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ORIGINAL ARTICLE

Glutamate induces neutrophil cell migration by activating class I metabotropic glutamate receptors

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Abstract Leukocytes are recruited at the site of infection or injury as a part of the innate immune system, and play a very critical role in fighting the invading microorganisms and/or healing wounds. Neutrophils are the most abundant leukocytes in healthy humans and are the principal cell types that arrive at the target site in the initial phase of this process. Previous studies from our laboratory have shown that the amino acid glutamate is a novel chemotaxis-inducing factor for human neutrophils. In this report, we provide evidences that clearly demonstrate that the glutamate-induced neutrophil cell migration activity is mediated by the class I metabotropic glutamate receptors. Our results further show that a specific integrin $\beta 2$ (ITG $\beta 2$) receptor, namely LFA1 ($\alpha_L\beta_2$) is activated upon glutamate treatment and is required for further downstream signaling events leading to increased migration of human neutrophil cells. Following glutamate stimulation, LFA1 is phosphorylated by the Src Kinase Lck at the Y735 residue, which triggers a downstream signaling cascade leading to activation of PI3K, Syk, Vav and finally the Rho family GTPase, Rac2. Interestingly, glutamate was previously found to be present in elevated levels in wound fluid. Furthermore, glutamate level was also found to go up following inflammation. Taken together, our study suggests a novel mode of neutrophil recruitment to the target site following an infection or injury.

Keywords Neutrophil recruitment · Chemotaxis · Glutamate receptor · Integrin

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Introduction

Circulating leukocytes are recruited at the site of infection, injury or inflammation to counteract the invading microorganism and/or to heal the tissue. At the beginning, the free-flowing leukocytes are captured by the activated endothelial cells on the blood vessel wall. This is then followed by leukocyte tethering and rolling, activation and firm adhesion to the endothelial cells, transendothelial diapedesis, and chemotactic migration to the target site (Ley et al. 2007; Luster et al. 2005; Kelly et al. 2007). Over the years, a number of soluble mediators/chemoattractants have been identified that have the ability to modulate one or more of these steps leading to leukocyte recruitment. For example, leukocyte recruitment and trafficking was found to be regulated by the bacterial by-product formyl methionyl

leucyl peptide (fMLP), complement proteolytic fragment C5a, the leukotriene B4 (LTB_4), platelet activating factor (PAF), the inducible and secreted chemokines such as TNFα, IL4, IL6, IL8 etc. (Medeiros et al. 1999; Olson and Ley 2002; Bartemes et al. 1999; Gabay 2006; Hickey et al. 1999; Patel 1999; Webb et al. 1993). In our previous report, we showed that the amino acid glutamate can act as a novel chemotaxis-inducing factor for human neutrophils (Gupta and Chattopadhyay 2009), the most abundant leukocytes that arrive at the infection or injury site at the very early stage of the process. Our results showed that glutamate stimulation induced Actin cytoskeleton polarization suggesting increase in migratory phenotype in human neutrophils. Transwell assays further revealed that indeed glutamate treatment enhanced cell migration ability in human neutrophils (Gupta and Chattopadhyay 2009).

Interestingly, glutamate was initially identified as the major excitatory neurotransmitter in the central nervous system (CNS), where it is required for synaptic development, memory and learning (Engelsen 1986; Fonnum 1984; Hudspith 1997; Matsugami et al. 2006; Nguyen et al. 2001; Ruediger and Bolz 2007). Later on, however, glutamate signaling was also identified in various other non-neuronal cell types such as bone osteoblasts, keratinocyte, taste buds, pancreas, liver, lung, kidney, intestine, testis, esophagus, adrenal, pituitary and pineal glands, heart, megakaryocytes and their precursors in the bone marrow, lymphocytes etc., indicating a global role for this amino acid (Chaudhari et al. 2000; Chenu et al. 1998; Franconi et al. 1996; Genever et al. 1999a, b; Gill et al. 1998; Hayashi et al. 2003; Hinoi et al. 2002, 2003, 2004; Hinoi and Yoneda 2001; Kiyama et al. 1993; Kristensen 1993; Storto et al. 2000; Yamada et al. 1998). Glutamate signaling is principally mediated by two receptor subtypes, (1) metabotropic glutamate receptor, and (2) ionotropic glutamate receptor, as well as glutamate transporters (Conn and Pin 1997; Dingledine et al. 1999; Gasic and Hollmann 1992; Hollmann and Heinemann 1994; Kew and Kemp 2005). Metabotropic glutamate receptors are associated with G protein coupled receptors and further classified into three classes (class I, II and III) based on sequence homology, second messenger coupling and pharmacology. Ionotropic receptors on the other hand are associated with ion channels permeable to specific cations and are further divided into three subclasses (NMDA, AMPA and Kainate) based on sequence homology and the type of agonist they bind to. Although, the physiological significance of glutamate signaling is very well established in the CNS, it is not so much for the peripheral tissues.

In the present study, we show that the glutamate-induced migration of human neutrophil cells is mediated by class I metabotropic glutamate receptors, mGluR1 and mGluR5. Our data further demonstrate that glutamate treatment activated the integrin $\beta 2$ receptor, LFA1, as well. Together, these two receptors mediate the downstream signaling events and are responsible for the increase in cell migration ability of human neutrophils. We have further shown that following exposure to glutamate, LFA1 becomes phosphorylated, and thereby activated by the Src kinase Lck at the Y735 residue. This in turn, leads to activation of PI3K, Syk, Vav and ultimately Rac2 leading to the enhanced cell migration ability.

Materials and methods

Reagents and antibodies

Glutamate, MCPG, MPEP, MK-801, PTX and fMLP were obtained from Sigma, DL-TBOA from Tocris Biosciences. Antibodies Rac2 (sc-96), pVav (sc-16408-R), Vav (sc017831), p-85 (Sc-1637), ITG$\beta 2$ (sc-8420), Lck (sc-433 and sc-28882) and Syk (sc-929) were from Santa Cruze Biotechnologies. Anti P-Tyr antibody was obtained from Cell Signalling Technologies (P-Tyr-100).

