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Review

Regulation of phagocyte migration and recruitment by Src-family kinases

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Abstract. Src-family kinases (SFKs) regulate different granulocyte and monocyte/macrophage responses. Accumulating evidence suggests that members of this family are implicated in signal transduction pathways regulating phagocytic cell migration and recruitment into inflammatory sites. Macrophages with a genetic deficiency of SFKs display marked alterations in cytoskeleton dynamics, polarization and migration. This same phenotype is found in cells with either a lack of SFK substrates and/or interacting proteins such as Pyk2/FAK, c-Cbl and p190RhoGAP. Notably, SFKs and their downstream targets also regulate monocyte recruitment into inflammatory sites. Depending on the type of assay used, neutrophil migration *in vitro* may be either dependent on or independent of SFKs. Also neutrophil recruitment in *in vivo* models of inflammation may be regulated differently by SFKs depending on the tissue involved. In this review we will discuss possible mechanisms by which SFKs may regulate phagocytic cell migratory abilities.

Keywords. Src-family kinases, neutrophil, macrophage, cell migration, cell adhesion, signal transduction, integrins.

Introduction

Evidence accumulated in the last 15 years assigns a central role to Src-family kinases (SFKs) in the regulation of a wide array of both non-myeloid and myeloid leukocyte responses, including cell proliferation and survival, cytokine secretion, phagocytosis, degranulation, reactive oxygen species generation, and integrin-dependent firm adhesion. The implication of SFKs in such diverse cell responses to environmental stimuli is supported by data highlighting the fact that different receptors, including immune receptors, growth factor receptors, integrins, trimeric G protein-coupled receptors, and selectin counter-receptors, transduce signals intracellularly via SFKs. Recent reviews have addressed the continually growing set of information relating SFKs with leukocyte responses and the reader is referred to these reports for a more comprehensive view of this topic [1–4]. The focus of this review will be limited to the involvement of SFKs in the regulation of phagocytic cell migration and recruitment into inflamed tissues. This issue has not yet been addressed in sufficient depth, but we believe that the available evidence suggests that SFKs play a relevant role in these responses and encourages studies to elucidate the mechanisms involved. Importantly, the road driven by the search for more effective Abl tyrosine kinase inhibitors for the treatment of chronic myeloid

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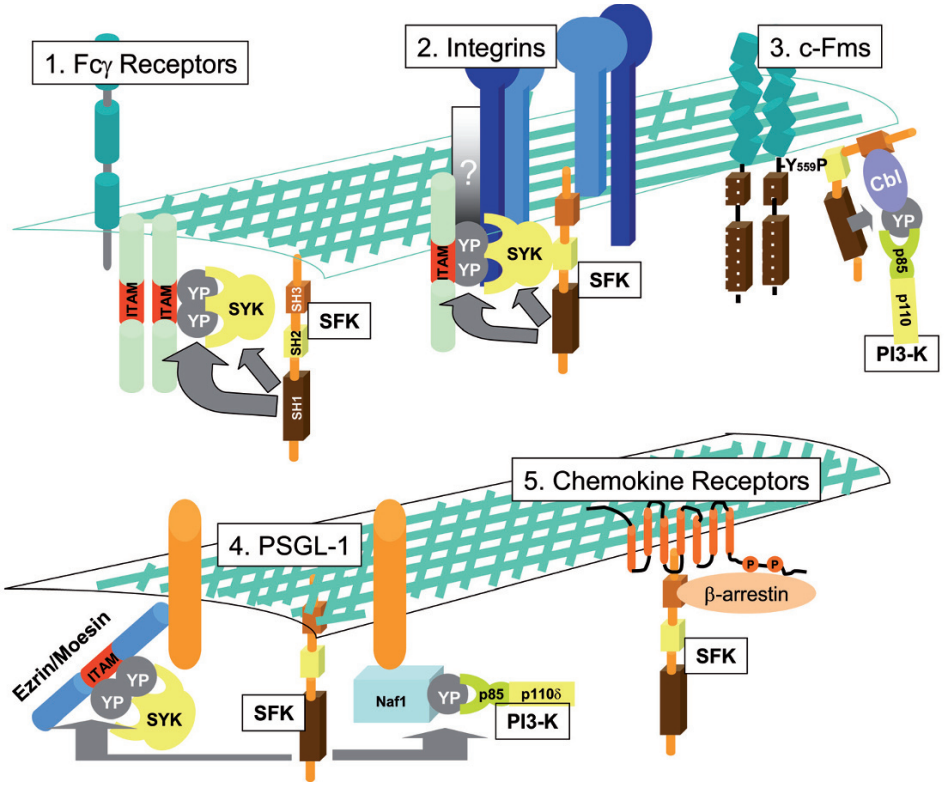

Figure 1. Signal transduction pathways implicating SFKs in phagocytic cells. Immune receptors and integrins share a common pathway based on phosphorylation of immunoreceptor tyrosine activation motif (ITAM)-containing adaptors by SFKs. Syk binds to phosphorylated tyrosine residues of ITAM-containing adaptors and is phosphorylated and activated by SFKs. Because SFKs have been reported to associate with Fcγ receptors [135–138], this association may represent the mechanism dictating proximity between SFKs and ITAM-containing adaptors. In the case of integrins, both SFKs and Syk have been reported to bind to the integrin β3 or β2 chain (see text). The grey box with a question mark indicates a putative molecule linking integrins to the ITAM-containing adaptors DAP12 and Fc γ−chain (see [12]). SFKs bind to c-Fms in myeloid leukocytes and recruit a c-Cbl/PI3-kinase complex to the membrane [18]. An SFK/c-Cbl/PI3-kinase complex has also been reported in the context of integrin signalling [31]. PSGL-1, the major leukocyte ligand for endothelial selectins, implicates SFKs for phosphorylation of its associated molecule Naf1 which in turn binds PI3-kinase [21]. PSGL-1 also triggers phosphorylation of ezrin and moesin, two ITAM-containing molecules associated with its cytoplasmic tail [20]; it is conceivable that ezrin and moesin phosphorylation is mediated by SFKs. Receptors for chemokines bind SFKs in phagocytes likely via binding to β-arrestin (see text). Grey arrows indicate phosphorylation. As indicated, interaction of SFK with integrins, c-Cbl and β-arrestin have been reported to occur via the SH3 domain.

leukemia has already led to the discovery of second-generation inhibitors displaying an either dual specificity for Abl and SFKs or SFK specificity alone [5]. These drugs could represent new weapons to control leukocyte recruitment and inflammation.

Src-family kinases in phagocytic cell signal transduction: general concepts

All eight SFK members (Src, Blk, Fgr, Fyn, Hck, Lck, Lyn and Yes) have been detected, albeit at variable degrees, in hematopoietic cells [1, 2]. Among phagocytic cells, granulocytes predominantly express Fgr, Hck and Lyn whereas monocyte/macrophages were reported to express also Fyn, Yes and Src [1, 2, 6–8]. As outlined above, SFKs have been implicated in signal transduction pathways triggered by different classes of surface receptors in both neutrophils and macrophages (Fig. 1).

