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Chapter 5

Actin polymerization induces shedding of FcγRIIIb from human neutrophils

ACTIN POLYMERIZATION INDUCES SHEDDING OF FcγRIIIb FROM HUMAN NEUTROPHILS

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ABSTRACT

FcγRIIIb (CD16) is a glycosyl phosphatidylinositol (GPI) anchored low-affinity IgG receptor, exclusively expressed on human neutrophils. FcγRIIIb associates with complement receptor 3 (CR3, Mac-1, CD11b/CD18), which may indirectly link FcγRIIIb to the actin cytoskeleton. Upon neutrophil activation or apoptosis, FcγRIIIb is shed from the cell surface. In all of these events, actin rearrangements play an important role. To establish a role for the actin cytoskeleton in the control of FcγRIIIb shedding, we treated human neutrophils with jasplakinolide, an actin-polymerizing peptide. We found that enhanced actin polymerization induces time- and dose-dependent shedding of FcγRIIIb. This effect was not restricted to FcγRIIIb, because the cell surface expression of CD43, CD44 and leucocyte (L)-selectin was also downregulated after induction of actin polymerization. This actin-dependent pathway is staurosporine-sensitive but does not appear to involve activation of protein kinase C (PKC) or CR3. These data show that the actin cytoskeleton can regulate protein ectodomain shedding from human neutrophils.

INTRODUCTION

The neutrophil represents the first line in the non-specific cellular host defense against a wide range of pathogens. When invading micro-organisms are opsonized with immunoglobulins (Ig) and complement fragments, the neutrophil will recognize and bind these particles through its Fc and complement receptors. This results in neutrophil activation followed by phagocytosis and killing of the pathogen. Human neutrophils express two types of low-affinity receptor for IgG: FcγRIIa (CD32) and FcγRIIIb (CD16) (Anderson and Looney, 1986). FcγRIIa is a membrane-spanning molecule, whereas FcγRIIIb is linked to the membrane by a glycosyl phosphatidylinositol (GPI) anchor (Selvarai et al., 1988; Qiu et al., 1990).

Despite the absence of a cytoplasmic tail, FcγRIIIb is capable of transducing signals into the cell that lead to actin filament assembly, phagocytosis and exocytosis of the content of secretory vesicles, specific and azurophilic granules (Huizinga et al., 1990d; Salmon et al., 1991). It is likely that the interaction of FcγRIIIb with transmembrane molecules allows the induction of intracellular signalling. Several studies have shown that the complement receptor 3 (CR3, Mac-1, CD11b/CD18), which is the major β2-integrin expressed on neutrophils, associates with FcγRIIIb on the neutrophil surface (Zhou et al., 1993; Todd III and Petty, 1997) and cooperates in the generation of the respiratory burst in neutrophils (Zhou and Brown, 1994). Studies with fibroblasts expressing CR3 and/or FcγRIIIb have shown that both molecules are necessary for complete phagocytosis (Krauss et al., 1994). When CR3 is cross-linked by antibodies or binds to extracellular matrix proteins, actin polymerization is induced (Löfgren et al., 1993; Walzog et al., 1994). The association of CR3 with the actin
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cytoskeleton is mediated via actin-binding proteins such as α-actinin, which shows increased binding to the cytoplasmic tail of the β2-subunit (CD18) when neutrophils are activated (Pavalko and la Roche, 1993).

Upon stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA) or during apoptosis, FcγRIIIb is shed from the cell surface by proteolytic cleavage (Huizinga et al., 1988; Dransfield et al., 1994; Homburg et al., 1995). It is currently unknown how this proteolytic event is regulated. Several studies have suggested a link between cytoskeletal rearrangements and the induction of shedding. During chemotaxis, which involves adhesion, spreading and enhanced motility of neutrophils, L-selectin and CD43 are shed from the cell surface (Kuijpers et al., 1992; Lopez et al., 1998). Similarly, antibody-induced cytoskeletal reorganization in pseudorabies virus-infected cells results in shedding of viral glycoproteins (Favoreel et al., 1997).

Shedding of cell surface proteins is not specific for adhesion molecules, but also involves growth factors and inflammatory mediators, such as TNF-α. It has been suggested that the TNF-α converting enzyme (TACE, ADAM17) is brought in proximity of its substrate via actin rearrangements (Werb and Yan, 1998). TACE is a disintegrin and metalloproteinase (ADAM) family member involved in shedding of a wide range of cell surface proteins (Peschon et al., 1998). In contrast to TNF-α, the protease responsible for FcγRIIIb shedding and its mechanism of activation is unknown. Studies with protease inhibitors have indicated that many cellular membrane proteins can be shed by (matrix) metalloproteinases (Hooper et al., 1997). However, FcγRIIIb shedding is inhibited not only by metalloproteinase inhibitors, but also by serine protease inhibitors (Bazil and Strominger, 1994).

