detected signal (Fig. 1F) as expected from its inability to inhibit SFKs [20]. In contrast, both STI and PP2 markedly inhibited c-Abl tyrosine phosphorylation (Fig. 1F). Comparing the effect of the two inhibitors on the total tyrosine phosphorylation signal (Fig. 1G) we found that, whereas treatment with PP2 or

deficiency of Hck and c-Fgr resulted in a marked inhibition of tyrosine phosphorylation of several proteins, STI did not affect this signal. We conclude that in BMDM SFKs phosphorylate a wide array of substrates, including c-Abl, and c-Abl downstream targets include a more limited number of proteins, some of which play a key role in regulating cell migration.

A closer view of cells at the margin of the wound showed that wild-type BMDM acquire an elongated morphology protruding large lamellipodia towards the wound space (Fig. 2H). In contrast, cells treated with imatinib remained rounded and showed no sign of polarization. Such a circular, multipolar morphology was reported to be a feature of macrophages with the genetic inactivation of c-Fgr and Hck or some of their substrates (see [2] for a discussion of this topic). PP2 caused a more profound alteration of BMDM morphology also inducing reduction of cell spreading and cell detachment from the adherent surface (Fig. 2H). We conclude that c-Abl is an essential component of a SFK-based signaling network regulating macrophage polarization and migration. In order to strengthen the conclusion that c-Abl regulates cell polarization and this requires its kinase activity, we examined the murine hematopoietic cell line Baf3 expressing wild Bcr-Abl or Bcr-Abl with the T315 mutation that inhibit imatinib binding. As shown in Fig. 2I, compared with the same cell line transfected with an empty vector, Bcr-Abl expressing cells are strongly polarized and both imatinib and PP2 reverted this phenotype to a circular morphology. Interestingly, the constitutive polarized phenotypes of Baf3 cells expressing Bcr-Abl with the T315 mutation was not reversed by imatinib, but only by PP2. These findings suggest that SFKs and c-Abl are both required to regulate cell polarization but, in line with their predominant role on tyrosine phosphorylation signals (Fig. 1G) and functional responses in macrophages (Fig. 2H), SFKs play an essential role also in cells expressing the constitutively active Bcr-Abl oncoprotein.

3.3. c-Abl and SFKs regulate Cdc42 and Rac activation

Because oncogenic forms of c-Abl have been reported to regulate actin dynamics in a manner dependent on the small GTPase Rac [15,21,22], we asked whether regulation of macrophage migration by c-Abl depended on Rac or Cdc42, another RhoGTPase implicated in actin polymerization and membrane protrusive activity. As shown in Fig. 3, the chemoattractant LPA increased the amount of active, GTP-bound Cdc42 and Rac in BMDM. Imatinib mesylate markedly reduced both GTP-bound Cdc42 and Rac levels 30 min following stimulation. Consistent with previous reports demonstrating that SFK-deficient macrophages display a reduced migratory ability [4,5], Cdc42 and Rac activation were lower both in unstimulated and LPA-treated *hck*−/−*fgr*−/− BMDM and further reduced by imatinib mesylate.

3.4. c-Abl regulates polarization and migration of normal and CML human neutrophils

Having implicated c-Abl in regulation of murine macrophage polarization and migration, we asked whether it also regulates human myeloid cell migratory ability. In a standard 3 μM pore transwell assay fMLP-induced neutrophil migration was markedly inhibited by both imatinib mesylate and by bosutinib, an inhibitor displaying a dual specificity for SFKs and c-Abl [23] (Fig. 4A). Inhibition of c-Abl also hampered the capability of fMLP to trigger a

polarized phenotype (Fig. 4B). Importantly, comparing neutrophils from control subjects and first diagnosis patient with Philadelphia-positive CML expressing Bcr-Abl, we found that fMLP-induced chemotactic migration of CML neutrophils was markedly higher (Fig. 4C).

4. Discussion

SFKs are essential components of a signal transduction pathway regulating macrophage cytoskeleton dynamics [2–7]. The genetic deficiency of SFKs [3,4] or some of their substrates and/or interacting proteins such as Pyk2, FAK and c-Cbl results in profound alterations in macrophage morphology, polarization and migration (reviewed in [2]).

Implication of SFKs in macrophage migratory ability has been linked to their role in signal transduction by integrins [2–4]. Integrin signaling in phagocytic cells has been shown to be similar to immune receptor signaling and based on a SFK/ITAM-containing adaptor/Syk module (reviewed in [7,24]). Noteworthy, c-Abl has been placed in between SFKs and Zap-70 in TCR-mediated signal transduction leading to T-cell activation [9] and the formation of the T-cell immunological synapse [10]. Additionally, c-Abl was recently implicated in regulation of β2 integrin-mediated neutrophil adhesion [25]. Our results extend these observations supporting the conclusion that c-Abl is an essential downstream target of a SFK-mediated integrin signaling network in phagocytic cells.

