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A critical 'threshold' of β_2-integrin engagement regulates augmentation of cytokine-mediated superoxide anion release

*,[1,2]Trevor R. Walker, [1,2,3]Marie-Helene Ruchaud-Sparagano, [1]Sarah R. McMeekin & [1]Ian Dransfield

[1]Rayne Laboratory, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG

1 Neutrophil adhesion regulates a number of processes involved in the pathogenesis of inflammatory diseases including rheumatoid arthritis. Neutrophil destructive potential can be modulated by adhesion, allowing alteration of inflammatory cell behaviour while preserving antimicrobial defences. β_2-Integrin-mediated neutrophil adhesion to albumin-coated latex beads (ACLB) allows modulation of integrin clustering and ligation and analysis of the effects of adhesion on neutrophil responses. Tumour necrosis factor-α (TNFα) enhanced neutrophil binding of different diameter ACLB equally, by almost four-fold, and independently of bead size. Adhesion of neutrophils to ACLB caused a size-dependent generation and release of O_2^- and also potentiated TNFα-induced O_2^- release.

2 Binding of ACLB was not affected by disruption of cytoskeletal integrity with nocodazole or cytochalasin D or following blockade of tyrosine kinase activity. In contrast, tyrosine phosphorylation and an intact cytoskeleton were essential for adhesion- and cytokine-induced O_2^- release from neutrophils. Inhibition of adhesion- and cytokine-induced O_2^- release by 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol[3,4-d]pyrimidine (PP2) indicated that a Src-family tyrosine kinase was the principal regulatory pathway mediating this response in neutrophils, a distal role for p38 MAPK was revealed by use of SB203580.

3 Tyrosine phosphorylation of c-Fgr, a Src-family tyrosine kinase, occurred following ACLB adhesion and exposure to TNFα, and was susceptible to inhibition by PP2. We suggest that activation of the key regulatory enzyme c-Fgr is achieved following ligation of a critical threshold of integrins following binding of large ($>3\,\mu$m) ACLB.
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Abbreviations: ACLB, albumin-coated latex beads; ECL, enhanced chemiluminescence; fMLP, formyl Met–Leu–Phe; PAF, platelet-activating factor; PAGE, polyacrylamide gel electrophoresis; PMN, polymorphonuclear leokocytes; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; PSGL-1, P-selectin-glycoprotein-ligand-1; TNFα, tumour necrosis factor-α

Introduction

The neutrophil granulocyte represents the vanguard of the cellular response to injury and infection (Haslett *et al*., 1989). Failure to recruit granulocytes results in inadequate clearance of microbes that can become life threatening (Anderson & Springer, 1987; Albelda *et al*., 1994). However, development of chronic inflammatory conditions with fibrotic repair mechanisms leading to compromised organ function is associated with excessive inflammatory cell recruitment or activation (Gailit & Clark, 1994). Thus, regulation of leukocyte recruitment and activation is critical for effective antimicrobial defences and efficient wound healing. Following rapid recruitment of neutrophils from the circulating pool of cells, a variety of microenvironmental stimuli induce rapid and specific mobilisation of granules that contain destructive enzymes and receptors for molecules of the innate and adaptive immune system, arming neutrophils for the recognition and removal of invading microorganisms (Borregaard & Cowland, 1997). This potent capacity for neutrophil-mediated destruction is very tightly regulated to ensure that inappropriate release of granule contents and superoxide, resulting in bystander damage to host tissue, is minimised. In addition, degranulation responses may be targeted to specific regions of the plasma membrane that are in close contact with target pathogens, further limiting potentially histotoxic effects (Owen & Campbell, 1995)

Many studies have examined 'priming' of neutrophil effector function by combinations of inflammatory mediators providing specific mechanisms for amplification of release of destructive granule contents. For example, although exposure of neutrophils to PAF has little effect on granule release alone, in combination with formyl Met–Leu–Phe (fMLP), granule secretion responses are greatly augmented (Aida & Pabst, 1990). Recent studies have demonstrated that adhesion receptors are also coupled to the intracellular signal transduction machinery, providing 'outside-in' signals that modulate

*Author for correspondence; E-mail: Trevor.Walker@ed.ac.uk
[2]Both authors contributed equally to this manuscript.
[3]Current address: Department of Pathology, School of Clinical and Laboratory Sciences, Medical School, The Royal Victoria Infirmary, Newcastle-upon-Tyne, NE1 4LP, U.K.
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leukocyte responses (Fashena & Thomas, 2000). Engagement of receptors that are involved in different stages of neutrophil recruitment have the potential to transduce signals that alter neutrophil behaviour, suggesting that the repertoire of receptors engaged on the neutrophil surface determines the functional responses in terms of adhesion, migration and secretion (Berton & Lowell, 1999). The functional alterations induced by adhesion receptors appear distinct from those caused by 'priming' agents, which have little effect on generation of reactive oxygen species by themselves (Aida & Pabst, 1990). As an example, ligation of neutrophil L-selectin and P-selectin-glycoprotein-ligand-1 (PSGL-1) has been shown to augment β_2-integrin-mediated adhesion and increase production of damaging reactive oxygen intermediates (Smolen *et al*., 2000). Selectin-dependent alterations in neutrophil function have been suggested to require co-stimulation with other agents, for example, platelet-activating factor (PAF) (Lorant *et al*., 1991; Ruchaud-Sparagano *et al*., 2000).

Studies in the 1980s demonstrated that adhesion to extracellular matrix components augmented release of degradative enzymes and toxic oxidant species (Nathan, 1987). Adherent neutrophils, in contrast to those in suspension, responded to secondary stimulation, for example, tumour necrosis factor-(TNFα) by the rapid and massive release of reactive oxygen species (Fuortes *et al*., 1999). Patients deficient in β_2-integrins failed to show adhesion-dependent amplification mechanisms, suggesting that ligation of β_2-integrins may induce specific signals that determine the extent of neutrophil activation (Nathan *et al*., 1989). Investigation of the mechanism by which integrin ligation promotes degranulation suggested that cytokines and integrins acted synergistically to decrease intracellular cAMP levels with protein kinase A acting as a negative regulator of granulocytes responses (Nathan & Sanchez, 1990). Tyrosine phosphorylation of key proteins through activation of tyrosine kinases is an important regulatory event in adhesion-dependent neutrophil activation (Fuortes *et al*., 1993). Use of the specific Src family kinase inhibitor, PP1 attenuated adhesion-dependent neutrophil degranulation, indicating a role for Src family tyrosine kinases (Mocsai *et al*., 1999). Further studies with double knockout p58(c-Fgr)−/−, p59/61(Hck)−/− animals also demonstrated defective adhesion-dependent responses, providing strong evidence that these kinases are involved. Examination of Fgr/Hck double knockout neutrophils showed a failure of spreading on extracellular matrix components, suggesting a strong link between the process of spreading and degranulation responses. Furthermore, one of the downstream targets of these Src family kinases, p72(Syk) was not phosphorylated (Lowell *et al*., 1996). Direct crosslinking of CD18 using antibodies fails to stimulate tyrosine phosphorylation of proteins that are important for the conversion of adhesion-dependent signalling and degranulation responses in Syk-deficient neutrophils (Yan & Berton, 1998). Activation of Src family kinases and Syk results in the activation of c-Cbl, Vav and Pyk-2 and redistribution of cytoskeletal proteins and components of the NADPH oxidase to a detergent insoluble fraction (Yan *et al*., 1997). Interestingly, neutrophil migration was not compromised in Syk-deficient animals, implying that migration is dependent upon signalling pathways from those involved in adherent activation. Furthermore, $hck^{-/-}$ $fgr^{-/-}$ $lyn^{-/-}$ neutrophils do not show impaired migration implying that β_2-dependent migration relies on different signalling pathways than those involved in adherent activation (Mocsai *et al*., 2002). Thus, integrin-mediated trans-endothelial migration may be possible without triggering damaging release of granule contents.