Preparation of human neutrophils

Human neutrophils of 90–95 % purity (as seen microscopically) were prepared from freshly drawn blood from healthy volunteers collected in heparinised vials, in endotoxin-free condition, as described (Badwey et al. 1982). Neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Paque, and hypotonic lysis of erythrocytes. The cells were resuspended in Krebs–Ringer HEPES buffer (118 mM NaCl, 4.8 mM KCl, 25 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4) containing 5.5 mM glucose or HBSS. Blood was collected from healthy volunteers in accordance with the guidelines of the Committee on Ethics in Experimentation, University of Calcutta, India. Except for the binding assays (Fig. 1), all the experiments were done with 8 μM glutamate, pH 7.0–7.5.

RNA isolation, reverse transcription and polymerase chain reaction assay

Total RNA was isolated from human neutrophils using the TRIzol RNA isolation reagent according to the manufacturer's instructions (Life Technologies). cDNA was synthesized from the purified, DNAse treated total RNA using random hexamer as primers. PCR using gene-specific primers for PCR reactions was used as described in previous reports (Ishiuchi et al. 2007; Wosik et al. 2004; Collard et al. 2002; Lee et al. 2010). PCR products were

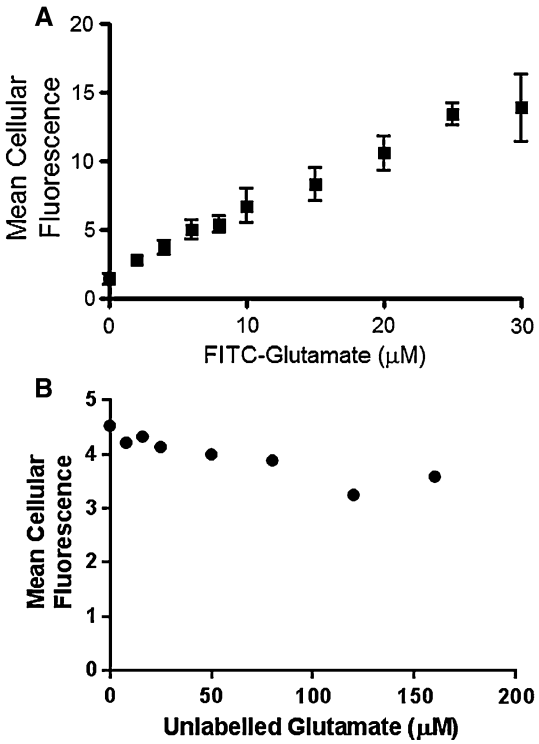

Fig. 1 Characterization of glutamate binding to human neutrophils. **a** FITC-glutamate binds to neutrophils in a dose-dependent manner. Neutrophils (5×10^5) were incubated with increasing concentration of FITC-glutamate for 45 min, and binding was assessed by FACS analysis. The data shown here are mean ± SEM of three independent experiments. $P < 0.0001$. **b** Inhibition of FITC-glutamate binding in the presence of excess unlabelled glutamate. Neutrophils (5×10^5) were incubated with FITC-glutamate (8 μM) in the presence of increasing concentration of unlabelled glutamate for 45 min. Binding was detected by FACS analysis. Result from one representative experiment is shown here

run on 1.5–2 % agarose gel and examined in a Gel Doc system (Biorad).

Chemotaxis assay

Chemotaxis assays were performed using transwell plate (corning) as described (Filippi et al. 2004). Glutamate or carrier (water) diluted in HBSS, (with Ca^2 and Mg^2) were placed in the lower chamber, and (4×10^5) neutrophils in HBSS were loaded in the upper wells. When stated, neutrophils were pre-incubated with indicated antagonists for 1 h at room temperature. The upper and lower wells were separated by a 3-μm pore size polycarbonate filter, which allows only neutrophil, but not macrophage, migration. The plate was incubated at 37 °C for 2 h. After recovery, the upper chamber side of the filter was wiped to remove the non migrated cells, and the filter was fixed and stained with Giemsa. The migrated cells on the reverse side of the filter were counted under an inverted microscope (Olympus).

Immunofluorescence

Immunofluorescence of the neutrophils was used to find the polarization of the actin cytoskeleton as described (Filippi et al. 2004). Neutrophils (3×10^5) were plated on glass coverslips coated with fibronectin (0.1 mg ml^{-1}). The cells were then stimulated by a uniform concentration of glutamate or carrier (water) for a specified time, fixed with 3.7 % paraformaldehyde for 20 min, followed by permeabilisation with 0.1 % Triton-X. The cells were then stained with 10 U ml^{-1}Alexa-488 Phalloidin for 20 min, washed 3 times with PBS. Finally, images were captured at 63× magnification in confocal microscope (Zeiss).

Preparation of FITC-tagged glutamate

FITC-glutamate was purified as described by Glaasker et al. (1996) with some modifications. Briefly, FITC in DMSO was mixed with 40-fold excess of glutamate at 4 °C for 2 h. The mixture was then run on C18 reverse phase HPLC column. At first, the column was run on 0.4 % glacial acetic acid, 0 % acetonitrile for 5 min, followed by increase to 15 % acetonitrile by 25 min and 30 % acetonitrile by 35 min. After that column was maintained at 30 % acetonitrile till 50 min, increased to 50 % acetonitrile by 51 min, and maintained the same concentration till 70 min. The fraction containing the FITC-glutamate was collected and used in the binding assays.