Since the beginning of the studies in this field, most of the attention has been turned to the role of SFKs in signal transduction by immune and integrin receptors. However, SFKs were later shown to participate in other signal transduction pathways triggered by different receptors including chemoattractant receptors, the receptor tyrosine kinase c-Fms, and the endothelial selectin counter-receptor P-selectin glycoprotein ligand 1 (PSGL-1). Importantly, recent studies supported the conclusion that signaling by immune and integrin receptors share common mechanisms implicating SFKs, immunoreceptor tyrosine activation motif (ITAM)-containing adapter molecules and Syk [9–11]. The issue of the convergence between immune receptors and integrin signaling has been recently reviewed in great detail [4, 12, 13].

Other receptors, such as trimeric G protein-coupled receptors for chemoattractants [14–17] and c-Fms [18, 19] seem to implicate only SFKs, but not Syk. In the case of PSGL-1-mediated signal transduction, SFKs were reported to participate in both an ITAM/Syk-dependent [20] and a putative Syk-independent pathway [21].

SFK-based signaling pathways highlighted in Figure 1 have been clearly implicated in key phagocytic cell responses including phagocytosis, release of reactive oxygen intermediates (ROIs) and granule constituents, firm adhesion to integrin ligands, macrophage differentiation and activation, and bone resorption (see [1, 3, 4, 13, 19, 22]). In this review we will focus on studies supporting the conclusion that SFKs regulate phagocyte migration *in vitro* and cell recruitment into inflammatory sites *in vivo*. Additionally we will discuss the possible molecular mechanisms responsible for regulation of phagocyte transmigration by SFKs.

Cross-talk between SFKs and Syk/ZAP-70 in leukocyte migration: a role for integrin signaling

Deficiency of β2 integrin expression results in marked alterations in leukocyte migratory ability *in vitro* and causes leukocyte adhesion deficiency type I (LADI), a syndrome characterized by impaired leukocyte recruitment in sites of inflammation *in vivo* and a reduced capability to eradicate infectious agents [23]. Integrin function depends on two distinct, albeit probably coordinated, events. "Inside-out" signals triggered by a wide variety of agonists of different surface receptors induce conformational changes in the integrin, thus increasing its binding affinity towards specific ligands. Following ligand binding, the integrin activates intracellular signaling pathways ("outside-in signaling") [24, 25]. Due to the early discovery of a wide array of inflammatory mediators that induce leukocytes, and especially neutrophils, to display a particularly high and directed migratory ability, signal transduction regulating leukocyte migration has mainly been studied in the context of chemoattractant receptor-induced responses. Notably, these same chemoattractants were later characterized as powerful agonists of "inside-out" signaling increasing integrin affinity for its ligands. A consistent model emerging from this set of data attributes to integrins the role of keeping leukocytes adherent to the substrate while they polarize and migrate along a chemotactic gradient. Within this model it is conceivable that β2 integrin deficiency results in a marked inhibition of leukocyte migration that, without integrins, behaves like a weightless astronaut.

Similarly, in the classic four-step model (selectin-mediated tethering and rolling, activation, integrin-dependent firm adhesion and trans-endothelial migration) of leukocyte recruitment into inflamed tissues, integrins have been thought to simply act as adhesive receptors which in a high affinity/avidity state arrest the leukocyte rolling along the endothelial surface and give it the time to sense a chemotactic gradient driving it into the subendothelial space. While anchoring of the cell to a substratum is certainly one of the mechanisms by which integrins regulate leukocyte migration, much less attention has been payed to their possible role as signal transduction devices. This contrasts with the established concept that integrin-derived signals regulate cytoskeletal dynamics underlying cell migration in non-hematopoietic cell types [26–28]. Studies in this field have been performed with cells either plated on integrin ligands in the absence of serum or other chemotactic factors, or plated on transwells coated with integrin ligands only on the underneath surface of the filter. This type of motility, which is considered to depend exclusively on integrin signaling, is referred to as haptokinesis or haptotaxis respectively, to distinguish it from chemokinesis and chemotaxis, types of motility induced by a uniform, or gradient-shaped, concentration of a chemoattractant [29]. Although haptokinesis and haptotaxis are unlikely to represent a physiologic mode of motility of leukocytes, we believe that integrin signaling may be critical in regulating some mode of leukocyte migration, working in concert with signals generated by chemoattractants.

Due to the recent evidence that integrin signal transduction requires SFKs, ITAM-containing adapter molecules and Syk (see above) we made a survey of the literature implicating SFKs and Syk/ZAP 70 in regulation of leukocyte migration (Table 1).

From this survey it is possible to conclude that, despite a few contradictory reports obtained with neutrophils (Table 1 and below), SFKs and Syk/ZAP70 play a positive role in regulation of migration of different leukocyte subtypes including B and T cells, mast cells, granulocytes and monocyte/macrophages. It must be noted that most of the studies highlighted in Table 1 exploited chemotactic assays, i.e. assays examining migration induced by chemokines or other chemoattractants, including stem cell factor (SCF), a ligand for the c-Kit receptor protein-tyrosine kinase. Therefore, the simplest interpretation of these findings would be to place the role of these kinases within trimeric G protein-coupled chemoattractant receptor and receptor protein-tyrosine kinase signaling. However, as we will discuss below, the implication of SFKs and/or Syk/ZAP70 in leukocyte migration may well derive from their role in signals generated by integrins

Table 1. Implications of Src-family kinases and ZAP-70/Syk in regulation of leukocyte migration.