We have exploited ligand-coated microparticles in a dynamic assay system to engage granulocyte β_2-integrins in order to further examine the relative contribution of adhesion-dependent signals to neutrophil effector function. Our data suggest that there is a critical 'threshold' requirement for integrin occupancy and clustering, necessary for the assembly and activation of downstream signalling cascades leading to augmentation of reactive oxygen species production and release. In particular, our findings suggest that integrin occupancy alone is unable to fully trigger neutrophil effector function and that additional signals associated with cytoskeletal rearrangements necessary for spreading are required.

Experimental procedures

Neutrophil isolation

Polymorphonuclear leukocytes (PMN) were isolated from peripheral blood of healthy donors as described previously (Dransfield *et al*., 1994). Briefly, after centrifugation of citrated whole blood at 300 *g* for 20 min and removal of platelet-rich plasma, leukocytes were separated from erythrocytes by dextran sedimentation using 0.6% dextran T500. PMN were then separated from mononuclear leukocytes using discontinuous isotonic Percoll gradients. PMN leukocytes were 95–98% neutrophils using morphological criteria, and viability was assessed by trypan blue exclusion.

Neutrophil adhesion to albumin-coated latex beads (ACLB)

β_2-Integrin-dependent adhesion of ACLB to freshly isolated neutrophils was measured as described previously (Stocks *et al*., 1995). Fluorescent latex beads were washed in HBSS and incubated with human serum albumin (10 mg ml^{-1}) for 15 min before washes in HBSS and resuspension at 0.5% in HBSS. Neutrophils, in the presence and absence of inhibitors as appropriate, were added to ACLB and agonist as described in figure legends. Cells (2.5×10^5) and ACLB (2.5×10^6), at a ratio of 1 : 10 for all bead sizes and to ensure that beads were in excess at all times, were then incubated at 37°C for 15 min in a shaking waterbath, at 110 beats min^{-1}. After fixation of the cells with 0.5 ml of 0.5% glutaraldehyde, nonadherent ACLB were removed by three washes and bead binding to neutrophils was measured by flow cytometry. ACLB binding to neutrophils without phagocytosis was confirmed using the above method, utilising nonfluorescent beads and probing cell and bead suspensions with a monoclonal human albumin antibody (HSA-11, 1 : 500) and a FITC-conjugated secondary antibody (1 : 500) for 30 min at 4°C following fixation, bead binding was then analysed by flow cytometry.

Measurement of superoxide release

Determination of release of superoxide anions by freshly isolated neutrophils was performed as described previously (Condliffe *et al*., 1996). Briefly, neutrophils were preincubated with inhibitors as described in figure legends prior to addition

of TNFα, in the presence of cytochrome *c* (1 mg ml^{-1}), for 15 min at 37°C. Following this, the reaction was stopped by centrifugation (300 *g*, 5 min, 4°C) and the superoxide dismutase-inhibitable reduction of cytochrome *c* was determined for each supernatant by measuring the peak absorbance between 535 and 635 nm using a scanning spectrophotometer. Results are expressed as nanomoles of superoxide anions generated per 10^6 neutrophils.

Scanning electron microscopy

Following neutrophil adhesion to ACLB as indicated in figure legends, suspensions were transferred to clean glass coverslips by centrifugation at 300 *g* for 3 min and cells fixed in glutaraldehyde (3% v v^{-1}) in sodium cacodylate buffer (0.1 M, pH 7.4) for 3 h. Coverslips were washed three times in sodium cacodylate buffer (0.1 M, pH 7.4) for 20 min each and transferred to osmium tetroxide (1% v v^{-1}) in sodium cacodylate buffer (0.1 M, pH 7.4) for 1 h. Following a 30-min wash in distilled water, samples were dehydrated by sequential washes in increasing concentrations of acetone for 1 h each (50% v v^{-1}, 70% v v^{-1}, 90% v v^{-1}), followed by three washes in acetone (100% v v^{-1}) for 1 h each. Critical point drying with carbon dioxide was carried out and samples had a sputter coating of gold : palladium (20 nm, 60 : 40 w w^{-1}) added prior to viewing by scanning electron microscopy.

Western blotting

Neutrophils were lysed, following stimulation as detailed in figure legends, in lysis buffer containing Tris HCl (100 mM, pH 8.0), NaCl (100 mM), EDTA (2 mM), Nonidet NP-40 (1% v v^{-1}), Na_3VO_4 (5 mM), NaF (50 mM), protease inhibitor cocktail for 30 min at 4°C. Samples were centrifuged for 5 min at 13,000 *g* and supernatants were reduced with electrophoresis sample buffer containing Tris HCl (0.25 M, pH 6.8), sodium dodecylsulphate (8% w v^{-1}), β-mercaptoethanol (10% w v^{-1}), glycerol (30% v v^{-1}) and bromophenol blue (0.02%). Samples for immunoprecipitation were incubated overnight at 4°C with shaking with a monoclonal antibody to c-Fgr following cell lysis, Pansorbin® cells were added for 1 h, immunoprecipitated pellets were washed and reduced in electrophoresis sample buffer. Reduced samples were subjected to 10% polyacrylamide gel electrophoresis (PAGE), Western blotted and probed with Rc20 (monoclonal antiphosphotyrosine antibody) and protein bands were visualised by enhanced chemiluminescence (ECL).

Statistical analysis

Analysis was carried out using the paired Student's *t*-test, differences considered to be significant when $P<0.05$.