Flow cytometry analysis to determine the binding of glutamate

Flow cytometry analysis was used to detect the binding of FITC-glutamate to neutrophils as described. Briefly, (5×10^5) neutrophils were incubated with various concentrations of FITC-glutamate, in 200 μl volume, for 45 min at room temperature in dark. After incubation, cells were washed twice with PBS and analysed on a BD science FACS Calibar. 10,000 neutrophil events were counted and the results are expressed as mean cellular florescence (MCF). Unlabeled glutamate (various concentrations) was incubated along with FITC-glutamate under identical conditions.

Lck, Syk, p-85, Vav1 and Rac activation assays

Rac2 activation assay was performed as previously described (Benard et al. 1999). Briefly, neutrophils (2×10^6) after stimulation for a specified time were lysed and the cell lysate was affinity precipitated by 10 μg of glutathione-*S*-transferase-p21 binding domain (GST-PBD). Proteins were blotted with specific rabbit Rac2 antibody (Gary Bokoch, Sc-96, Santa Cruz) to determine

the Rac2-GTP level. Cell lysates were also probed for total Rac2. In experiments determining tyrosine phosphorylation of Lck, Syk, Vav1 and p-85, neutrophil lysates (2×10^6) were immunoprecipitated with 5 μg of respective antibodies, mouse Vav1 (Sc-17831) and mouse p-85 (Sc-1637). Immune complexes were captured by Protein-A Sepharose (GE Amersham) and immunoblotted using antibody against phosphor-tyrosine (cell signaling/Santa Cruz). The pulled down Protein-A Sepharose complexes were also immunoblotted using antibody against the same protein to determine the total pulled down protein in the immune complex.

Cloning of GST-β2 and kinasing of the purified protein

The cytoplasmic tail of integrin β2 (46 amino acid) was amplified by PCR from purified neutrophils, using the primers—P1, 5′-ATA ACC GGA TCC AAG GCT CTG A-3′(forward) and P2, 5′-GAC CAA CTC GAG CTA ACT CTC A-3′(reverse). The cDNA fragment was cloned in *Bam*HI and *Xho*I sites of pGEX4T1 vector (GE Pharmacia) and the sequence was verified. The fusion protein was expressed in BL21DE3 *E. coli* strains and was purified using Glutathione–Sepharose beads (GE Pharmacia). Only GST protein was also purified for use as controls. The purified proteins were further used for kinasing reactions.

After appropriate time of stimulation, equal amounts of proteins were immunoprecipitated with 5 μg of Lck antibody for 2 h followed by capture of the immunocomplex by protein-A Sepharose for an additional 1 h. The pulled down complex was incubated with 8 μg GST or GST-β2 (substrate) in the kinase buffer [25 mM Hepes (pH 8.0) containing 0.2 mM Na_3VO_4, 10 μM ATP, 5 mM $MgCl_2$, 3 mM $MnCl_2$, 2 μM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM dithiothreitol] in the presence of 10 μCi of [γ-^{32}P]ATP at 30 °C for 30 min. The reaction was terminated by the addition of SDS-sample buffer. The sample was resolved in SDS-PAGE and labeled proteins were visualized by phosphoimager (GE Pharmacia).

The pH of the glutamate solution (sigma) used for the above experiments was kept between 7.0 and 7.5. The glutamate solution was prepared under endotoxin-free condition.

Results

Glutamate signaling in human neutrophils is mediated by class I metabotropic receptors

Direct binding of glutamate with human neutrophils was examined using fluorescent (FITC)-tagged glutamate. Incubation of neutrophils with increasing concentration of FITC-glutamate resulted in dose-dependent binding of glutamate as detected by flow cytometry. Binding of glutamate reached saturation at concentration of 25 μM (Fig. 1a). Only FITC showed weak background binding. Background autofluorescence of neutrophils and FITC was negated to show the actual binding. To determine the specificity of binding of glutamate, neutrophils were further incubated with a constant concentration of FITC-glutamate (8 μM) in the presence of increasing concentration of unlabelled glutamate. A modest inhibition of binding of FITC-glutamate to the neutrophil cells was observed, suggesting a specific mode of binding (Fig. 1b).

To identify the receptor responsible for the glutamate binding and downstream signaling events, we first screened purified human neutrophils for the presence of various glutamate receptor transcripts by reverse transcriptase-PCR assay. Our data showed that both class I metabotropic receptors (mGluR1 and mGluR5) transcripts are expressed in human neutrophils (Fig. 2a). To further support our observation, we examined the plausible involvement of these receptors for the glutamate signaling with available pharmacological inhibitors like AP5 (NMDA), CNQX (AMPA/Kianate), (+)MK-801 (NMDA), MCPG (class I/II metabotropic receptor), MPEP (class I metabotropic receptor—highly selective for mGluR5) and pertussis toxin (G protein coupled receptor, class II/III metabotropic receptor). Human neutrophils were preincubated with the above-mentioned inhibitors for the indicated time and then stimulated with glutamate for different time periods and then migration ability and/or actin polymerization was monitored. Our result showed that inhibition of ionotropic glutamate receptors [inhibitors used AP5, (+)MK-801 and CNQX] does not have any effect on the cell migration or actin polymerization process (Fig. 2b and data not shown). However, inhibition of only class I, but not class II or III metabotropic glutamate receptors, blocked the cell migration inducing ability of glutamate (Fig. 2b, c). Furthermore, pre-incubation with competitive glutamate transporter inhibitor DL-TBOA failed to exert any effect on glutamate-induced cell migration ability of neutrophils (Fig. 2d) indicating lack of involvement of glutamate transporters in the process. Altogether, these results suggest that the glutamate-mediated increase in cell migration activity of human neutrophils is exclusively mediated by the class I metabotropic glutamate receptors mGluR1 and mGluR5 (Table 1).