Kinase	Cell type	Comments and References
Fgr	Neutrophils, macrophages, cell lines	Neutrophils deficient in both Fgr and Hck or dendritic cells with the sole deficiency of Fgr display a normal or even more robust chemotactic response to chemokines [61]. In response to the chemotactic peptide fMLP, hck-/-fgr-/- [60] or hck-/-fgr-/-lyn-/- [55] neutrophils migrate normally through 3 μm pore transwell filters but hck-/-fgr-/- fail to migrate through 1 μm transwell filters [60]. hck-/-fgr-/- macrophages display a reduced chemokinetic and chemotactic ability [32, 33]. Expression of full length Fgr in COS-7 cells markedly enhances haptokinetic migratory ability [36]. Receptors for chemokines (CCR3, CXCR1) directly [15] or indirectly [16] interacts with Fgr and Hck in granulocytes and the urokinase plasminogen-activator-receptor, a powerful chemoattractant, forms a membrane signaling complex containing the Src-family kinases, Fgr, Fyn, Lyn and Hck, as well as β2 integrins, in monocytes [6].
Hck	Neutrophils, macrophages, cell lines	Hck expression in the monocytic cell line U937 enhances cell migration [34]. Neutrophils expressing a constitutively active Hck display an enhanced capacity to migrate towards fMLP [37]. A constitutively active p59Hck isoform induces small GTPase-dependent plasmamembrane protrusions in non-myeloid and myeloid cells, and p61Hck regulates podosome formation [35, 84]. Tyrosine residues of ELMO1 – a protein regulating Rac activation by the Dock180/Crk pathway –, that are phosphorylated by Hck, are essential to promote fibroblast migration [92].
Lyn	Neutrophils, mast cells, hematopoietic precursors, cell lines	Neutrophil stimulation with chemoattractants activates a Lyn/Shc/p85-p110 phosphatidylinositol 3-kinase pathway (PI-3K) [93, 94]. Expression of a constitutively active form of Lyn in rat basophilic leukaemia 2H3 cells expressing the Src-family kinase inhibitor Csk, in which fibronectin-induced signalling is suppressed, restores podosome formation and cell migration [95]. Stem cell factor (SCF)- and IgE-induced chemotaxis is reduced in Lyn-deficient mast cells [96, 97]. Reduced SCF-induced cell migration in cell lines expressing some SCF receptor (c-Kit) mutants, correlates with decreased Lyn activation [98]. Interaction of the chemokine Stromal cell-derived factor (SDF/CXCL12) with its receptor (CXCR4) triggers a signalling pathway implicating Lyn and PI-3K in HL-60 cells and hematopoietic progenitors and SDF-induced cell migration is defective in Lyn-deficient bone marrow cells [99]. Down-regulation of Lyn expression by siRNA reduces CXCR4-dependent cell migration in hematopoietic precursors increasing integrin-dependent cell adhesion in parallel [100]. Also Lyn-deficient neutrophils and macrophages display an hyperadhesive integrin signalling-dependent phenotype [72]. Dominant negative Lyn mutants inhibit the CrkL-dependent enhancement of SDF-induced hematopoietic cell chemotaxis and CrkL phosphorylation [101].
Fyn	Mast cells, cell lines	SDF/CXCL12-induced cell migration in CXCR4 expressing pre-B lymphoma cells involves formation of complexes between CXCR4, the tyrosine phosphatase SHP2, Cbl and Fyn [102]. SCF-induced cell migration is reduced in Fyn-deficient mast cells [103, 104]. Chemoattractant receptor engagement in a T cell line induces Fyn-dependent PI-3K activation, and overexpression of a Fyn mutant form inhibits chemoattractant-induced cell migration [105].
Lck	Lymphoid cell lines	Uncoupling of interaction between CD4 and Lck suppresses the chemotactic migration of a T cell line [106]. Lck expression is required for SDF/CXCL12-induced cell migration and signal transduction in CXCR4-expressing Jurkatt T cells [107, 108].
Syk	Lymphocytes and lymphoid cells, cell lines	MIP-1β/CCL4-CCR5 interaction induces formation of a signaling complex which includes Syk [109]. Expression of Syk in COS-7 cells induces α4β1/Rac2-dependent cell migration [46]. Syk regulates SDF/CXCL12-induced polarization in B cell lines [110]. Syk is required for cytoskeleton remodelling and cell migration of the macrophage celll line RAW264.7 in response to the chemokine CX3CL1 (Fractalkine) [47]. Syk regulates lamellopodia formation and turnover, and cell migration in neutrophils and myelomonocytic cell lines [48, 68].

Table 1 *(Continued)*

Kinase	Cell type	Comments and References
ZAP-70	Lymphocytes and lymphoid cells	Overexpression of a dominant-negative ZAP-70 protein in a T cell line, blocks LFA-1-dependent cell migration in response to SDF/CXCL12 in an ITAM-dependent, but TCR/CD3 complex-independent manner [111]. SDF/CXCL12-induced Jurkat T cell and T lymphocyte migration requires kinase active ZAP-70 [112, 113]. ZAP-70 expressing chronic lymphocytic leukaemia B-cells (B-CLL) display enhanced migration towards CCR7 ligands (MIP-3β/exodus-1/CCL19 and 6Ckine/exodus-2/CCL21) if compared to ZAP-70 negative B-CLL [114].

and acting in concert, as it occurs in non hematopoietic cells stimulated with growth factors, with chemoattractant-derived signals.

Regulation of macrophage migration and recruitment by SFKs

The first strong evidence suggesting a link between a chemoattractant receptor, β2 integrins and SFKs in regulating mononuclear phagocyte migration derives from studies showing that the urokinase type plasminogen activator-receptor (uPA-R), the β2 integrins LFA-1 and CR3, and the SFK members Hck, Fgr, Lyn and Fyn form a large molecular complex in human monocytes [6]. The finding that cell stimulation with uPA triggers tyrosine phosphorylation responses suggested a functional role of this complex in regulation of monocyte migration. Interestingly, this complex was reported to have size and detergent solubility properties similar to those of detergent-resistant, glycosylphosphatidylinositol (GPI)-anchored-, sterol- and sphingolipid-enriched membrane microdomains referred to as "membrane-rafts". However, and somewhat surprisingly, the possibility that signal transduction by integrins in phagocytic cells requires these microdomains has not been further addressed. A definite demonstration of the role of SFKs in macrophage migration had to await the generation of mice with the genetic inactivation of SFKs expressed at high levels in this cell type. Due to a highly redundant function of these kinases in regulation of macrophage responses [30], examining only cells from mice with the triple inactivation of the *hck*, *fgr* and *lyn* genes Meng and Lowell could demonstrate, in the first pivotal study on this topic, that SFKs are essential for the recruitment of monocytes into the peritoneal cavity in the thioglycolate-induced peritonitis model [31]. *hck-/-fgr-/-lyn-/-* primary peritoneal macrophages also displayed a profound defect in their capability to spread over surface-bound fibronectin, despite expressing several integrin subunits at the same level of wild type cells. These findings prompted studies to address the migratory ability of SFK deficient macrophages in conventional *in vitro* assays. The chemokinetic ability of *hck-/-fgr-/-* macrophages was reduced in wound-healing assays or in transwell migration assays through porous membranes coated with fibronectin or matrigel [32]. A later study reported that *hck-/-fgr-/-* macrophages also displayed a defective chemotactic response to zymosan-activated serum [33].The concept that SFKs regulate macrophage migration also derives from studies complementary to those exploiting cells deficient in SFKs. Overexpression of wild type or constitutively active Hck increased the chemotactic migration of the myelomonocytic cell line U937 [34]. Similarly, overexpression studies in non myeloid cell lines, showed that kinase active Hck and Fgr are able to induce lamellopodial protrusions typical of polarized and actively migrating cells and to markedly increase haptokinetic migration, respectively [35, 36]. In a very interesting study, Ernst and colleagues generated mice expressing a constitutively active mutant of Hck carrying a substitution of the C-terminal regulatory tyrosine (Y499) with phenylalanine [37]. The mice with this "gain-of-function" Hck mutation acquired a lung pathology characterized by monocyte and eosinophil infiltration within the lung interstitium and the airways, and macrophages from these mice displayed an enhanced migratory ability in wound-healing assays.