Materials

Latex beads (1–10 μm) were purchased from Polysciences Inc. (Warrington, PA, U.S.A.), Dextran T500, Percoll and ECL reagents were purchased from Amersham Pharmacia Biotech (Buckingham, U.K.), cytochrome *c* and monoclonal anti-human albumin (HSA-11) were purchased from Sigma (Poole, U.K.), protease inhibitor cocktail was purchased from Life Tech (Paisley, U.K.), Pansorbin®, GF109203X, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol[3,4-d]pyrimidine (PP2), 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3), nocodazole and cytochalasin D were purchased from Merck Biosciences (Nottingham, U.K.), monoclonal antibodies against c-Fgr and phosphotyrosine were purchased from Upstate Biotech (Milton Keynes, U.K.), antibody 44 was a generous gift from Professor N Hogg, Leucocyte Adhesion Laboratory, ICRF (London, U.K.), antibody MHM23 was from Abcam (Cambridge, U.K.) and all other reagents were of analytical grade.

Results

ACLB binding induced superoxide (O_2^-) release is dependent upon particle size

Our previous studies have shown that binding of ACLB to neutrophils occurs in a β_2-integrin dependent manner following analysis by flow cytometry (Stocks *et al*., 1995). We utilised different bead sizes (1–10 μm) in order to ligate and localise integrins differentially on the neutrophil plasma membrane upon adhesion, in order to ascertain whether a 'threshold' of integrin occupancy would induce a signalling event resulting in superoxide generation and release. In the absence of stimulation, a similar proportion of neutrophils (approximately 15%) were found to bind beads of either 1, 3 or 10 μm sizes. Stimulation with TNFα (20 ng ml^{-1}) promoted ACLB binding to 75–80% of neutrophils in a manner that was independent of particle size (Figure 1a). In Mg^{2+}-depleted buffer, binding of ACLB and O_2^- release was inhibited to control levels either in the presence and absence of TNFα, confirming that adhesion and adhesion-induced superoxide release were integrin-dependent (Figure 1). In contrast, when we examined O_2^- release following bead binding a differential effect of particle size was observed. Although binding of either 1 or 3 μm beads failed to induce O_2^- release above that observed under control conditions, 10 μm beads induced a significant increase in O_2^- release from 4.5 ± 0.7 nmol 10^{-6} cells under control conditions to 12.6 ± 0.9 nmol 10^{-6} cells (Figure 1b, $P<0.01$). Incubation of TNFα stimulated neutrophils with either 1 or 3 μm ACLB induced a small increase in O_2^- release when compared with TNFα alone. However, O_2^- release was significantly increased (approximately three-fold) in the presence of 10 μm ACLB (Figure 1b, $P<0.01$). This effect of 10 μm beads on O_2^- release was not simply additive with TNFα, implying that β_2-integrin-mediated adhesion to a relatively large continuous surface acts synergistically with TNFα to augment effector function. In Mg^{2+}-free buffer, the effect of ACLB binding to PMNs in the presence and absence of TNFα on O_2^- release and bead binding was inhibited thus confirming a requirement for functional integrins to mediate these adhesion-induced responses. The effect of the phorbol ester PMA, which activates protein kinase C directly and independently of integrins or cell surface receptors induced 68.2 ± 4.7 nmol 10^{-6} cells O_2^- release, which was unaffected by ACLB binding (data not shown). Figure 2 shows the effect of β_2-integrin-specific function blocking antibodies MHM23 and 44 to inhibit significantly fMLP-stimulated neutrophil binding to 1 μm ACLB by 89.9 ± 2.6 and 85.1 ± 1.5% respectively, thus confirming that neutrophil adhesion to ACLB is β_2-integrin-dependent.

Scanning electron microscopy confirmed that ACLB (1–10 μm) were bound to the surface of neutrophils and not

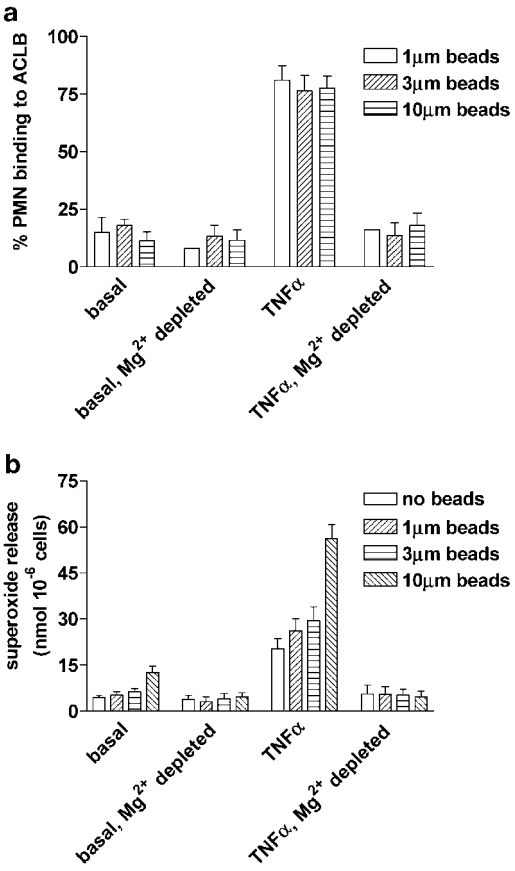

Figure 1 Binding of ACLB to human neutrophils induces superoxide release. (a) Cells in HBSS were treated with vehicle (open bars) or TNFα (20 ng ml^{-1}, horizontally hatched bars) or in Mg^{2+}-depleted HBSS (vehicle, obliquely hatched bars; TNFα, filled bars) for 15 min prior to addition of various sizes of ACLB (1–10 μm) as indicated, for a further 15 min at 37°C and shaken at 110 beats min^{-1}, and ACLB binding analysed by flow cytometry. Results are expressed as % PMN binding to ACLB ± s.e.m., results are from three experiments each performed in duplicate. (b) Cells were treated as indicated in (a), reactions were carried out at 37°C under shaking conditions and superoxide release determined by cytochrome *c* reduction as detailed in Methods. Results are expressed as nmol superoxide released 10^{-6} cells ± s.e.m., from 24 experiments carried out in duplicate.

phagocytosed following adhesion (Figure 3a–c), implying that loss of smaller beads from the external cell surface by phagocytosis does not account for their inability to induce O_2^- release. A monoclonal Ab specific for albumin was used to detect surface-bound but not internalised beads by flow cytometry. These experiments confirmed that ACLB (1–10 μm) were localised to the cell surface and that the extent of binding was similar for each size of ACLB (Figure 4a). Neutrophils treated with TNFα showed increased surface binding of ACLB, independently of size (Figure 4b), both observations being consistent with our results obtained with fluorescent ACLB binding to neutrophils (Figure 1a).