Glutamate activates Rac2, Vav and PI3K in human neutrophils

The Rac members of Rho family of small GTPases are well-known regulators of leukocyte chemotaxis and

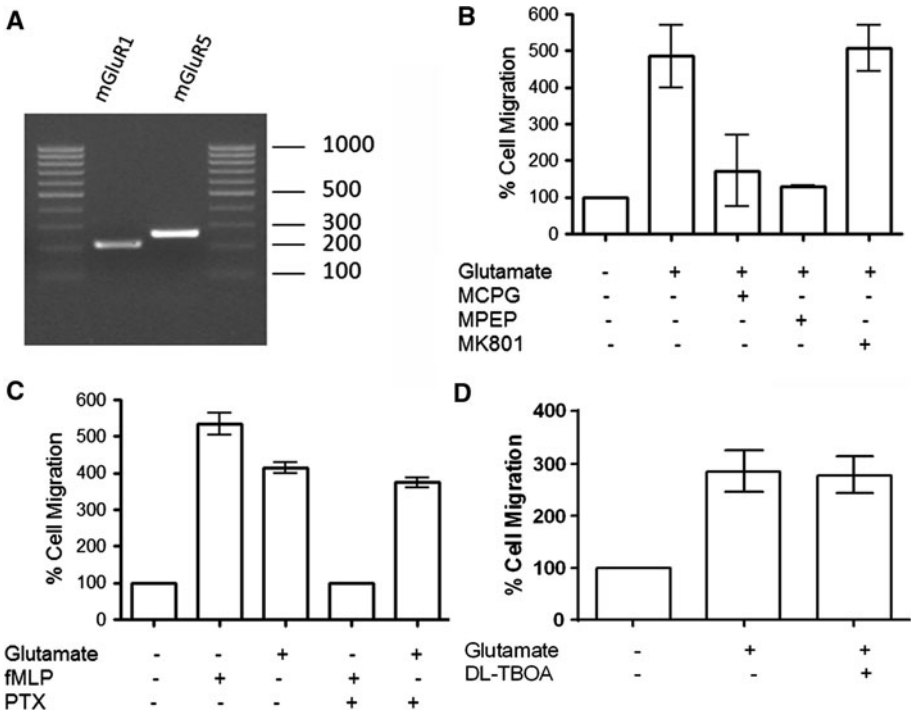

Fig. 2 Glutamate-induced increase in neutrophil cell migration is mediated by class I metabotropic glutamate receptors. **a** RT-PCR analysis showing expression of class I metabotropic glutamate receptors (mGluR1 and mGluR5). Glutamate-mediated increase in neutrophil cell migration is inhibited by class 1 metabotropic receptor antagonists (MCPG and MPEP), but not by NMDA receptor antagonist MK-801 (**b**), class II/III metabotropic antagonist PTX (**c**), or by glutamate transporter inhibitor DL-TBOA (**d**). Neutrophils were pre-incubated for 1 h with the inhibitors and then stimulated with glutamate. The data shown here are mean ± SEM of three independent experiments. $P < 0.05$

Table 1 Involvement of class I metabotropic glutamate receptor in glutamate signaling

Antagonists	Target	Effect on glutamate-mediated neutrophil cell migration/actin polymerization
AP-5	NMDAR	None
MK-801	NMDAR	None
CNQX	AMPA/Kainate	None
MCPG	Class I/II mGluR	Inhibits glutamate-mediated neutrophil migration
MPEP	Selective for mGluR5—Class 1 mGluR	Inhibits glutamate-mediated neutrophil migration
PTX	Class II/III mGluR	None

influence cell migration by controlling the formation of lamellipodia and filopodia (Roberts et al. 1999). Rac proteins (Rac1, 2, and 3) belong to the Rho family of GTPase and are one of the most important regulators of actin cytoskeleton remodeling (Chung et al. 2000; Tapon and Hall 1997). Rac proteins are "on" when GTP bound and "off" when GDP bound (Heyworth et al. 1994). In order to gain insight on the glutamate-induced cell migration process, we examined the status of Rac2 in glutamate-induced human neutrophils. Our results showed that glutamate stimulation activated Rac2 in neutrophils as evidenced by increased Rac2-GTP level (Fig. 3a). Notably, Rac GTPases are activated by Vav protein, the Rho/Rac guanine nucleotide exchange factor (GEF), which in turn, is activated by phosphorylation at the Tyrosine residue. As such, we looked into the activation status of the Vav protein by examining its phosphorylation status. As expected, the level of phosphorylated Vav was elevated, indicating its increased activation status following glutamate stimulation (Fig. 3b).

Interestingly, the Vav proteins work in close collaboration with the Phosphatidylinositol 3 kinase (PI3K) protein. Indeed, several studies have demonstrated that various PI3K isoforms are required for neutrophil recruitment and activation of Rac2 in neutrophils (Koyasu 2003; Liu et al. 2007; Pinho et al. 2007). Upon facing various stimuli, PI3K becomes activated through phosphorylation of the p85 subunit. Therefore, to check involvement of PI3K, we examined the level of phospho-p85 in glutamate-treated human neutrophils at various time points. Our data revealed that PI3K becomes phosphorylated and thereby activated in human neutrophils following glutamate treatment (Fig. 3c).