Macrophage chemokinetic migration and neutrophil chemotaxis in response to different chemoattractants depends on the c-Abl kinase activity (Figs. 2 and 4). Notably, c-Abl was recently shown to positively regulate chemokine-induced T cell migration [10]. These results are in line with the evidence that Bcr-Abl increases spontaneous, chemokinetic migration of both $CD34^+$ hematopoietic precursors [16] and Baf3 cells [11]. Additionally, the findings that CML neutrophils display an enhanced migratory ability may explain alteration in adhesion and trafficking of CML cells [26].

Several reports implicated c-Abl in regulation of cytoskeleton dynamics underlying membrane ruffling, filopodia formation and lamellipodia protrusion [10,15,21,22,27]. Consistent with their essential role in regulating actin polymerization, Rho GTPases have been placed downstream the Abl/Arg family of tyrosine kinases in signal transduction to the cytoskeleton (see [21,22,25,27]). Consistent with these studies, we found that stimulation of macrophage chemokinesis by LPA was accompanied by Cdc42 and Rac activation and this depended on both c-Abl kinase activity and SFK expression (Fig. 3).

Stimulation through both chemoattractants and integrin adhesive receptors play a coordinated, essential role in mediating myeloid cell migration (see [2]). We hypothesize that the most upstream component of the integrin signaling pathway is represented by a complex formed by a SFK (either Hck or c-Fgr) and c-Abl. Signals via this complex seem to be indispensable also for responses to chemoattractants. These findings suggest that Abl-specific or Abl-Src dual specific inhibitors may represent new drugs to control leukocyte recruitment into inflammatory lesions.

Acknowledgments

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Structured summary

MINT-7296608: *Integrin beta-1* (uniprotkb:P09055) *physically interacts* (MI:0914) with *Hck* (uniprotkb: P08103), *Abl* (uniprotkb:P00520) and *Fgr* (uniprotkb:P14234) by *anti bait coimmunoprecipitation* (MI:0006)

MINT-7296596: *Integrin beta-1* (uniprotkb:P09055) *physically interacts* (MI:0914) with *Fgr* (uniprotkb: P14234) and *Abl* (uniprotkb:P00520) by *anti bait coimmunoprecipitation* (MI:0006)

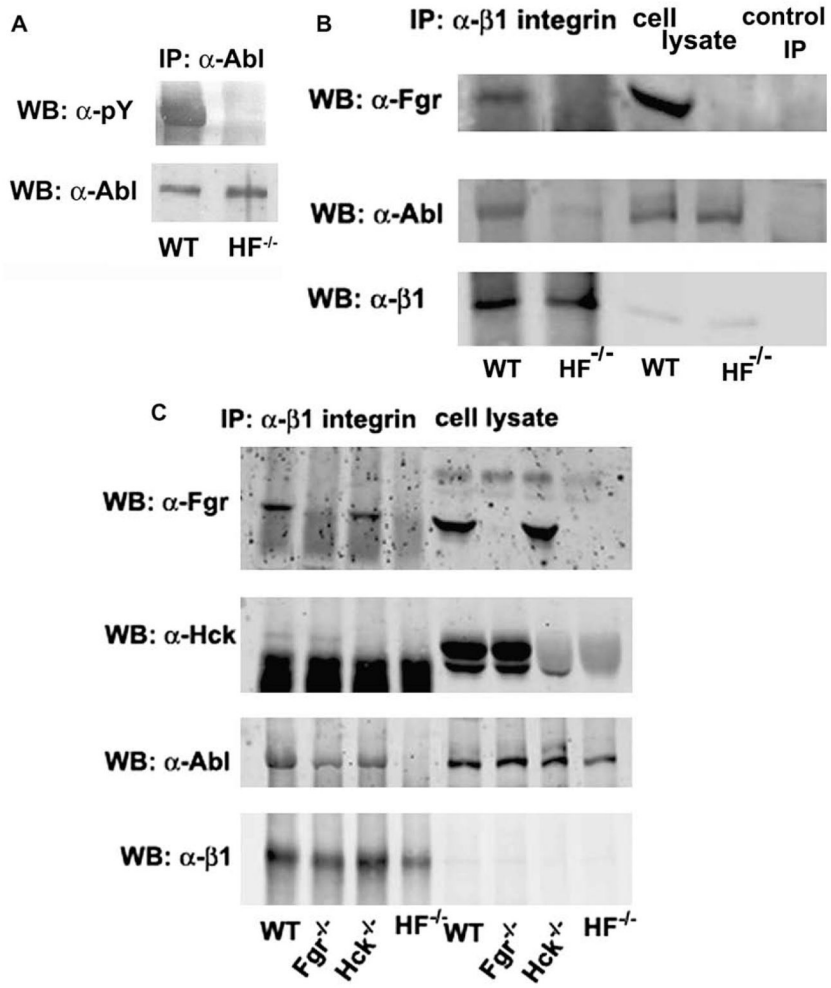

Fig. 1.