ACLB-induced O_2^- release requires cytoskeletal integrity

These data led us to consider the hypothesis that localisation and clustering of occupied integrins above a certain 'threshold' level generates signals that result in potentiation of TNFα-induced O_2^- release. We therefore used nocodazole (5 μM) and

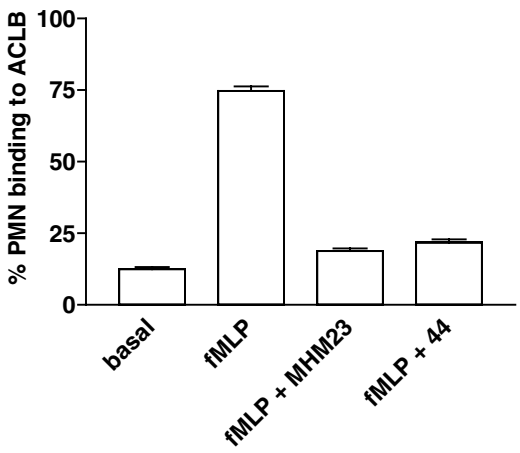

Figure 2 Binding of ACLB to neutrophils is β_2-integrin-dependent. Cells in HBSS were treated as indicated with β_2-integrin-blocking antibodies MHM23 and 44, at maximally effective concentrations as determined in functional assays (data not shown), for 15 min prior to addition of fMLP (10 nM). ACLB (1 μm) were added for a further 15 min at 37°C and shaken at 110 beats min^{-1}, and ACLB binding was analysed by flow cytometry. Results are expressed as % PMN binding to ACLB ± s.e.m., results are from three experiments each performed in duplicate.

cytochalasin D (5 μM), which are known to be selective concentrations at disrupting either microtubule dynamics or actin microfilaments respectively (Howard *et al*., 1981; Zaslaver *et al*., 2001). Interestingly, these treatments had no significant effect on binding of ACLB (1–10 μM) to neutrophils, indicating that integrin functionality was not compromised (Figure 5a and 6a). Both nocodazole and cytochalasin D were found to inhibit partially TNFα and ACLB-potentiated TNFα-induced O_2^- release (Figure 5b and 6b), confirming a role for intact cytoskeletal elements in providing scaffolding for the formation of signalling complexes to mediate O_2^- generation in response to both cytokines and adhesion events.

Tyrosine phosphorylation is required for ACLB-induced O_2^- release

Binding of ACLB to neutrophils appears to be PKC-independent, as the specific inhibitor GF109203X had no significant effect on ACLB (10 μm) binding in the presence and absence of TNFα (Figure 7a). In addition, O_2^- release induced by TNFα in the presence of bound ACLB (10 μm) was not significantly inhibited by GF109203X whereas PMA-induced O_2^- release was inhibited by 75 ± 14% (Figure 7b). These data suggest that PKC activation and resulting serine and threonine phosphorylation of its substrates is not required for integrin-mediated potentiation of superoxide release. In contrast, a role for tyrosine kinases in mediating the signalling events that regulate superoxide release in neutrophils subsequent to receptor activation, integrin occupancy and clustering was suggested by the ability of genistein (50 μM) to inhibit completely O_2^- release induced by TNFα in the presence and absence of bound ACLB (Figure 7b). Genistein, a selective tyrosine kinase inhibitor (IC_{50} 2.6 μM) (Liles *et al*., 1995) had no effect on ACLB binding to neutrophils under control and TNFα-stimulated conditions (Figure 7a) ruling out an effect on integrin ligation directly. Importantly, as shown in Figure 7b, genistein was not able to block superoxide release

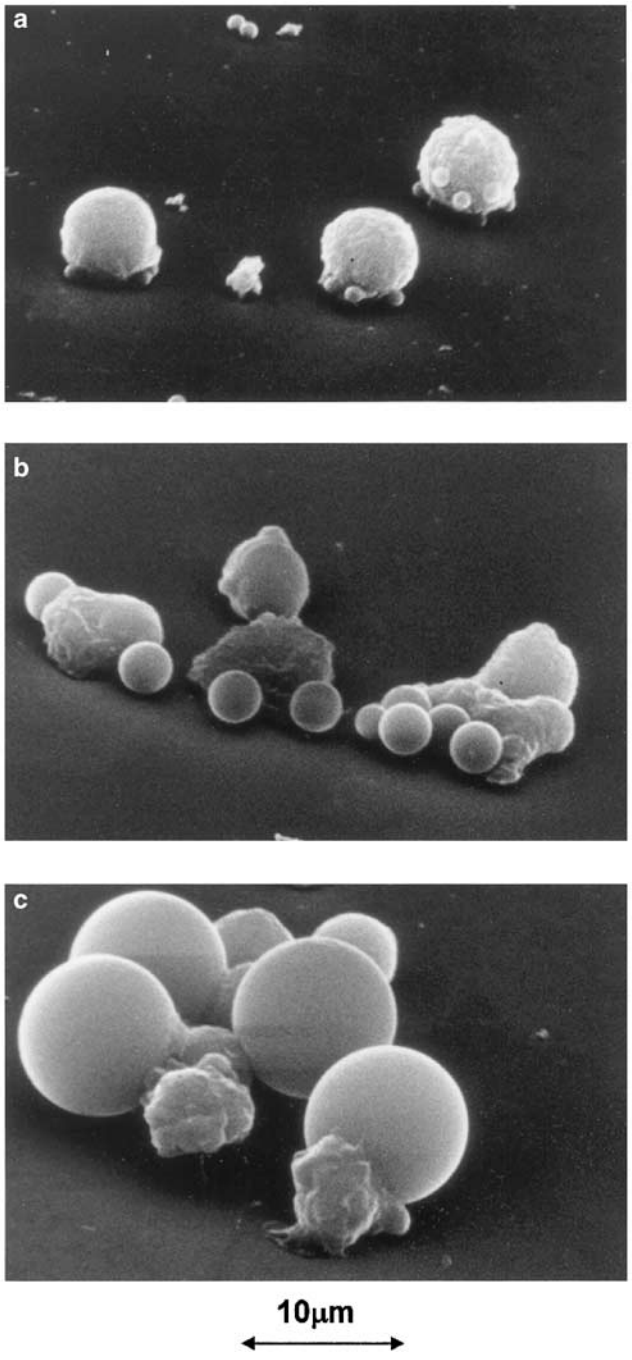

Figure 3 Scanning electron microscopy of neutrophils adhering to ACLBs. Cells were incubated with ACLB (a) 1 μm, (b) 3 μm, (c) 10 μm for 15 min at 37°C and shaken at 110 beats min^{-1}, cell suspensions were transferred to glass coverslips, processed and examined by scanning electron microscopy as described in Experimental procedures.