Glutamate treatment activates integrin beta-2 (ITGβ2) receptor in human neutrophils

Glutamate was previously shown to trigger cellular adhesion to laminin and fibronectin in T cells expressing

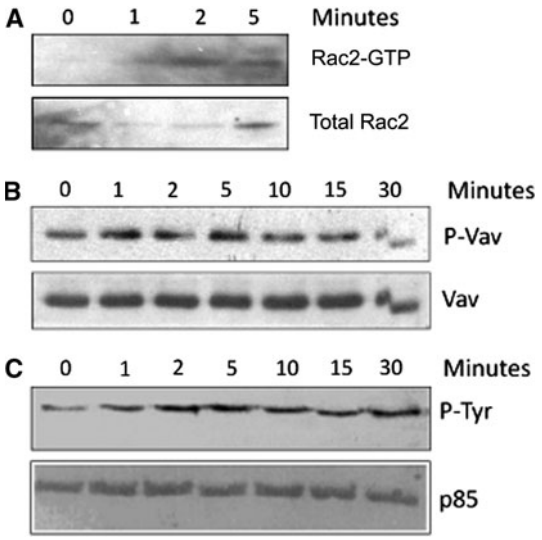

Fig. 3 Glutamate stimulation activates Rac, Vav and PI3K in human neutrophils. Level of phosphorylated Rac2 (**a**), Vav (**b**) and p85 (**c**) was monitored in neutrophil cells following glutamate treatment at indicated time intervals, as described in "Materials and methods" and in the Text. Phosphorylated form of these proteins indicates their active form

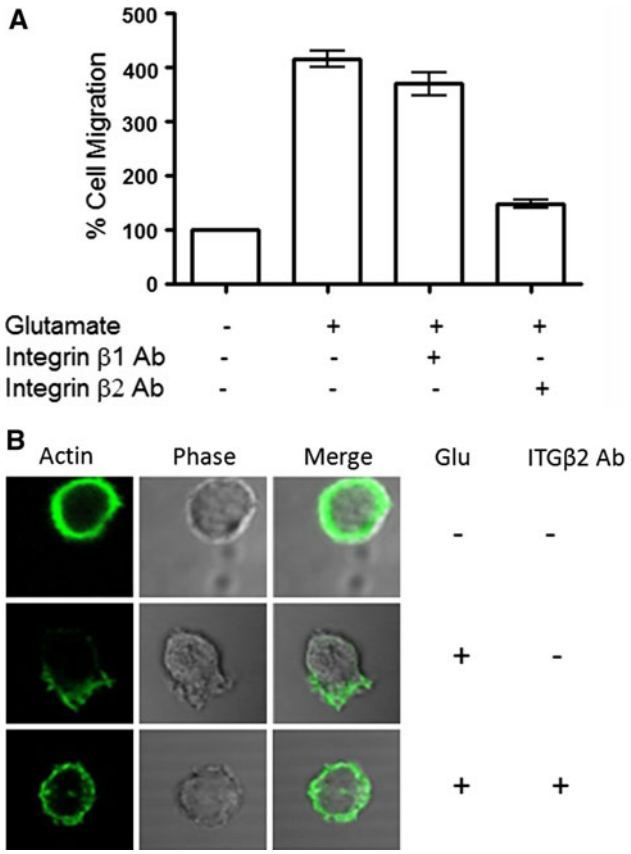

Fig. 4 Integrin $\beta 2$ is required for glutamate-mediated stimulation of cell migration activity in human neutrophils. **a** Glutamate-induced increase in cell migration is blocked by pre-incubation with antibody against integrin $\beta 2$, but not integrin $\beta 1$. Neutrophil cells were pre-incubated with integrin $\beta 2$ blocking antibody (20 μg/ml) or integrin $\beta 1$ blocking antibody (20 μg/ml) for 30 min, and then stimulated with glutamate. **b** Glutamate induces cytoskeleton changes involving integrin $\beta 2$ receptor. Neutrophils pretreated with integrin $\beta 2$ blocking antibody (20 μg/ml) for 30 min, were washed and subjected to glutamate stimulation for 1 min, stained with Alexa Phalloidin and viewed under microscope (as described in "Materials and methods"). The data shown here are mean ± SEM of three independent experiments. $P < 0.005$

functional glutamate receptor GluR3 and this was mediated by integrin $\beta 1$ receptor (Ganor et al. 2003). Furthermore, Piao et al. (2009) have recently shown that Glioma cells with functional AMPA receptors promoted cell invasion via integrin $\beta 1$-dependent cell adhesion to ECM. Together, all these findings indicate a plausible role for integrins in glutamate-induced cell migration in human neutrophils. Consequently, we performed transwell migration assay with inhibitors to receptors (a) integrin $\beta 2$ and (b) integrin $\beta 1$. Inhibitors to integrin $\beta 1$ (20 μg/ml, Sc-18841L) and $\beta 2$ (20 μg/ml, Sc-18862L) were blocking antibodies (Santa Cruz Biotechnology). Preincubation with the inhibitors (30 min) followed by glutamate treatment for 2 h showed a substantial decrease in migration with integrin $\beta 2$ blocking antibody only, but not with $\beta 1$ blocking antibody (Fig. 4a). This experiment clearly indicates integrin $\beta 2$ (ITG$\beta 2$) to be the major integrin receptor involved in glutamate signaling in human neutrophils. Moreover, polarization of actin cytoskeleton, the cardinal feature of chemotaxis, was also abrogated in human neutrophils followed by pretreatment with $\beta 2$ blocking antibody (20 μg/ml) (Fig. 4b).

Glutamate-induced phosphorylation of ITG$\beta 2$ cytoplasmic tail at Y735 is mediated by protein tyrosine kinase Lck

The cytoplasmic tail domains of integrin β subunits contain a number of phosphorylation sites, especially Ser and Thr, which have been shown to be differentially phosphorylated under various conditions. To decipher the signaling cascade following glutamate-mediated activation of ITG$\beta 2$, we first examined the phosphorylation status of the molecule. Interestingly, we found an immediate phosphorylation of Tyr residue upon glutamate stimulation (Fig. 5a).