In this study we have analyzed the link between cytoskeletal rearrangements and FcγRIIIb shedding from the human neutrophil. We have used the cyclic peptide jasplakinolide, isolated from the marine sponge Jaspis johnstoni, which promotes polymerization of globular actin (G-actin) into filamentous actin (F-actin) (Crews et al., 1986; Bubb et al., 1994). This results in increased association of actin with the Triton-insoluble cytoskeleton, leading to cellular rigidity and loss of integrin-mediated adhesion of human neutrophils (Sheikh et al., 1997). Our present results show that increased actin polymerization is sufficient to induce shedding of FcγRIIIb, CD43, CD44 and L-selectin from neutrophils.

MATERIAL AND METHODS

Materials
Phorbol 12-myristate 13-acetate (PMA), staurosporine, herbimycin A and cytochalasin D (cyto D) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Tumor necrosis factor alpha (TNF-α), PP1 and Ro31-8220 were purchased from Calbiochem-Novabiochem Co., San Diego, CA, USA. Jasplakinolide was purchased from Molecular Probes, Leiden, The Netherlands. The following monoclonal antibodies (mAb) were obtained from the CLB, Amsterdam, The Netherlands: CLB-Fcgran1 (FcγRII, CD16), DFT1 (CD43), NK1-P2 (CD44), CLB-B13.9 (CD66b), murine control IgG1 and fluoresceine-isothiocyanate (FITC)-labeled goat-anti-mouse-Ig. The mAb Leu-8 (CD62L) was obtained from Becton and Dickinson, San Jose, CA, USA. Fibronectin was from the CLB, Amsterdam, The Netherlands.
Isolation of neutrophils

Peripheral blood was obtained from healthy volunteers. Granulocytes were purified from buffy coats of 500 ml of blood anticoagulated with 0.4% (w/v) trisodium citrate, as described before (Roos and de Boer, 1986). In short, mononuclear cells and platelets were removed by density centrifugation over isotonic Percoll with a specific gravity of 1.076 g/ml. Erythrocytes were removed by a 10-min treatment with ice-cold lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA). The remaining granulocytes were washed twice in phosphate-buffered saline (PBS) and were resuspended in incubation medium [132 mM NaCl, 6 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 1.2 mM K₂HPO₄, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) human serum albumin (pH 7.4)] at concentrations of 2x10⁶/ml or 10⁷/ml. The purity and viability of the neutrophils was over 95%.

Cell treatment in suspension

Neutrophils (10⁷/ml) were preincubated in a shaking waterbath for 5 min at 37°C, jasplakinolide or vehicle DMSO was added, and the incubation was continued for 60 minutes at 37°C. Subsequently, the neutrophils were treated with PMA or vehicle DMSO for 10 minutes at 37°C. At the end of the incubation period the cells were briefly centrifuged and cell-free supernatants were collected for quantitation of soluble FcyRIIIb by ELISA (see below). Cell pellets were fixed in ice-cold PBS containing bovine serum albumine (0.1% w/v) and were used for flow cytometry. Various kinase inhibitors (staurosporine, 100 nM; Ro31-8220, 10 uM; herbimycin A, 1 uM; PP1, 10 uM) were also tested in this system. For time-course studies, neutrophils (10⁷/ml) were preincubated in a shaking waterbath for 5 min at 37°C, jasplakinolide (10 µM) was added, and samples were taken at various times as indicated in the figures and processed as described above. Cells activated with PMA (200 ng/ml) for 10 min at 37°C served as positive controls for FcyRIIIb shedding.

Flow cytometry

The level of expression of FcyRIIIb, CD43, CD44, L-selectin and CD66b on the neutrophil surface was determined by FACScan analysis. Fixed neutrophils were washed once with ice-cold PBS containing bovine serum albumine (0.1% w/v) and were then incubated with mAb (5 µg/ml) for 45 minutes at 4°C. The cells were subsequently stained with fluoresceine-isothiocyanate (FITC)-labeled goat-anti-mouse-Ig for 30 minutes at 4°C. After washing, the cells were resuspended in PBS containing bovine serum albumin (0.1% w/v), and their fluorescence was measured by FACScan (Becton and Dickinson, San Jose, CA, USA).