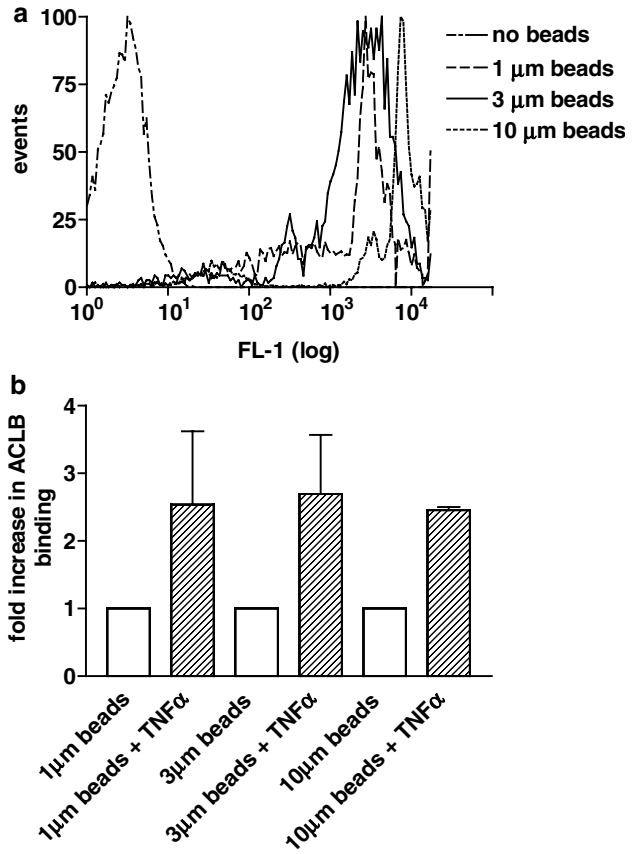

Figure 4 Neutrophil cell surface-bound ACLBs detected by flow cytometry. (a) Flow cytometric analysis of ACLB binding was carried out as in Figure 1, using nonfluorescent beads and a monoclonal human albumin antibody to detect binding of beads to the neutrophil surface. (b) ACLB binding in the presence and absence of TNFα (10 ng ml^{-1}) was carried out and analysed as detailed in (a). Results are expressed as fold increase in ACLB binding $\pm$ s.e.m., above control fluorescence detected with cells in the absence of ACLB, results are from individual observations from three experiments.

induced through direct activation of protein kinase C by PMA, ruling out a direct effect on assembly of the NADPH oxidase. Further experiments using the Src family tyrosine kinase inhibitor PP2, at a known selective concentration (IC_{50} 0.6 μM) (Salazar & Rozengurt, 1999), revealed inhibition of O_2^- release induced by binding of ACLB (10 μm) in the presence or absence of TNFα to below control levels (Figure 8a). PP2 had no effect on PMA-induced O_2^- release and PP3, an inactive structural analogue of PP2, also had no effect on ACLB, TNFα or PMA-induced O_2^- release (data not shown). These data suggest an obligatory role for an Src-family tyrosine kinase in mediating O_2^- release induced by both ACLB binding, through integrin occupancy and clustering, and by TNFα. Our observations also implicate an Src-family tyrosine kinase at a point of convergence of the signalling pathways initiated by the TNFα receptor and β_2-integrins, which act synergistically to induce O_2^- release in neutrophils.

As p38 MAPK has previously been implicated in regulating release of primary and secondary granules from neutrophils in an Src-family tyrosine kinase-dependent manner (Mocsai *et al*., 2002), we examined whether p38 MAPK was a potential downstream target of Src-family kinases in the pathway mediating O_2^- release. Adhesion of neutrophils to ACLB (10 μm) caused a modest increase in the active phosphorylated form of p38 MAPK, whereas TNFα induced a significant increase in the active form of p38 MAPK, which was not further increased upon subsequent adhesion to ACLB (Figure 8b). A role for p38 MAPK as a potential downstream target of Src-family kinases in the pathway mediating O_2^- release as identified was identified by the ability of SB203580 (10 μM), a concentration known to be maximally effective in

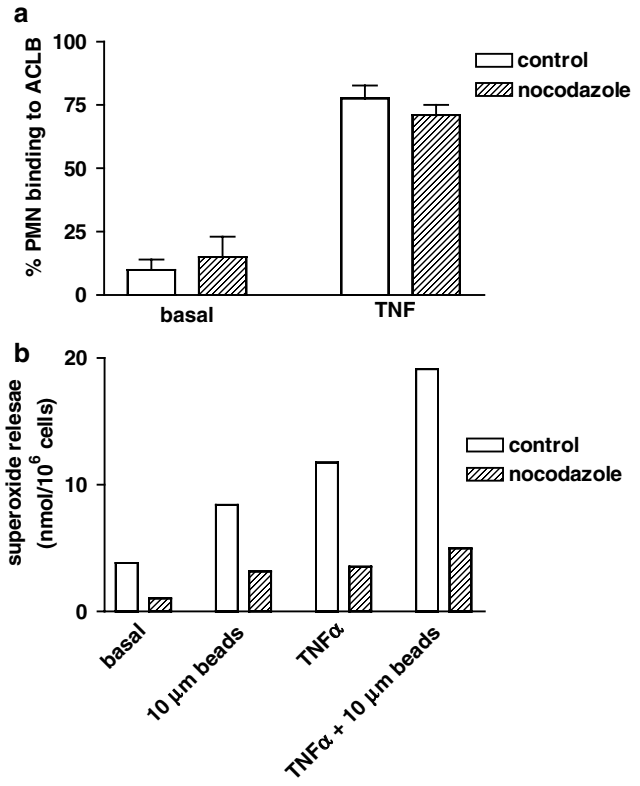

Figure 5 Release of superoxide upon ACLB binding requires intact microtubules. (a) Cells were treated with nocodazole (5 μM, hatched bars) or vehicle (open bars) for 10 min after addition of TNFα (20 ng ml^{-1}) and ACLB (10 μm) for 15 min as indicated and superoxide release measured as described before. Results are expressed as nmol superoxide released 10^{-6} cells $\pm$ s.e.m., from three experiments carried out in duplicate. (b) Cells were treated as for (a) and binding to ACLB (10 μm) was analysed by flow cytometry as described before. Results are expressed as % PMN binding to ACLB $\pm$ s.e.m., from three experiments carried out in duplicate.