Importantly, cytoplasmic Protein Tyr Kinases (PTKs) such as Src family and Syk family play a major role in transmitting signals following receptor activation in leukocytes. Therefore, we looked into the activation status of these two families of PTKs in human neutrophils following glutamate treatment. We found that both Lck and Syk PTKs get Tyr phosphorylated, and thereby activated when neutrophils were stimulated with glutamate (Fig. 5b, c). Moreover, a significant decrease in Tyr

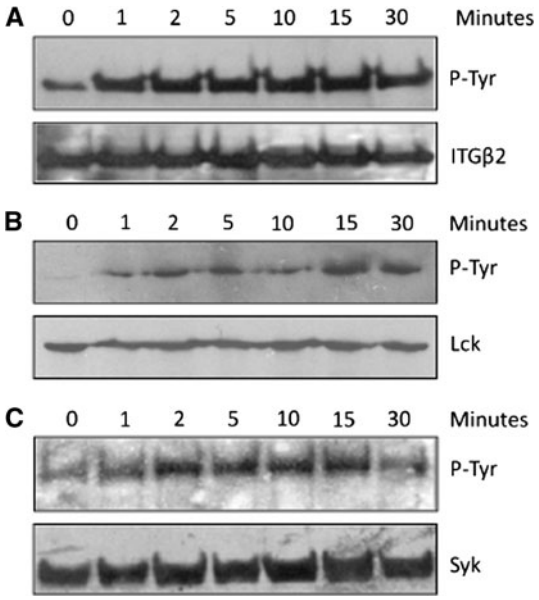

Fig. 5 Glutamate treatment activates ITGβ2 receptor and the protein tyrosine kinases Lck and Syk. **a** Activity of ITGβ2 receptor after glutamate treatment was assayed by following its phosphorylation status. Neutrophils (2×10^6) were left untreated or treated with 8 μM of glutamate for specified time and then lysed. After lysis of cells, equal amounts of proteins were immunoprecipitated with integrin β2 antibody. The immunoprecipitated material was separated on SDS/PAGE and blotted with indicated antibodies. Level of phosphorylated Lck (**b**) and Syk (**c**) increased in human neutrophils following glutamate treatment. Lysates from neutrophils (2×10^6) untreated or treated with 8 μM glutamate for indicated times were immunoprecipitated with respective antibodies and examined for their phosphorylation status

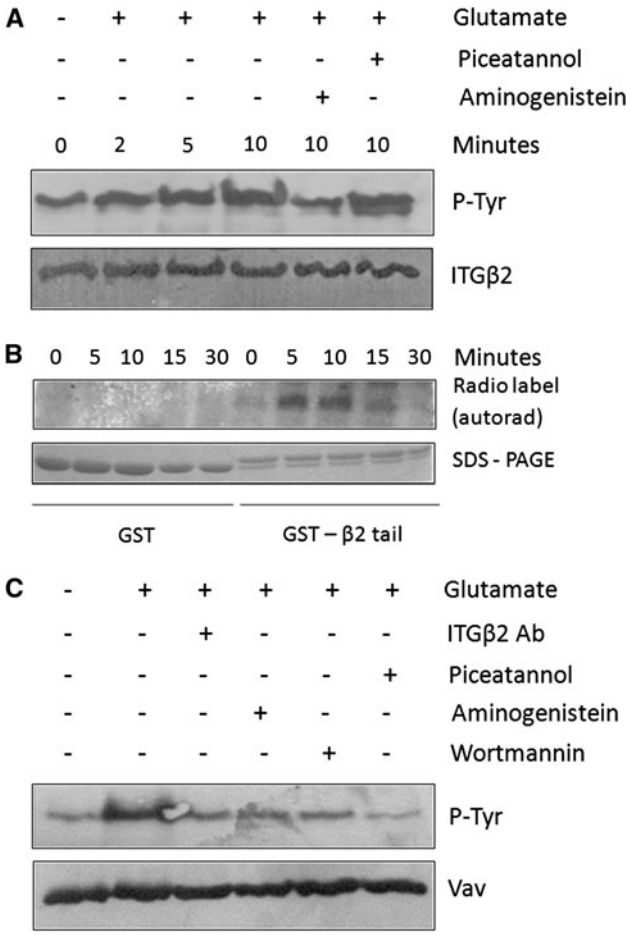

Fig. 6 Lck phosphorylates the cytoplasmic tail and thereby activates the ITGβ2 receptor following glutamate stimulation in human neutrophils. **a** The tyrosine phosphorylation of integrin β2 cytoplasmic tail is mediated by the kinase Lck and not by Syk. Neutrophils (2×10^6) were pretreated with Syk inhibitor piceatannol (250 μM), Lck inhibitor aminogenistein (250 μM) or diluents (DMSO) for 30 min at room temperature. This was followed by incubation with glutamate (8 μM) for the specified time. After lysis of cells, equal amounts of proteins were immunoprecipitated with integrin β2 antibody. The immunoprecipitates were separated on 7.5 % SDS/PAGE and blotted with indicated antibodies. **b** Lck phosphorylates Y735 residue of integrin β2 tails. Neutrophils (2×10^6) cells were treated with 8 μM glutamate for varying times and lysed. Thereafter, Lck was pulled down and incubated with substrate proteins (GST and GST-β2 tail) in the presence of γ^{32} ATP for 30 min and further resolved in 12 % SDS-PAGE. The labeled proteins were then visualized by phosphorimager. *Lower panel* indicates Coomassie staining of the kinase gel to confirm equal loading. **c** PI3K, Lck and Syk act upstream of Vav. Neutrophils (2×10^6) were pretreated with Syk inhibitor piceatannol (250 μM), Lck inhibitor aminogenistein (250 μM) PI3K inhibitor Wortmannin or ITGβ2 blocking antibody (20 μg/ml) for 30 min at room temperature. After lysis of cells, equal amounts of proteins were immunoprecipitated with anti-Vav antibody. The immunoprecipitates were separated on SDS/PAGE and blotted with the indicated antibodies

phosphorylation status of ITGβ2 was observed when neutrophils were preincubated with Lck inhibitor (aminogenistein) (Fig. 6a). However, no such inhibitory effect was found when neutrophils were pretreated with the Syk inhibitor (piceatannol) (Fig. 6a). To further establish Lck as the Kinase for ITGβ2, we did an in vitro kinase assay using purified cytoplasmic tail domain of ITGβ2 and immunoprecipitated Lck. For this matter, human neutrophils were stimulated with glutamate for various time periods and then Lck was immunoprecipitated. These Lck were then incubated with purified cytoplasmic tail domain of ITGβ2 in the presence of γ-P^{32} ATP. Our data showed that indeed Lck can directly and specifically phosphorylate ITGβ2 at the cytoplasmic tail domain (Fig. 6b). Notably, the cytoplasmic amino acid stretch of ITGβ2 tail has only one tyrosine residue, at Y735. Taken together, our results clearly showed that glutamate-induced phosphorylation of ITGβ2 is mediated by the PTK Lck at Y735 residue.