c-Abl is a substrate of SFKs in BMDM and binds to c-Fgr or Hck complexed to the β1 integrin chain. (A) BMDM, isolated and cultivated as described in Section 2, were lysed and anti-c-Abl immunoprecipitates (IP) separated on SDS/PAGE gels. Proteins were blotted on nitrocellulose and blots probed with Abs of the indicated specificity (WB). One representative experiment of 2–4 performed is reported. (B and C) BMDM lysates were incubated with anti-β1 integrin Ab and immunoprecipitates (IP) processed as described above with Abs of the indicated specificity (WB). WT, wild-type BMDM; $HF^{-/-}$, *hck−/−fgr −/−* BMDM. Two representative experiment of three performed are reported.

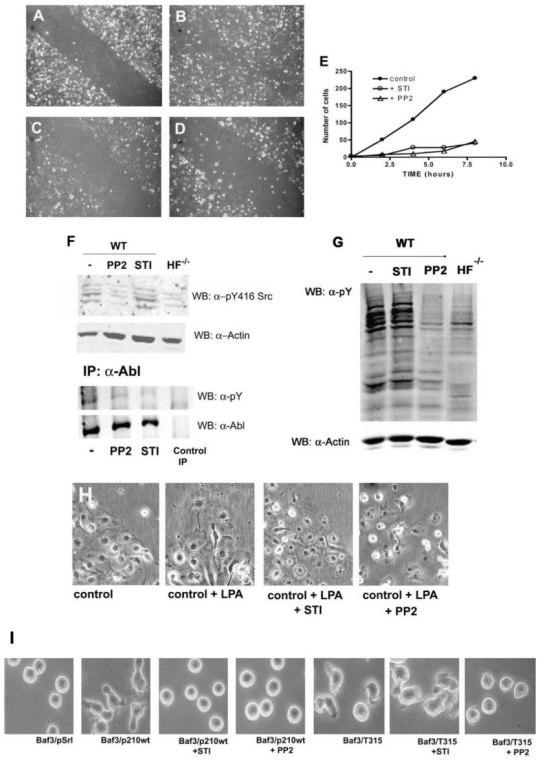

Fig. 2.

Macrophage migration requires the c-Abl kinase activity. (A–D) BMDM monolayers were wounded with the tip of a pipette and, after washing, added with medium supplemented with 100 ng/ml LPA without (A, B) or plus 10 μM imatinib mesylate (STI) (C) or 10 μM PP2 (D). Monolayers were photographed immediately after the wound (A) or after 6 h from wounding (B–D). (E) Cells migrated into the wound were quantified after different time from wounding. (F) BMDM were incubated with control medium (−) or medium supplemented with 10 μM imatinib mesylate (STI) or 10 μM PP2 for 30 min before lysis. To detect phosphorylation of SFKs, lysates were separated on SDS/PAGE gels, proteins blotted on nitrocellulose and blots probed with Abs of the indicated specificity (WB). To detect c-Abl tyrosine phosphorylation, BMDM were lysed in RIPA buffer as described in Section 2 and immunoprecipitated (IP) with anti-Abl Abs before blotting. (G) BMDM were incubated with control medium (−) or medium supplemented with 10 μM imatinib mesylate (STI) or 10 μM PP2 for 30 min before lysis. (H) Phase contrast images of cells at the margin of the wound after 2 h are shown. These were taken at 40× magnification with an Olympus IX50 microscope and acquired with an Olympus c-7070 wide zoom Digital compact camera. Inhibitors were used at the concentration reported above. One typical of several experiment performed is reported. (I) Baf3 cells expressing Bcr/Abl are strongly polarized. Baf3 cells transfected with an empty vector (Baf3/pSrl) or a vector containing wild-type Bcr/Abl (Baf3/p210wt) or Bcr-Abl with the T315 mutation (Baf3/T315) were cultivated and incubated with STI (10 μM) or PP2 (10 μM) as described in Section 2. Morphology of cells either untreated or treated with STI or PP2 is shown. Cells were plated in 24 well plates in RPMI 1640 medium containing 10% FBS and phase contrast images at 40× magnification taken as described in the legend.