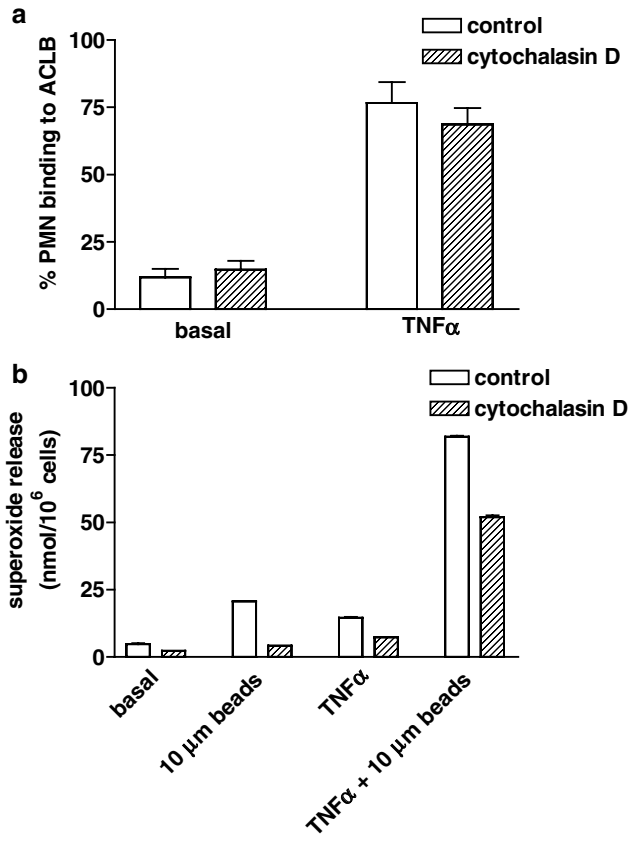

Figure 6 Release of superoxide upon ACLB binding requires intact actin microfilaments. (a) Cells were pretreated with cytochalasin D (5 μM, hatched bars) or vehicle (open bars) for 10 min for 37°C after addition of TNFα (20 ng ml^{-1}) and ACLB (10 μm) for 15 min as indicated, superoxide release measured as described before. Results are expressed as nmol superoxide released 10^{-6} cells $\pm$ range, from one experiment, representative of two others with similar results. (b) Cells were treated as for (a) and binding to ACLB (10 μm) analysed by flow cytometry a described before. Results are expressed as % PMN binding to ACLB $\pm$ s.e.m., from three experiments carried out in duplicate.

neutrophils and to have no effect on the formation of the NADPH oxidase (Cuenda *et al*., 1995; Lal *et al*., 1999), to partially inhibit O_2^- release induced by TNFα and bound ACLB (10 μm) from neutrophils (Figure 8c).

Binding of ACLB induces tyrosine phosphorylation of Fgr

In view of the above data suggesting involvement of Src family kinases in mediating the ACLB-induced O_2^- response of neutrophils, we next examined patterns of tyrosine phosphorylation in neutrophils. Adhesion of ACLB (10 μm) to neutrophils induced tyrosine phosphorylation of a protein of approximately 56–58 kDa, as detected in whole-cell lysates (Figure 9a). Tyrosine phosphorylation of this protein was dependent on ACLB particle size, implicating the extent of integrin occupancy and clustering as a regulating factor in the level of tyrosine kinase activity and phosphorylation status of this protein. Treatment of neutrophils with TNFα only induced a modest increase in tyrosine phosphorylation of this protein above control levels, but in the presence of TNFα and adhesion to ACLB (10 μm), there was a dramatic potentiation of the tyrosine phosphorylation of this 56–58 kDa protein, apparently correlating with the level of induced O_2^- release (Figure 9a).

Immunoprecipitation of one potential candidate, the Src-family tyrosine kinase Fgr showed that this enzyme became tyrosine phosphorylated in response to TNFα, adhesion to ACLB (10 μm) also resulted in tyrosine phosphorylation of Fgr and in combination resulted in potentiation of this phosphorylation (Figure 9b). Furthermore, tyrosine phosphorylation of Fgr following activation by TNFα and adhesion to ACLB was sensitive to inhibition by PP2, consistent with the suggestion that either autophosphorylation of c-Fgr or phosphorylation of c-Fgr *via* a related tyrosine kinase was required for translation of adhesion-dependent signals to augmentation of superoxide release (Figure 9b).

Discussion

In this study we have shown that the ability of PMN to initiate a respiratory burst and release of superoxide can be potentiated by β_2-integrin-mediated adhesion to ACLB, in a size-dependent manner. The novel approach in which ACLB have similar ligand density has allowed exposure of cells to regulated levels of adherent substrate of defined surface area,

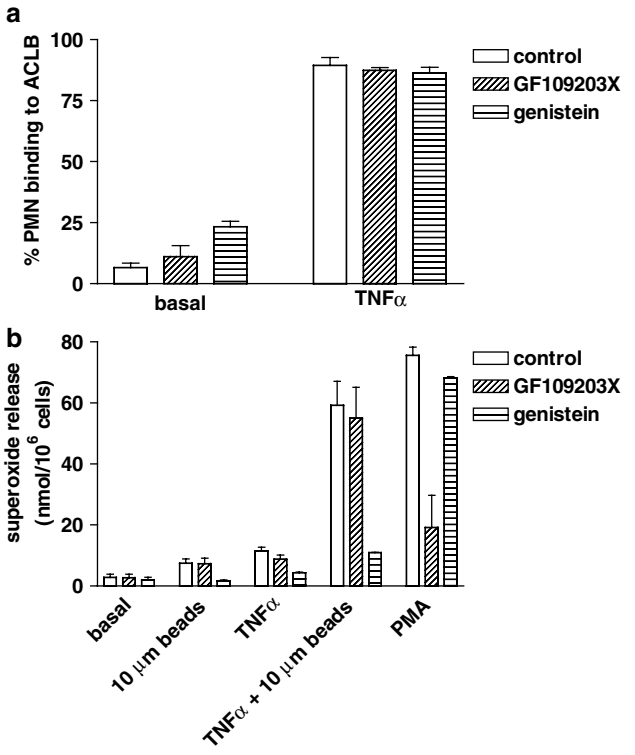

Figure 7 Release of superoxide upon ACLB binding requires tyrosine phosphorylation. (a) Cells were treated with vehicle, TNFα (20 ng ml^{-1}) for 20 min prior to addition of GF109203X (oblique hatched, 5 μM) or genistein (horizontally hatched, 50 μM) for 10 min. Cells were then adhered to ACLB (10 μm) under shaking conditions at 37°C for 15 min and ACLB binding analysed by flow cytometry. Results are expressed as % PMN binding to ACLB ± s.e.m., from three experiments carried out in duplicate. (b) Cells were treated with vehicle, TNFα (20 ng ml^{-1}) or PMA (10 nM) and inhibitors as detailed in (a) and superoxide release measured as described before. Results are expressed as nmol superoxide released 10^{-6} cells ± s.e.m., from three experiments carried out in duplicate.