Immunoprecipitation from cells

Neutrophils were labeled with ¹²⁵I-iodide according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL, USA). After labeling, the cells were resuspended in incubation medium and treated with jasplakinolide and PMA as described above. Immunoprecipitation of FcyRIIIb was performed from the cell-free medium with mAb 5D2 covalently coupled to CNBr-activated Sepharose 4B as described before (Huizinga et al., 1990c). Immunoprecipitated proteins were treated with N-glycanase according to the manufacturer's instructions (Genzyme, Boston, MA, USA). Immunoprecipitated, deglycosylated proteins were subjected to SDS-PAGE and autoradiography.
Quantitation of soluble FcγRIIIb in cell-free supernatants

Soluble FcγRIIIb (sFcγRIIIb) in neutrophil supernatant samples was measured by sandwich ELISA as described before (Koene et al., 1996). In short, 96-well ELISA plates were coated with the FcγRIIIb mAb 5D2. Detection of sFcγRIIIb in the samples was performed with a biotin-labeled, polyclonal rabbit-anti-human-FcγRIIIb antibody and streptavidin poly-horseradish peroxidase. Substrate buffer was added, and the color reaction was allowed to proceed for about 15 min and was stopped by addition of H₂SO₄. Absorbance at 450 nm was measured in a Titertek multiscan ELISA reader (Flow Laboratory, Rockville, MD, USA). The concentration of sFcγRIIIb in each sample was calculated from a standard curve obtained by serial dilutions of human plasma containing 5 nM sFcγRIIIb (Huizinga et al., 1990c).

Quantitation of lactoferrin

Lactoferrin in neutrophil supernatant samples was measured by ELISA as described before (Teeling et al., 1998). In short, 96-well ELISA plates were coated with a rabbit antibody against human lactoferrin, and detection of lactoferrin in the samples was performed with a peroxidase-labeled bovine-antibody against human lactoferrin. After addition of substrate buffer, the color reaction was allowed to proceed for about 10 min and was stopped by addition of H₂SO₄. The absorbance at 450 nm was measured, and the concentration of lactoferrin in each sample was calculated from a standard curve obtained by serial dilutions of purified human lactoferrin.

Adhesion assay

Fibronectin (20 μg/ml) was coated on a 24-well plate (Costar, Cambridge, MA, USA) for 60 min at 37°C. The plate was washed with PBS, and 500 μl of a neutrophil suspension (2x10⁶/ml) was added to each well. The plate was then incubated for 5 min at 37°C. Neutrophils were treated with DMSO (1% v/v), TNF-α (10 ng/ml), jasplakinolide (10 μM) or cyto D (0.5 μg/ml), and at various times aliquots from the plate were taken. The cells in these aliquots were lysed in PBS supplemented with Triton X-100 (1% v/v). The plate was washed once with PBS, and the adherent cells were then lysed as well. The amount of the cytosolic enzyme lactate dehydrogenase (LDH), representative for the number of neutrophils present in the samples, was measured as described (Bergmeyer, 1970). The amount of LDH in a lysate from 2x10⁶ neutrophils was arbitrarily set at 100%.
RESULTS

To study the role of the actin cytoskeleton in ectodomain shedding, we incubated human neutrophils with jasplakinolide to induce actin polymerization. Downregulation of various cell surface molecules was measured by flow cytometry, and soluble FcγRIIIb was measured by sandwich ELISA. When actin polymerization was induced by jasplakinolide, the cell-surface expression of FcγRIIIb was downregulated in a dose-dependent fashion (Fig.1A).