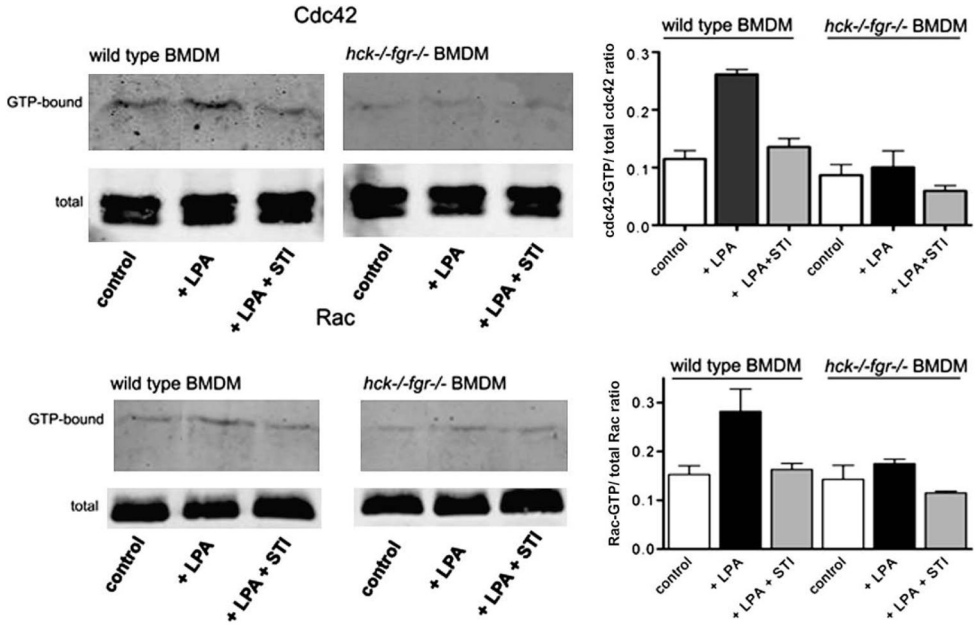

Fig. 3.

SFKs an c-Abl regulate Cdc42 and Rac activation. BMDM were isolated, cultivated and lysed as described in Section 2. GTP-bound Cdc42 and Rac were assayed after 30 min of incubation with 100 ng/ml LPA without or with 10 μM imatinib mesylate as described in Section 2 and mean result of three independent experiments are shown.

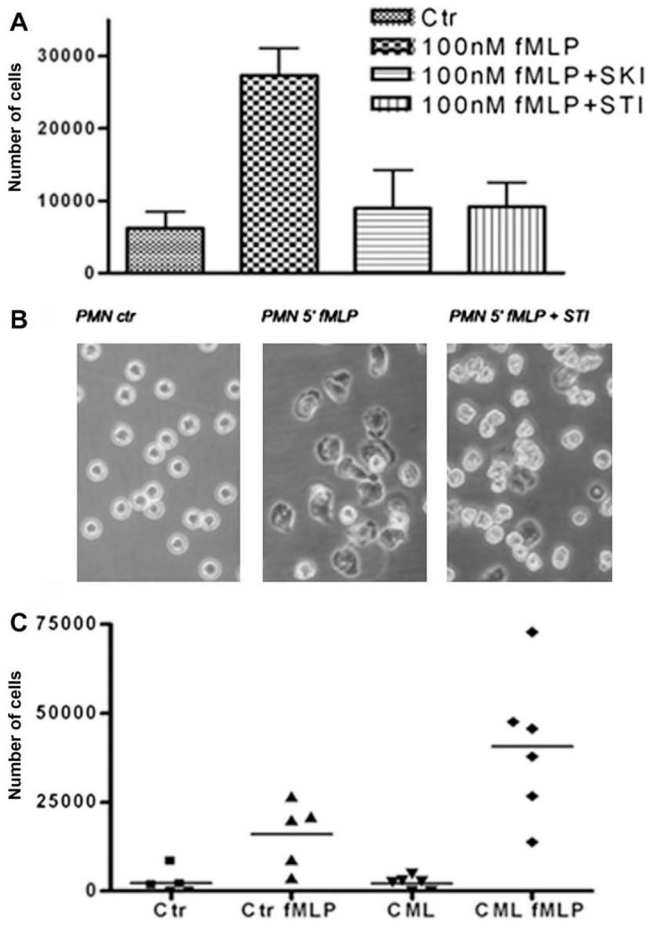

Fig. 4.

c-Abl regulates human neutrophil migration and polarization. (A) Neutrophils were incubated with 1 μM imatinib (STI) or bosutinib (SKI) for 30 min before transfer to the upper compartment of 3 μm pore transwells. Neutrophils migrated to the lower compartment of the transwell that contained or not (Ctr) 100 nM fMLP were quantified after 60 min. Mean results ± S.D. of three independent experiments are reported. (B) Control or imatinib (STI)-treated neutrophils were stimulated with 100 nM fMLP for 5 min. (C) Neutrophils from healthy donors or first diagnosis CML patients were assayed as in (A).