Hck and Fgr are essential component of an integrin-dependent signaling network regulating macrophage cytoskeleton dynamics Exploiting the availability of cells from mice with a deficiency in SFKs, some proteins acting as substrates of, or interacting with, SFK members expressed in mononuclear phagocytes have been unequivocally identified (see [3] for a recent review and Table 2). In *hck-/-fgr-/-* peritoneal macrophages (PEM) plated on fibronectin, phosphorylation of paxillin, cortactin, tensin, Pyk2 and Syk was markedly reduced compared to wild type cells [32]. Both *hck-/-fgr-/-lyn-/-* PEM plated on fibronectin, and *hck-/-fgr-/-* PEM and bone marrow-derived macrophages (BMDM) maintained in culture in serum-containing medium displayed a profound reduction in tyrosine phosphorylation of c-Cbl [31, 33]. Additionally, c-Cbl interacted with Fgr and Lyn [31]. Pyk2

Table 2. Components of the SFK-mediated signalling pathway regulating phagocytic cell migration.

Component	Comments and References
Syk	Syk tyrosine phosphorylation requires the SFK members Hck and Fgr in both neutrophils [55] and macrophages [32]. Syk was reported to be implicated in regulation of migratory ability of murine macrophages and human monocytic and neutrophilic cell lines [46–48, 68].
Pyk2/FAK	Pyk2 is a substrate of Hck and Fgr and interacts with Fgr in murine macrophages and Src in osteoclasts [32, 36, 41]. Both *pyk2-/-* and *FAK-/-* macrophages display morphological alterations similar to *hck-/-fgr-/-* macrophages and reduced migratory ability [38, 39].
c-Cbl	c-Cbl is a substrate of SFKs and interacts with Fgr and Lyn in murine macrophages and Src and Pyk2 in osteoclasts [31, 33, 41]. Macrophages with the deficiency of c-Cbl or Cap, a c-Cbl binding protein, display alterations of morphology and migratory ability similar to *hck-/-fgr-/-*, *pyk2-/-* and *FAK-/-* macrophages [33, 40].
Phosphoinositide 3-kinases (PI3K)	The γ isoform of PI3K, which is activated by G protein-coupled receptors, is required for neutrophil and macrophage polarization and their migratory response to chemoattractants [115–118]. However, class IA PI3K, which are regulated by tyrosine phosphorylation signals, have been also reported to regulate phagocytic cell migration. Studies with cells deficient of the p85 regulatory or the p110δ catalytic subunit, or exploiting selective inhibitory antibodies, implicated class IA PI3K in regulation of macrophage migration *in vitro* [116, 119, 120]. Deficiency of p110δ [21] or a dominant negative form of p85 [121] cause a reduced recruitment of neutrophils into the peritoneal cavity or the lung, respectively, *in vivo*. c-Cbl-associated, membrane localized PI3K activity is markedly reduced in *hck-/-fgr-/-lyn-/-* triple mutant macrophages and this correlates with reduced cell migratory ability [31].
p190RhoGAP	The Rho inhibitor p190RhoGAP associates with Pyk2 in murine macrophages and is a substrate of Hck and Fgr [36]. An excessive RhoA activation, resulting from deficiency of the transcriptional repressor proto-oncogene BCL16, leads to alterations of murine macrophage morphology and motility similar to those found in SFK, Pyk2/FAK- or c-Cbl-deficient cells [43].
Vav	Vav proteins act as guanine nucleotide exchange factors (GEFs) for Rac and Cdc42 [122]. SFKs and Syk are required to phosphorylate Vav in neutrophils [55, 60, 63]. Despite Vav deficiency does not affect neutrophil chemotactic activity [62, 63], a dominant inhibitory Vav mutant blocks M-CSF-induced murine macrophage chemotaxis [123]. Both SFK- and Vav proteins-deficiency results in reduced sustained adhesion to integrin ligands *in vitro* and *in vivo* [62, 78].
WASp	Wiskott-Aldrich Syndrome protein (WASp) is a key regulator of the Arp2/3 complex and actin polymerization. WASp is a substrate of Hck and Fgr and tyrosine phosphorylated WASp exhibits an enhanced capability to trigger actin polymerization in macrophages [124, 125].
Paxillin	Paxillin, a cytoskeletal adaptor protein recruiting actin-binding and signalling proteins to integrin-based adhesion sites [126], is a substrate of SFKs in macrophages [32, 127, 128].
Cortactin	Cortactin, an F-actin binding protein interacting with the Arp3 subunit of the Arp2/3 complex and regulating its actin-nucleating activity [129], is a substrate of Hck and Fgr in murine macrophages and Fgr-transfected cell lines [32, 36].
c-Abl	Abl kinases regulate cytoskeleton dynamics in multiple cell types [130, 131]. SFKs cross-talk with Abl in regulating haematopoietic cell migration and activation [99, 132, 133]. Cortactin, whose phosphorylaton requires SFKs, is also an Abl/Arg substrate [134]

interacted with p190RhoGAP and Fgr in BMDM, and although formation of Pyk2/p190RhoGAP complexes is independent of Fgr and Hck expression, Fgr and Hck deficiency resulted in a reduced tyrosine phosphorylation of Pyk2 and p190RhoGAP [36]. The role of some of these SFK substrates or interacting proteins in the regulation of macrophage cytoskeleton dynamics and migration has been clearly established. In fact, as we will discuss below, the phenotype of macrophages deficient of Pyk2 or c-Cbl, or with a putative reduction in the inhibitory activity of p190RhoGAP towards Rho, is very similar to the phenotype of SFK deficient macrophages.

Early studies showed that, after short periods of plating over fibronectin-coated surfaces, *hck-/-fgr-/-* BMDM and PEM were much less spread than wild type cells and displayed a rounded morphology [32]. After prolonging the time of cultivation for a few days, mutant cells spread fully, but showed profound alterations in organization of the actin-based cytoskeleton. Wild type BMDM assumed a characteristic elongated, bipolar morphology and phalloidin staining revealed the distribution of filamentous actin mainly along a leading edge at the extremity of one of the two poles of the cell. In marked contrast, *hck-/-fgr-/-* BMDM displayed either a multipolar or a fully circular

morphology, and fluorescently-labelled phalloidin stained multiple lamellipodia and several thin filopodia around the cell periphery. Intriguingly, very similar morphological alterations were detected in Pyk2 deficient macrophages monitored by video microscopy after stimulation with SDF1α [38]. This experimental approach allowed Okigaki and colleagues to show that while wild type macrophages became polarized, form lamellopodia at one cell pole and move the cell body in the direction established by the leading edge, *pyk2-/-* macrophages extended multiple lamellopodia in different directions and showed a reduced ability to follow the leading edge. Additionally, in response to localized SDF1α stimulation, staining with fluorescently labelled phalloidin showed F-actin concentrated at the edge of the cell facing the chemokine gradient in wild type macrophages, while in *pyk2-/-* macrophages F-actin accumulated at multiple sites along the cell periphery.