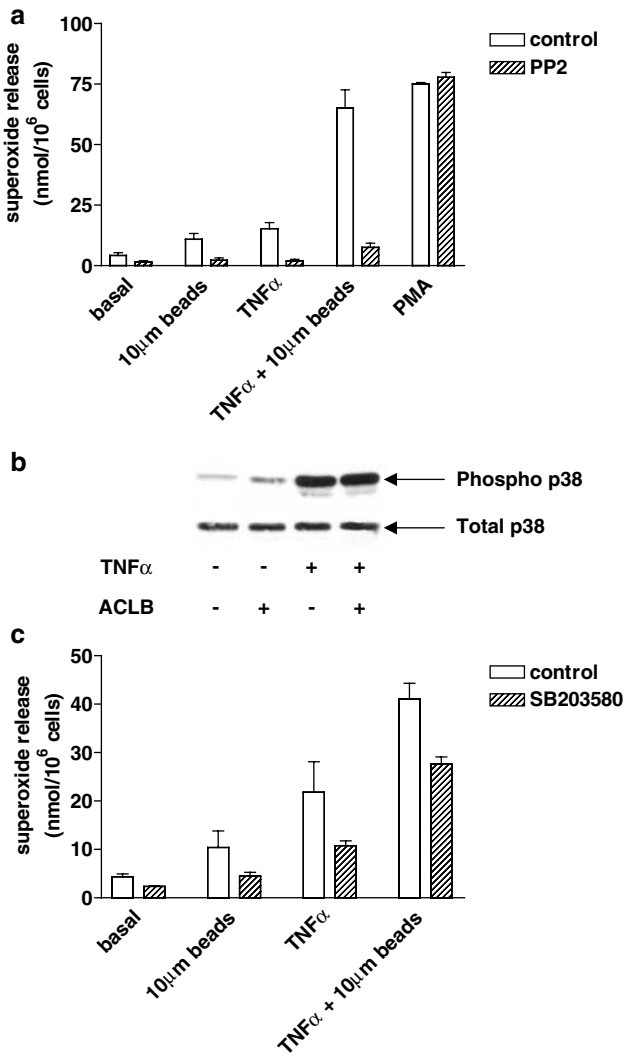

Figure 8 Adhesion-induced superoxide release requires Src family kinases and p38 MAP kinase. (a) Cells were treated with PP2 (5 μM, hatched bars) or vehicle (open bars) for 15 min prior to addition TNFα (20 ng ml^{-1}) and ACLB (10 μm) as indicated for 15 min and superoxide release measured as described before. Results are expressed as nmol superoxide released 10^{-6} cells ± s.e.m., from three experiments carried out in duplicate. (b) Cells were treated with TNFα (20 ng ml^{-1}) and ACLB (10 μm) as indicated and whole-cell lysates analysed by Western blotting using p38 MAPK and phospho-p38 MAPK antibodies. (c) Cells were treated with SB203580 (10 μM, hatched bars) or vehicle (open bars) for 10 min prior to addition of TNFα (20 ng ml^{-1}) and ACLB (10 μm) for 15 min and superoxide release measured as described before. Results are expressed as nmol superoxide released 10^{-6} cells ± s.e.m., from three experiments carried out in duplicate.

enabling limitation of the extent of integrin engagement associated with neutrophil adhesion. As previously demonstrated, β_2-integrin-dependent ACLB adherence to neutrophils was found to be enhanced in the presence of TNFα (Young *et al*., 1990), suggesting that an 'inside-out' modulation of integrin activity accounts for promotion of adhesion (Condliffe *et al*., 1996). Increased integrin adhesion following cytokine stimulation has been previously shown to occur independently of changes in cell surface expression of β_2-integrins (Vedder & Harlan, 1988; Schleiffenbaum *et al*., 1989). Consistent with this suggestion, our data show that neutrophil adhesion to both 1 and 10 μm ACLB was promoted to the same extent by TNFα, suggesting that differential integrin ligation was not the major factor involved in the regulation of superoxide responses. We present evidence that particle size and by implication localisation of integrins involved in adhesion plays a critical role in potentiation of TNFα-induced superoxide release. Binding of larger 10 μm particles were able to induce superoxide release and importantly to potentiate the TNFα dependent superoxide responses, presumably through ligating and clustering sufficient integrins to single sites of adhesion to form a competent signalling complex. In contrast, multiple 1 μm β_2-integrin ligand-coated particles would ligate numerous patches of integrins over the neutrophil surface, but we hypothesise that insufficient clustering fails to form signalling complexes and an inability to trigger superoxide release. Importantly, these smaller beads remain bound to the neutrophil plasma membrane and are not internalised, as shown by scanning electron microscopy and flow cytometry; therefore, the inability of smaller beads to induce O_2^- release is not due to phagocytosis and phagosomal membrane closure resulting in termination of integrin signalling. These data demonstrate that a 'threshold' effect of integrin engagement and clustering occurs in neutrophils that may determine the extent of response to cytokines and adhesion-dependent signals.

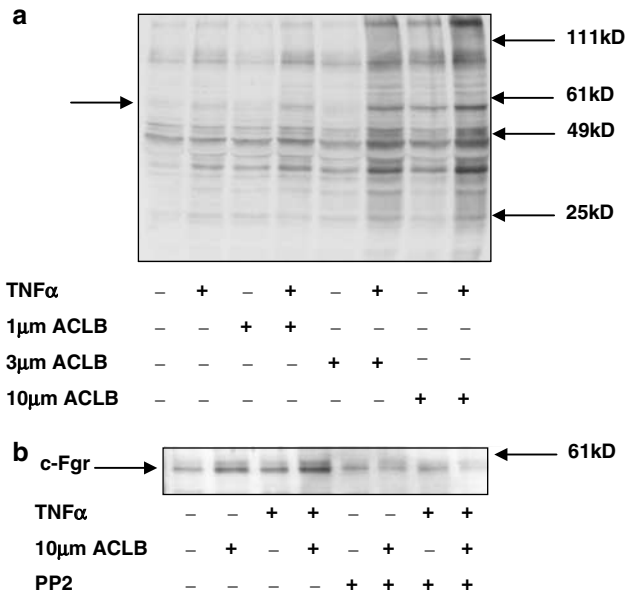

Figure 9 Adhesion induces tyrosine phosphorylation of Fgr. (a) Cells were incubated with TNFα (20 ng ml^{-1}) for 15 min prior to addition of ACLB (1–10 μm) for 15 min at 37°C as indicated. Western blots of whole-cell lysates were probed with Rc20 (antiphosphotyrosine) and visualised with ECL, molecular weight markers are as indicated. The tyrosine-phosphorylated protein of interest is indicated with an arrow. (b) Cells were incubated with TNFα (20 ng ml^{-1}) for 15 min prior to addition of ACLB (10 μm) for 15 min at 37°C as indicated. Cells were immunoprecipitated with a monoclonal antibody to c-Fgr, Western blotted, probed with Rc20 (antiphosphotyrosine) and visualised with ECL, c-Fgr is indicated.