It should be noted that, inhibition of Lck and Syk PTKs, as well as PI3K blocked the activation of Vav protein (Fig. 6c). This suggests that Lck, Syk and PI3K all act upstream of Vav protein and therefore upstream of Rac2.

The heterodimeric integrin $\beta 2$ receptor for glutamate is LFA-1

From our findings, it is clear that the ITG$\beta 2$ receptor is a very critical player involved in glutamate-induced neutrophil cell migration. The ITG$\beta 2$ family comes in a combination of $\alpha_M\beta_2$ (Mac-I), $\alpha_L\beta_2$ (LFA-I), $\alpha_X\beta_2$ and $\alpha_D\beta_2$. Out of these four, $\alpha_M\beta_2$ and $\alpha_L\beta_2$ are the most prominent members of the $\beta 2$ family expressed on the neutrophils. To further characterize the nature of ITG$\beta 2$ receptor, we performed functional assays in the presence of blocking antibodies to subunits α_L and α_M. Human neutrophils were stimulated with glutamate following pre-incubation with blocking antibodies to either ITG$\beta 2$ or subunits α_L and α_M. After lysing the cells, Vav, p85 and Syk were immunoprecipitated with corresponding antibodies and examined for their phosphorylation/activation status. Our data revealed that there was substantial inhibition of increase in phosphorylation levels of Vav (Fig. 7a), p85 (Fig. 7b) and Syk (Fig. 7c) upon pre-incubation with anti-ITG$\beta 2$ antibody and anti-α_L antibody, but not with anti-α_M antibody. These data comprehensively prove that the most important ITG$\beta 2$ receptor required for the glutamate-induced neutrophil migration is LFA-I ($\alpha_L\beta_2$).

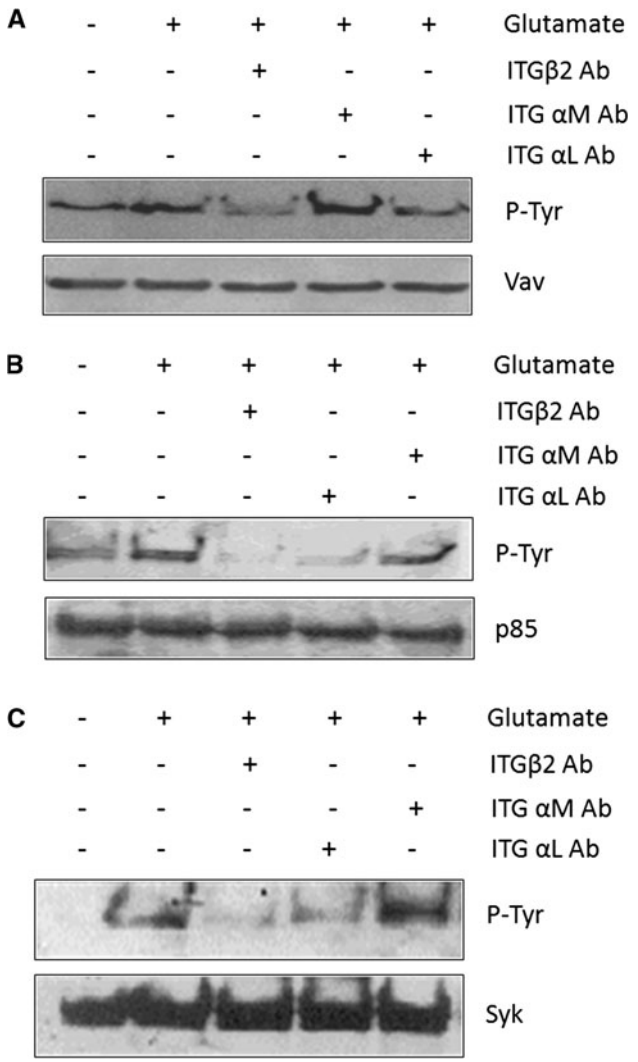

Fig. 7 The heterodimeric integrin $\beta 2$ receptor for glutamate signaling is LFA-1. Neutrophils were either treated with 8 μM glutamate alone or pretreated with integrin $\beta 2$ blocking antibody (20 μg/ml), integrin αL blocking antibody (20 μg/ml) or integrin αM blocking antibody (20 μg/ml) for 30 min at room temperature, followed by glutamate treatment for 5 min. After lysis of cells, equal amounts of proteins were immunoprecipitated with Vav1 (**a**), p85 (**b**) and Syk (**c**) antibodies. The immunoprecipitates were separated on SDS/PAGE and blotted with indicated antibodies. Only pre-incubation with ITG$\beta 2$ and αL antibodies, but not αM antibody, were able to inhibit the increase in phosphorylation and thus activation of PI3K, Syk and Vav following glutamate treatment

Discussion

In the present study, we have unraveled a glutamate signaling pathway in human neutrophils, a non-neuronal cell type, leading to increased cellular migration (Fig. 8). Our results show that (1) the amino acid glutamate stimulates the cell migration ability of human neutrophils by activating class I metabotropic glutamate receptors, (2) which in turn transduces the signal downstream by activating a specific integrin $\beta 2$ (ITG $\beta 2$) receptor, namely LFA-1, (3) through Src Kinase Lck-mediated Tyrosine phosphorylation at Y735 of the cytoplasmic tail domain, (4) active LFA-1 then stimulates Syk and PI3K kinases, leading to activation of Vav and ultimately Rac2, and (5) active Rac2 further induces actin polymerization causing increased cell migration in human neutrophils. Interestingly, glutamate level was previously found to rise following inflammation (Lawand et al. 2000; Omote et al. 1998). Not only that, elevated level of glutamate was also observed in wound fluid (Albina et al. 1993). Taken together, our results therefore suggest a novel mode of neutrophil recruitment mediated by the amino acid glutamate at the infection or injury site.