The decreased migratory ability of *pyk2-/-* macrophages *in vitro* was shown to correlate with a deficit in macrophage tissue infiltration in the carrageenan-induced inflammatory model *in vivo*. A phenotype similar to that of *pyk2-/-* macrophages was recently described in macrophages deficient in FAK [39]. *FAK-/-* BMDM extended and retracted numerous short lived protrusions all around the cell periphery and displayed a reduction in kinetics of adhesion formation and disassembly. A generalized defect in chemotaxis, random motility and invasion through matrigel of *FAK-/-* macrophages correlated with a reduced recruitment of monocytes into the peritoneal cavity in the thioglycollate-induced peritonitis model. Similarly to *hck-/-fgr-/-*, *pyk2-/-* or *FAK-/-* macrophages, macrophages with the genetic inactivation of c-Cbl displayed a more flattened, apolar, circular morphology [33]. Comparison of F-actin distribution in *hck-/-fgr-/-* with *c-cbl-/-* macrophages showed that both mutant cells displayed a large number of thin filopodia around the cell periphery, and exhibited a reduced chemokinetic and chemotactic activity. Notably, SFKs regulated not only c-Cbl tyrosine phosphorylation but also its capability to bind and redistribute a phosphatidylinositol 3-kinase (PI-3 kinase) activity to a detergent insoluble fraction [31]. It is striking that silencing expression of Cap, a protein binding to and redistributing c-Cbl to lipid raft microdomains in the murine macrophage cell line RAW264.7, was recently reported to result in a marked increase of cell spreading, rounding and formation of several peripheral filopodia-like protrusions [40]. Cap silencing also caused a reduction in phosphorylation of c-Cbl at early time points after adhesion and in cell migratory ability in an *in vitro* wound healing assay. Examination of Cap knockout mice and mice that, after irradiation, were reconstituted with bone marrow cells deficient in Cap led to the important finding that deficiency of Cap in macrophages protected mice from-high-fat diet induced insulin resistance and this correlated with decreased infiltration of the adipose tissue by macrophages.

The findings summarized above suggest that SFKs, Pyk2/FAK and c-Cbl constitute a signaling complex playing a critical role in regulating integrin-dependent cytoskeleton dynamics implicated in macrophage migration. Genetic deficiency of one of these molecules caused very similar morphological alterations and resulted in a decreased murine macrophage migratory ability both *in vitro* and *in vivo*. Notably, SFKs, Pyk2 and c-Cbl also form a molecular complex regulating osteoclast adhesion and migration [41, 42]. In macrophages, the Rho inhibitor p190RhoGAP interacts with this complex via direct or indirect binding to Pyk2 [36]. Strikingly, Pixley and colleagues found that deficiency of the proto-oncogene BCL6 in macrophages resulted in an excessive RhoA activation, a decreased translocation of p190RhoGAP at the plasma membrane and an altered cell morphology which has several features in common with that described above for SFK-, Pyk2/FAK- or c-Cbl-deficient cells [43]. In fact, *Bcl6-/-* macrophages are less polarized and display a multipolar stellate or an apolar circular morphology; additionally they exhibit a reduced chemokinetic and chemotactic activity.

It is important to note that although the above summarized studies point to a role for an SFK/Pyk2/FAK/c-Cbl/p190RhoGAP signaling complex in regulating macrophage polarization and membrane extension at a leading front, a more insightful knowledge of steps of cell migration regulated by this complex is still missing. In order to move, a cell must generate a protrusive force at the leading edge and, following adhesion of the protruded lamellopodium to the substratum, translocate the cell body forward and, after disruption of cell-substratum attachments, detach the cell rear [44]. In migrating cells, integrin-based sites of adhesion must therefore constantly form at one side and disassemble at the other one. An important aspect of the integrin function in cell migration is not related to their capability to bind substrate components, to deliver signals triggering actin polymerization and cell protrusion or to be the target of inhibitory signals that disassemble focal adhesions at the cell rear, but concerns the actual fate and redistribution of integrins in the migrating cell [44]. As discussed by Lauffenburger and Horwitz, a fraction of the integrins from the rear adhesions are internalized in endocytic vesicles which then fuse with the cell surface thus allowing the recycled integrins to

diffuse towards the leading front to support new adhesion sites. Surprisingly, the role of SFKs in the regulation of this important step of cell migration has not been addressed. In fact, SFKs regulate membrane traffic [45] and, as far as phagocytic cells are concerned, granule-plasma membrane fusion is a neutrophil function which has been reported to be SFK-dependent in several studies (see below).

The recent evidence that integrin signaling in myelomonocytic cells implicates SFKs, ITAM-containing adaptors and Syk (see above and Fig. 1) raises the issue of the role of Syk in regulation of macrophage cytoskeleton dynamics and migration. As outlined in Table 1, several studies with both myeloid and lymphoid cells assigned a role to ZAP-70/Syk in regulation of leukocyte migration. In murine macrophages, both integrin ligand-induced haptokinetic and chemokine-induced chemokinetic migration were reported to be Syk-dependent [46, 47]. Additonally, Syk redistributes to the leading front in response to the chemokine CCL2/MCP-1 in the human monocytic cell line THP-1 [48]. Although Syk binding to phosphorylated ITAM tyrosine residues of adaptor molecules is essential for full Syk activation and downstream signaling (reviewed in [12]), Syk interacts with SFKs, integrins and c-Cbl in leukocytes (see [11, 49–51]). Altogether these findings suggest that also in leukocytes integrins are the site of organization of a signaling platform whose components cross-talk to induce an early reciprocal activation followed by the phosphorylation of critical downstream targets. We believe that SFKs are upstream of the full formation of this signaling complex. SFKs constitutively bind to different β integrin chains [52]. As far as myeloid cells are concerned, Src was reported to bind to the β3 integrin in osteoclasts constitutively [11] and we found that Fgr and Hck associated with β1 in BMDM (unpublished observation). Syk also interacted with β3 [11] or β2 integrins in osteoclasts or myelomonocytic cells [50, 51, 53, 54], respectively, but, depending on the type of cells or the assay conditions used, this association was reported to be either constitutive [50, 53, 54] or secondary to integrin engagement [11, 51]. Independently of the mechanism of Syk recruitment into the integrin-organized signaling complex that, as outlined above, can be direct, i.e. via binding to the integrin β chain, or indirect, i.e. via binding to SFKs or c-Cbl, Syk phosphorylation requires SFKs [11, 32, 55]. These last findings suggest that Syk acts downstream of SFKs in integrin signal transduction in phagocytic cells. Although the simplest way to interpret the above summarized findings is that, due to their proximity, SFKs phosphorylate and activate Syk, new recent and exciting data showed that integrin-stimulated Syk activation requires ITAM-containing adapters [4, 9–12] whose tyrosine residues are phosphorylated by SFKs [10].