Interestingly, our studies show that integrin engagement *per se* does not trigger enhancement of superoxide responses to a second agonist. It is now well established that integrin engagement leads to the assembly of adhesion-dependent signalling complexes, containing components such as Src family kinases (Lowell & Berton, 1999). Use of specific inhibitors of cytoskeletal regulation such as nocodazole and cytochalasin D revealed that augmented O_2^- release could be blocked without influencing particle binding. These inhibitors also showed that there is a small degree of O_2^- release from neutrophils in suspension, being mediated by constitutive signalling events and cytoskeletal dynamics or may involve transient neutrophil/neutrophil interactions under these conditions. Previous reports have shown that cytoskeletal disruption with cytochalasin D (5 μM) could inhibit leukocyte motility and phagocytosis implying the necessity of cytoskeletal function for cellular responses (Howard *et al*., 1981). Our data therefore suggest that a competent cytoskeleton may be required for delivery, formation and functional coupling of signalling complexes to the site of particle adhesion (van Spriel *et al*., 2001), necessary for enhanced production of reactive oxygen species in response to TNFα. Previous studies indicate a critical requirement for cytoskeletal structures in spreading neutrophil adhesion (Sheikh *et al*., 1997) and there is a close association between the cytoskeleton and superoxide release from neutrophils (el Benna *et al*., 1994). Since we have demonstrated that neutrophil adhesion to ACLB occurs independently of cytoskeletal reorganisation and microtubule reassembly this may represent a useful model to allow dissociation of integrin-mediated adhesion events from the process of cell spreading. Our data suggest that in addition to integrin-mediated recruitment of effector molecules (Yan & Berton, 1998) further cytoskeletal reorganisation is required for the formation of a competent signalling complex that mediates superoxide generation in response to both cytokines and adhesion. One possibility is that active participation of the microfilament and microtubule networks within neutrophils directs movement of signalling proteins or effector molecules to sites of integrin interaction (van Spriel *et al*., 2001).

Tyrosine kinase activation has been reported to be required for cytoskeletal rearrangement essential for neutrophil spreading and strengthening of adhesion and migration (Gaudry *et al*., 1992; Takami *et al*., 2001). Experiments using the selective protein kinase C inhibitor GF109203X demonstrate that protein kinase C does not have a role in ACLB binding and TNFα-induced O_2^- release. TNFα has been shown to induce tyrosine phosphorylation of a number of proteins in neutrophils in an adhesion-dependent manner, resulting in recruitment of proteins to adhesion contacts (Fuortes *et al*., 1993). For neutrophils adherent to 10 μm ACLB, we have demonstrated an obligate role for tyrosine phosphorylation since genistein, a selective tyrosine kinase inhibitor (Liles *et al*., 1995), resulted in almost complete inhibition of O_2^- release induced by TNFα. However, ACLB binding was not affected, implying that genistein inhibits assembly of signalling complexes and cytoskeletal reorganisation required for spreading adhesion in neutrophils as suggested by Fuortes *et al*. (1993). Inhibition of O_2^- release by genistein from unstimulated neutrophils in suspension indicates that a degree of constitutive signalling occurs under these conditions and involves the same pathways that are involved in adhesion- and cytokine-induced activation. This small degree of 'constitutive' activation may reflect neutrophil/neutrophil adhesive interactions in suspension as unstimulated O_2^- release was also inhibited by removal of Mg^{2+} and may therefore involve integrin activation. We have demonstrated that Fgr, a Src-family tyrosine kinase, undergoes tyrosine phosphorylation upon adhesion of ACLB and that adhesion-induced O_2^- release requires Src-family tyrosine kinase activity and integrin functional competency. Whether Fgr undergoes autophosphorylation or is phosphorylated by a related tyrosine kinase remains to be confirmed. These findings are consistent with studies in murine neutrophils, for which the Src family kinases Fgr, Hck and Lyn are critical (Mocsai *et al*., 2000).

A number of candidate downstream effectors of Src-family tyrosine kinases have been identified but their respective roles have not yet been fully characterised. The tyrosine kinase Syk is recruited to the cytoskeleton in an Src-dependent manner following β_2-integrin ligation and neutrophils from Syk$^{-/-}$ mice did not undergo adhesion or activation following ligation of β_2-integrins (Mocsai *et al*., 2002). Activation of p38 MAPK and subsequent granule release from neutrophils by fMLP has also been shown to be a downstream target of Src-family kinases and Syk (Mocsai *et al*., 2002). This study and others (Waterman *et al*., 1996) have shown that TNFα induces activation of p38 MAPK in neutrophils, and also adhesion caused a partial activation of this signalling enzyme. Direct inhibition of p38 MAPK partially reduced release of primary and secondary granules, thus implicating this pathway as a possible regulatory event (Mocsai *et al*., 2002). Our data show partial inhibition of adhesion-induced superoxide release in the presence and absence of TNFα by the p38 MAPK inhibitor

SB203580, implicating this pathway as a possible downstream target of Fgr. In agreement, SB203580, in a concentration-dependent manner, has also been shown to only partially inhibit fMLP- and PMA-induced O_2^- release without affecting assembly of the NADPH oxidase (Lal *et al*., 1999) However, the p38 MAPK pathway must act in parallel with a distinct signalling pathway that is insensitive to SB203580 to regulate the extent of adherent neutrophil responses. Interestingly, ligation of L-selectin on neutrophils has been shown to lead to β_2-integrin regulation and activation of p38 MAPK and ERK1/2, demonstrating a potential mechanism where adhesion to L-selectin can recruit and amplify signals transduce *via* β_2-integrin activation (Green *et al*., 2002).

A sequential model in which engagement of β_2-integrins using monoclonal antibodies induced integrin-dependent cytoskeletal rearrangement resulting in signalling complex formation between cytoskeletal proteins and tyrosine kinases, leading to *de novo* actin-polymerisation and tyrosine kinase activation, has been proposed (Yan & Berton, 1998). Based on our observations, we would suggest that integrin ligation with ACLB promotes activation events through β_2-integrins that involve microtubule and microfilament reorganisation and redistribution of the MTOC and the Golgi within neutrophils. The kinetics of adhesion-dependent signalling protein recruitment are complex but require in part a reorganised cytoskeleton to provide a framework for the targeted delivery of membrane vesicles containing specific membrane proteins and enzymes, together with Src-family tyrosine kinases to regulate the extent of neutrophil activation. The ability of neutrophils to release O_2^-, although to a limited extent, in the presence of agents that disrupt actin assembly and microtubules suggest that key signalling components may be dynamically moved to and away from sites of integrin activation. Tyrosine phosphorylation and activation of Fgr is an obligatory step in the regulation of neutrophil O_2^- release and may integrate activation inputs from both integrins and TNF receptors to mediate the potentiation of O_2^- release from neutrophils.

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