Notably, LFA-1 activation was found to be mediated by Src family kinase Lck via phosphorylation of Y735 at the cytoplasmic tail domain. To our knowledge, this is the first report demonstrating tyrosine phosphorylation of Y735 residue of ITG$\beta 2$ cytoplasmic tail by a Src family kinase (Lck). Other reports have only shown serine/threonine phosphorylation of the integrin $\beta 2$ tails (Nurmi et al. 2007; Valmu et al. 1999). Importantly, a previous report showed that a tyrosine-based sorting signal, at Y735 residue, is important for recycling of internalized integrins to the cell surface and ligand-supported migration in transfected Chinese hamster ovary cells (Fabbri et al. 1999). Together these suggest a role for Lck-mediated phosphorylation of Y735 residue of ITG$\beta 2$ cytoplasmic tail in the integrin

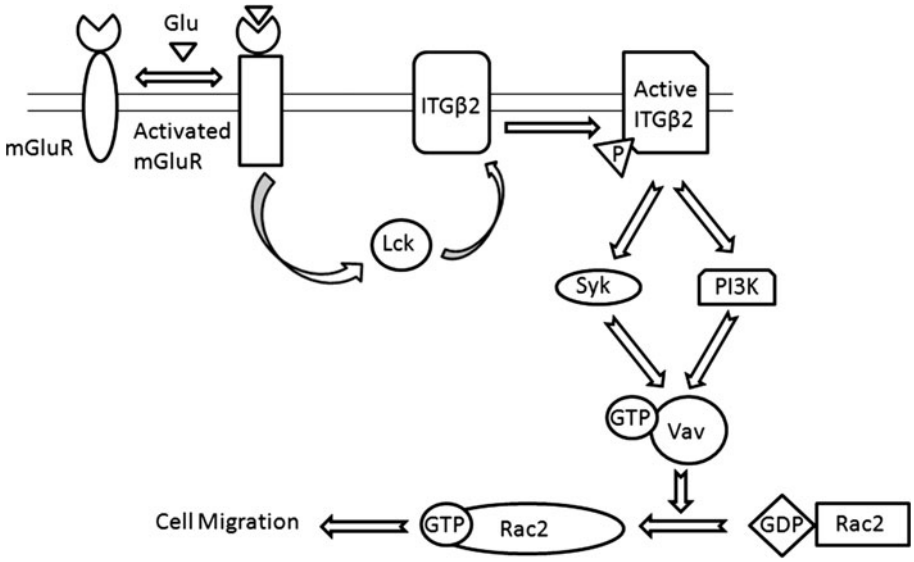

Fig. 8 Model depicting glutamate-mediated regulation of human neutrophil cell migration. In response to glutamate, the metabotropic receptors become activated, leading to phosphorylation and activation of the PTK Lck. In turn, Lck then phosphorylates the ITGβ2 receptor (LFA-1) tail leading to activation of Syk, PI3K and Vav1, finally causing activation of Rac2. This stimulates actin polymerization and increases the cell migration ability of human neutrophils

recycling process. Notably, our results showed that both Src family kinase member Lck and the Syk family kinase member Syk are activated upon glutamate stimulation in human neutrophils. However, only inhibition of Lck, but not Syk, caused significant inhibition of ITGβ2 phosphorylation/activation. Interestingly, inhibition of both Lck and Syk caused inhibition of Vav phosphorylation/activation. Our results thus showed that only Lck is responsible for phosphorylation of LFA1. Nonetheless, both Lck and Syk can regulate the downstream signaling events following glutamate stimulation. This suggests that Lck and Syk might both have overlapping as well as separate distinct function(s) in glutamate-induced signaling events in human neutrophils. Further support for this notion comes from the fact that Lck was previously found to activate Syk in human natural killer (NK) cells. Moreover, Src and Syk family PTKs can interact and synergize in inducing various substrate phosphorylation and thus activation events (Couture et al. 1996; Kurosaki et al. 1994; Pisegna et al. 2002; Ting et al. 1995).

Based on our results and observations, we propose a novel role for glutamate signaling in recruitment of human neutrophils at the injury or infection site. Here, we show that following an infection, injury or inflammation, glutamate is released into the extracellular space, which in turn acts as chemoattractant and recruits neutrophils to the target site. At first, glutamate activates the class I metabotropic glutamate receptors. This leads to activation of LFA-1 ($\alpha_L\beta_2$) integrin receptor through phosphorylation of the cytoplasmic tail domain caused by the Src family Receptor Tyrosine Kinase Lck. Along with Lck, Syk protein kinase also becomes activated following glutamate stimulation. Activation of Lck, Syk, as well as PI3K leads to activation of Vav protein, which in turn activates Rac2 and thereby stimulates cell migration. Interestingly, activated PMNs were previously shown to release glutamate causing decrease in endothelial barrier function (Collard et al. 2002), paving the way for neutrophil transendothelial migration. Therefore, it is possible that after being activated by glutamate and/or other soluble signals released by various skin cell types and/or damaged nerve endings present at the target site, neutrophils further release glutamate to attract more neutrophils and/or other leukocytes forming an autocrine/paracrine loop.

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Conflict of interest The authors declare that they have no conflict of interest.

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