Besides those discussed above, other substrates or interacting partners of SFKs may be implicated in the signaling network regulating phagocytic cell migration. Table 2 highlights a few of the proteins that are likely to play the most significant role. Interestingly, one of these proteins is the non receptor tyrosine kinase c-Abl. SFKs were reported to cross-talk with c-Abl within signaling pathways regulating different leukocyte responses (Table 2; see [56, 57] for reviews). Our own studies showed that c-Abl is phosphorylated in an Fgr/Hck-dependent manner in murine macrophages and c-Abl inhibitors reduce macrophage migratory ability (AB and GB, unpublished observation). In this context, it is worth noting that, although the issue of leukocyte migration was not specifically addressed in that study, imatinib mesylate (a c-Abl inhibitor formerly known as STI-571) was reported to be an effective treatment for a murine form of autoimmune arthritis [58].

Are Src family kinases implicated in regulation of neutrophil chemotactic movement and recruitment?

The evidence that SFKs regulate macrophage migration prompted studies to address whether the movement of the highly motile neutrophil towards a chemotactic gradient also depends on expression or function of SFKs. *In vitro* migration assays through transwells with 3 μm diameter pores in response to fMLP, an assay that has represented the gold standard to examine neutrophil chemotaxis, clearly showed that both *hck-/-fgr-/-* and *syk-/-* neutrophils do not display any alteration in chemotactic activity [17, 55]. These early findings were subsequently confirmed in both fMLP and chemokine-induced migration assays [59, 60]. Interestingly, neutrophils and dendritic cells from *hck-/-fgr-/-* mice were reported to be even more responsive to chemokines, a finding that was demonstrated to depend on the reduced tyrosine phosphorylation of the immunoreceptor tyrosine inhibitory motif (ITIM)-containing surface molecule PIR-B, and the consequent reduced recruitment of the inhibitory tyrosine phosphatase SHP-1/2 in mutant cells [61]. In line with these reports, studies with cells deficient of Vav proteins, guanine nucleotide exchange factors (GEFs) for Rho GTPases that are substrates of SFK/Syk (see [55, 60, 62]), reported no, or only a partial, alteration in fMLP-induced neutrophil chemotactic ability [62, 63]. Altogether these findings suggest that when neutrophils migration occurs through relatively large pores, as in standard transwell assays, in response

to an optimal chemoattractant gradient, the role played by SFKs and Syk in its regulation is marginal. In other words, despite the fact that integrins are essential for migration, as suggested by the markedly defective fMLP-induced migratory ability of β2 integrin-deficient neutrophils in the same type of assay (see [55]), the role they play seems to depend not on their SFK/Syk-mediated signaling ability but, as discussed above, on their action as adhesion receptors that anchor the cell to a substrate. The findings summarized above also suggest that implication of SFKs in trimeric G protein-coupled receptors signaling (Figure 1) has not relevance in the context of chemotactic migration, but other neutrophil responses to chemoattractants, such as degranulation [14, 16, 59, 64, 65] and ROIs generation [60]. A few *in vivo* studies on neutrophil recruitment in inflammatory lesions also supported the conclusion that SFKs and Syk are dispensable for cell migration. In fact, neutrophil recruitment into the peritoneal cavity during a thioglycollate-induced sterile peritonitis, into CXCL1/MIP-2-containing Matrigel sponges injected subcutaneously or into the skin and during the development of an LPS/TNF-induced local Shwartzman reaction, was not affected by deficiency of either SFKs or Syk [55, 59, 61, 65].

Studies with neutrophils expressing constitutively active Hck either alone or together with other SFKs as a consequence of inactivation of the inhibitory C-terminal Src kinase (Csk), only partly confirmed findings with SFK-deficient cells. Neutrophils with active Hck display a higher migratory ability in transwell assays [37]. Although this finding would apparently contradict the normal migratory ability of SFK deficient cells, one possible interpretation is that activation of one SFK may impinge on a signaling pathway that is intrinsically SFK-independent, but can be optimized by SFKs. However, Csk deficient neutrophils migrate less than wild type cells in similar assay conditions and this correlates with a tighter spreading to integrin ligands [66]. Thus, depending on either differences in the extent of integrin engagement, due for example to differences in the ligand used, or the SFK member involved, signals leading to cytoskeleton rearrangements dictating cell spreading might prevail over cytoskeleton dynamics regulating cell migration. That caution must be used before drawing definite conclusions on the role of SFKs in regulating neutrophil migration is suggested by the recent evidence that, although Hck and Fgr deficiency in mouse and treatment with an SFK inhibitor in human neutrophils had no impact on migration through 3 μM diameter pores in response to fMLP, they resulted in a complete block of migration through 1 μM diameter pores [60]. Additionally, neutrophil polarization assays disclosed a requirement for SFKs in recruitment of the Rac GEF P-Rex-1 to the leading front of migrating neutrophils [67] and the recruitment of Syk in complex with the Rac GEF Vav1 to the front lamellopodium of neutrophil-like differentiated HL-60 cells [68]. Interestingly, this last study reported that expression of a Syk kinase-dead mutant or a Syk mutant lacking the Vav binding site in differentiated HL-60 cells resulted in a severe impairment of migration of cells adherent to fibrinogen towards an fMLP gradient. Thus, classical integrin-dependent SFK/Syk-mediated signals may regulate neutrophil polarization and the membrane protrusion force required for transmigration through narrow apertures. In contrast to the reports discussed above [55, 59, 61, 65], results obtained with a few *in vivo* models of inflammation supported the conclusion that SFKs regulate neutrophil recruitment. In a model of lipopolysaccharide (LPS)-induced shock, enhanced survival of $hck^{-/-}fgr^{-/-}$ mice correlated with a reduced accumulation of neutrophils in the liver parenchyma [69]. In addition, marked alterations in the recruitment of granulocytes to the lung were reported resulting from changes in either SFK expression or activity in granulocytes. In a model of allergic airway inflammation eosinophil accumulation into the lung was markedly defective in $hck^{-/-}fgr^{-/-}$ and $fgr^{-/-}$ mice [70]. Inactivation of Csk in granulocytes, which results in hyper-activated SFKs, caused progressive development of pulmonary inflammation in mice [66]. Similarly, knock-in mice expressing constitutively activated Hck also developed spontaneous pulmonary inflammation [37]. These strains of mice manifested increased susceptibility to LPS–induced endotoxic shock, which is associated with a marked increase in the number of granulocytes infiltrating the liver. Interestingly, exaggerated accumulation of myeloid cells in the lung, as well as other organs, is a typical feature of me^{V}/me^{V} mice, which express very low levels of the tyrosine phosphatase SHP-1 [71]. Noteably, reduced recruitment of SHP-1 to the immunoreceptor tyrosine-based inhibitory motifs (ITIM)-containing surface molecules SIRP1α and PIR-B in *lyn-/-* neutrophils resulted in exaggerated integrin-dependent cell activation [72]. Thus in myeloid leukocytes either increased positive signaling, through expression of constitutively activated Src-family kinases, or loss of inhibitory signaling in SHP-1 mutant mice, resulted in the exaggerated inflammatory responses *in vivo*. Consistently with all these findings, Src-family kinase inhibitors protected rodents against allergic or LPS-induced acute lung inflammation [73, 74]. Interestingly, recent studies reported that accumulation of syk-/- neutrophils in the tissue in a model of reverse-passive Arthus reaction was markedly reduced [68].

The above summarized studies suggest that, in agreement with controversial reports on the role of SFK in regulating neutrophil migration *in vitro*, SFKs may be either implicated in or dispensable for neutrophil recruitment in sites of inflammation. So far, the emerging picture is that recruitment into the peritoneum seems to be SFK-, as well as Syk-, independent [55, 61]. In contrast, recruitment of neutrophils, eosinophils and monocytes into the lung and the liver requires activity of SFKs [37, 66, 69, 70, 73, 74]. Finally, neutrophil recruitment into the skin has been reported either to be regulated [66] or not [59, 65] by SFKs.

Differences in the role played by SFKs in regulating neutrophil recruitment into inflamed tissues may be explained by variations in vessel hemodynamic parameters and structure. Leukocyte recruitment does not simply reflect the cell migratory ability detected in *in vitro* assays because it implicates additional and complex events including leukocyte interaction with the endothelium and the actual transmigration through the endothelial barrier. In the following section we will discuss recent evidence highlighting a few critical steps of the interaction between the leukocyte and the endothelium that may be regulated by SFKs.

Src-family kinase-mediated signaling in leukocyte interacting with the inflamed endothelium

Leukocyte recruitment into inflamed tissues is viewed as a multistep cascade of adhesive interactions between leukocytes and the endothelial cell. These involve selectin-mediated leukocyte tethering and rolling, integrin-dependent firm adhesion and, ultimately, transmigration across the endothelial layer [24, 25]. The evidence that neutrophil recruitment into inflammatory sites in some *in vivo* models of inflammation appears to be regulated by SFKs raises the issue of the possible mechanisms by which SFKs exert this action (Fig. 2).

An early step in neutrophil-endothelium interaction is the binding of PSGL-1 with its endothelial counter-receptors P- and E-selectin. Besides mediating leukocyte rolling, PSGL-1 triggers intracellular signals regulating interaction of β2 integrins with ICAM-1; this interaction mediates "slow rolling" and primes leukocytes for arrest on the endothelial surface [21, 24]. A very recent report identified a new signaling pathway triggered by PSGL-1 in neutrophils and based on SFK-mediated phosphorylation of Nef-associated factor 1 (Naf1), a protein constitutively associated with the PSGL-1 cytoplasmic domain [21]. Tyrosine phosphorylated Naf1 recruits the p85-p110δ isoform of PI3-kinase and this results in "inside-out" signals which are essential for full activation of the LFA-1 binding capacity by trimeric G protein-coupled chemoattractant receptors. Importantly, inhibition of this pathway diminished integrin-dependent neutrophil adhesion to the endothelium and neutrophil recruitment *in vivo*. Concordant with this study, SFKs have been recently implicated in PSGL-1-induced, Mac-1-mediated firm adhesion of neutrophils to adherent plateles [75]. In the light of the paradigm that SFKs and Syk act in concert in signal transduction by different myelomonocytic surface receptors (Fig.1 and above), it is striking that PSGL-1-dependent slow rolling on ICAM-1 was shown to be dependent on Syk and a PSGL-1-Syk signaling pathway converges with a chemokine-$G\alpha_i$ pathway in neutrophil recruitment *in vivo* [76]. Intriguingly, besides regulating its interaction with the endothelium, a PSGL-1-Syk pathway was shown to regulate neutrophil transcriptional activity [20]. Altogether, these findings suggest that SFKs and Syk are implicated in selectin-mediated priming of neutrophil adhesion, recruitment and, potentially, in transcription of pro-inflammatory mediators.

Another possible step in neutrophil-endothelium interaction regulated by SFKs is integrin-mediated arrest and firm adhesion. Neutrophils deficient in Hck and Fgr failed to spread over integrin ligands upon triggering with TNFα or fMLP [77]. Additionally, in assay conditions allowing rapid activation of integrin adhesiveness by chemoattractant receptors, mutant neutrophils adhered as wild type cells, but spread over a smaller area and rapidly detached from the adherent surface [78]. This study also reported that per cent of *hck*$^{-/-}$*fgr*$^{-/-}$ neutrophil arresting on the inflamed striated muscle venules larger than 60 μm is reduced. Interestingly, inactivation of Vav1/3, a known substrate of the integrin-mediated SFKs/Syk signaling pathway (see above) results in a neutrophil phenotype very similar to that reported for *hck*$^{-/-}$*fgr*$^{-/-}$ neutrophils [62]. These findings suggest that a classical integrin-dependent "outside-in" signaling pathway may contribute to strengthen neutrophils adhesion in certain vascular regions.

Exciting recent studies highlighted a possible additional step of neutrophil recruitment implicating SFKs, i.e. a transendothelial cell pathway of emigration regulated by the formation of podosomes. Almost ten years ago, reinvestigating pathways of neutrophil emigration through the venular endothelium, Feng and colleagues concluded that this can also occur by a transcellular route through both endothelial cells and pericytes [79]. This study showed that adherent neutrophils projected cytoplasmic processes into the underlying endothelium. Recently, it was demonstrat-

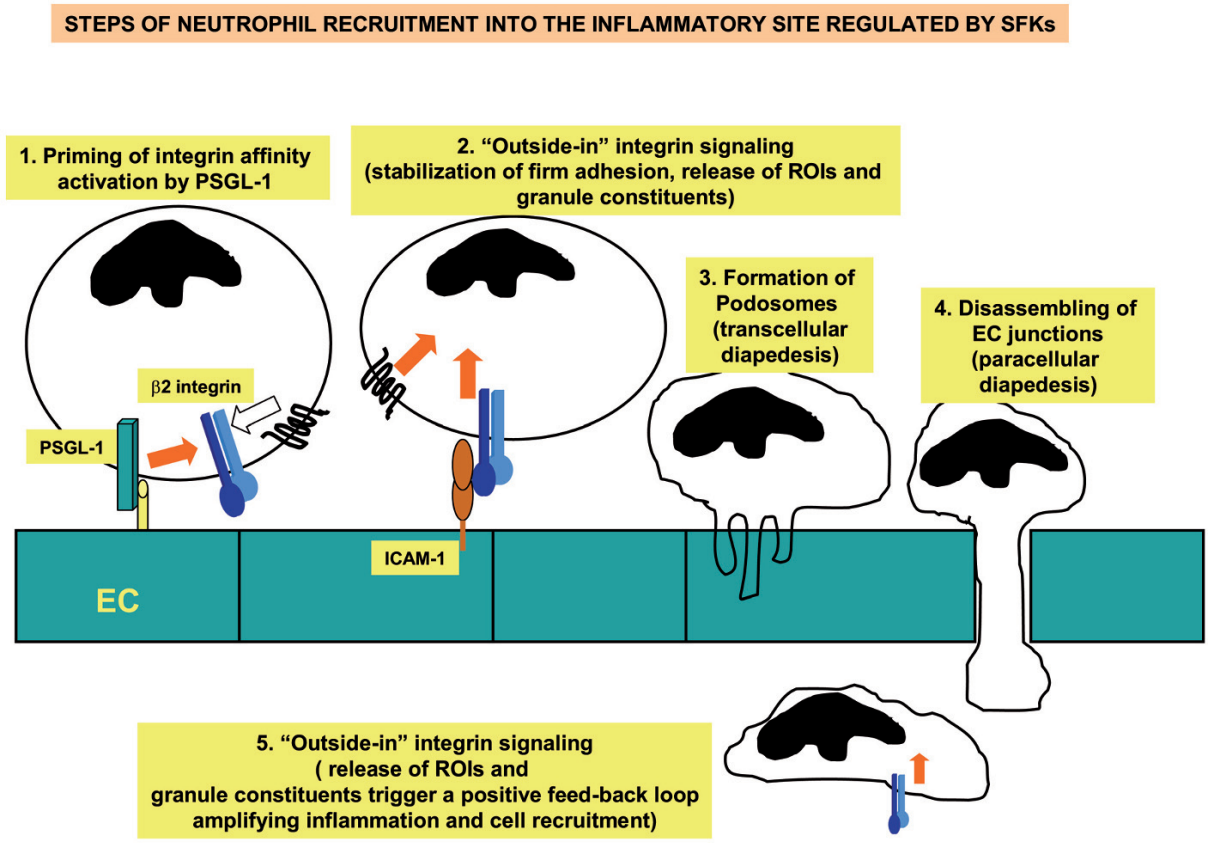

Figure 2. Steps of leukocyte recruitment in which SFKs may play a regulatory role. Red arrows indicate pathways implicating SFKs. White arrows indicate pathways that are likely SFK-independent. Upon binding to P- or E-selectin, PSGL-1 triggers signals priming β2 integrin activation and dictating "slow rolling" [21, 76]; trimeric G protein-coupled chemoattractant receptors then induce full activation of integrin affinity and leukocyte arrest on the endothelial surface. SFKs have been implicated in PSGL-1 signal transduction following binding of P-selectin [21, 75]. The role of SFKs in E-selectin-induced PSGL-1-dependent signal transduction has not been addressed, but Syk, a tyrosine kinase downstream of SFKs in several signalling pathways (see text), regulates E-selectin-induced, β2 integrin-dependent slow rolling [76]. Following full activation of β2 integrin affinity, integrin-induced "outside-in" signaling may stabilize leukocyte arrest to the endothelial surface via formation of integrin-based cytoskeletal and F-actin multimolecular complexes [62, 78]. Additionally, outside-in signalling regulating integrin-dependent neutrophil degranulation and release of ROIs may regulate endothelial cell (EC) "activation" (see text) and favour the paracellular pathway of diapedesis (step 4 in the Figure) by inducing disassembly of endothelial cell junctions. Integrin signalling in phagocytic cell requires SFKs (see [3]). SFKs have also been implicated in degranulation and ROIs generation in response to chemoattractants [14, 16, 59, 60, 64, 65]. Although this issue has not been addressed in neutrophils, SFKs regulate podosome formation and transcellular diapedesis in lymphocytes [80, 84]. This pathway of leukocyte transmigration may play a relevant role in the microvascular circulation [80]. Once emigrated in the subendothelial space, SFK-dependent integrin "outside-in" signaling may regulate, via generation of ROIs and release of granule consitutents (see above), a positive feed-back circuit amplifying inflammation and cell recruitment.

ed that lymphocytes use podosomes as protrusive structures able to mediate transcellular diapediesis [80]. Interestingly, this form of cell passage through the vasculature accounts for approximately one third of lymphocyte emigration through lung and dermal microvascular endothelial monolayers, whereas it is less important for transmigration through venules. Podosomes are cylindrical plasma membrane evagination with a core of filamentous actin and a ring structure consisting of integrins and cytoskeletal proteins such as vinculin and talin [81], which were originally characterized in osteoclasts and v-Src transformed cells [82, 83]. SFKs regulate podosome formation [80, 81] and the lysosome-associated isoform of Hck plays an essential role in the biogenesis of podosomes in macrophages [84]. It is therefore tempting to speculate that one of the mechanisms underlying the reduced recruitment of neutrophils and macrophages deficient of SFks into inflammatory sites [31, 69] is a reduced capacity to form and stabilize podosomes.

It is important to note that SFKs regulate important neutrophil effector functions including superoxide anion generation (see [60, 77] and references contained therein) and degranulation [14, 16, 59, 64, 85]. Release of reactive oxygen intermediates or granule constituents may regulate expression of counter-receptors for leukocyte integrins by the vascular

endothelium or tightness of the endothelial cell layer [86, 87]. In this context, it is of great interest that neutrophil emigration through venular walls was reported to occur preferentially through vessel sites expressing lower amounts of basement membrane constituents; these sites are closely associated with gaps between pericytes [88]. Importantly, emigrating neutrophils enlarge these sites via release of serine proteases. Therefore, SFK-mediated neutrophil activation leading to release of preformed or *de novo* synthesized mediators may trigger a positive feedback loop based on induction of changes in the vascular endothelium that favour cell recruitment.

Conclusions

In this report we reviewed evidence suggesting that SFKs are important regulators of myelomonocytic cell migration and recruitment. Although mechanisms by which this regulation may be exerted are still elusive, it may be anticipated that modulation of SFK activity may represent a new important strategy to control inflammatory reactions. In this context, it is important to note that recent evidence also points to a role for SFK-mediated signals in the regulation of the tightness of endothelial cell junctions [89, 90]. Notably, inhibition of endothelial SFKs results in decreased neutrophil transmigration. Hence, inhibitors of SFKs may act inhibiting both leukocyte responses and endothelial changes that converge in regulating cell recruitment in inflammatory reactions. The search for more effective inhibitors for the treatment of chronic myeloid leukemia is a very active field of pharmaceutical research and has led to the discovery of drugs able to specifically inhibit c-Abl or displaying a dual specificity for c-Abl and SFKs [5, 91]. Hence, it may be anticipated that identification of new drugs regulating leukocyte recruitment in inflammatory responses may result from these studies. Interestingly, two of these drugs, Dasatinib and Bosutinib, initially selected for their capability to inhibit both c-Abl and SFKs, were recently also shown to be powerful inhibitors of Syk [91]. Due to the special role played by the cross-talk between SFKs and Syk in immune receptor and integrin signaling (Fig. 1 and above) it is tempting to propose that these two drugs could represent powerful weapons to control some form of inflammatory pathologies.

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