Figure 1.
Surface downregulation of FcγRIIIb, CD43, CD44 and L-selectin by jasplakinolide treatment of neutrophils. Human neutrophils (10⁷/ml) were incubated with increasing concentrations of jasplakinolide for 70 minutes at 37°C. As a control for shedding, neutrophils were incubated with PMA (200 ng/ml) for 10 minutes at 37°C. (A) Surface expression of FcγRIIIb, CD43, CD44 and L-selectin was measured by flow cytometry. The expression shown (mean fluorescence intensity) represents the mean ± SD of four independent experiments. (B) After 70 minutes of treatment with jasplakinolide (or 10 min with PMA), supernatants were collected and soluble FcγRIIIb was measured by ELISA. The results shown represent the amount of soluble FcγRIIIb (pmol/ml) ± SD measured in three independent experiments. (Inset) Autoradiograph of SDS-PAGE of immunoprecipitated, deglycosylated soluble FcγRIIIb after either PMA (lane 2) or jasplakinolide (lane 3) treatment of ¹²⁵I-labeled neutrophils. As a control, a lysate of neutrophils is shown (lane 1). Exposure time of the film was 7 hours, and molecular mass markers are shown in kDa. The arrow indicates the 22 kDa soluble FcγRIIIb.
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This effect may represent a general phenomenon because the surface expression of other molecules that are known to be shed upon neutrophil activation, such as CD43, CD44 and L-selectin, was also downregulated upon jasplakinolide treatment (Fig.1A). The reduced cell-surface expression of FcγRIIIb was accompanied by release of soluble FcγRIIIb in cell-free supernatants (Fig.1B). Immunoprecipitated, deglycosylated soluble FcγRIIIb shed from either jasplakinolide-treated or PMA-treated neutrophils migrated with a similar apparent molecular mass (22 kDa) on SDS-PAGE (inset Fig.1B). As a control, a lysate of untreated neutrophils is shown with the membrane-bound FcγRIIIb that still carries the GPI-anchor (24 kDa). This suggests that the proteinase involved in PMA-induced FcγRIIIb shedding is also activated upon actin polymerization.

To investigate whether jasplakinolide-induced FcγRIIIb shedding is accompanied by release of specific granule contents from neutrophils, we analyzed the CD66b surface expression. CD66b is a membrane marker for specific granules (Borregaard and Cowland, 1997), which is upregulated after activation of neutrophils with PMA. Neither basal CD66b expression nor PMA-induced CD66b upregulation was affected by jasplakinolide (Fig.2A). This finding was confirmed by quantitation of lactoferrin, a soluble protein that is, like CD66b, present in specific granules (Borregaard and Cowland, 1997). Lactoferrin is released upon PMA but not upon jasplakinolide treatment of neutrophils (Fig. 2B). These results show that jasplakinolide does not induce release of specific granules.

The kinetics of jasplakinolide-induced FcγRIIIb shedding are very different from PMA-induced FcγRIIIb shedding (Fig.3). Whereas jasplakinolide-induced FcγRIIIb shedding takes 30-60 minutes, a similar amount of soluble FcγRIIIb is already released after only 10 minutes of stimulation with PMA (Fig.1B). This difference in kinetics suggests that different
Jasplakinolide-induced FcγRIIIb shedding

Figure 3.
Kinetics of FcγRIIIb release by jasplakinolide. Human neutrophils (10⁷/ml) were incubated with DMSO (1% v/v, O) as control or jasplakinolide (10 μM, ●) for 60 min at 37°C. (A) At different times FcγRIIIb expression was determined by flow cytometry. As a control for FcγRIIIb shedding, neutrophils were incubated with PMA (200 ng/ml, ▲) for 10 min at 37°C. The expression shown (mean fluorescence intensity) represents the mean ± SD of five independent experiments. (B) Soluble FcγRIIIb was determined by ELISA in cell-free supernatants after various incubation times. The results shown represent the mean amount of soluble FcγRIIIb (pmol/ml) ± SD of five independent experiments.

signalling pathways lead to FcγRIIIb shedding. We tested whether PKC or other protein kinases play a role in the jasplakinolide-induced FcγRIIIb shedding by using a set of kinase inhibitors. The protein kinase C (PKC) inhibitor Ro31-8220 did not block jasplakinolide-induced FcγRIIIb shedding whereas the Ser/Thr kinase inhibitor staurosporine did (Fig.4A). In contrast, PMA-induced FcγRIIIb shedding was inhibited by staurosporine as well as by Ro31-8220 (Fig.4B). We also tested a possible involvement of tyrosine kinases in jasplakinolide-induced FcγRIIIb shedding by using the inhibitors herbimycin A and PP1. Pretreatment of neutrophils with these tyrosine kinase inhibitors did not inhibit the jasplakinolide-induced FcγRIIIb shedding (data not shown). Together, these data suggest that a staurosporine-sensitive kinase, but not PKC, mediates the jasplakinolide-induced FcγRIIIb shedding.

In addition to activation of the neutrophils, also apoptosis has been described to be accompanied by loss of FcγRIIIb from the cell surface (Homburg et al., 1995). It could be argued that jasplakinolide-induced FcγRIIIb shedding may represent the onset of apoptosis. However, no Annexin-V binding, as measured by flow cytometry, was found in neutrophils treated with jasplakinolide (data not shown).

FcγRIIIb associates with CR3, the main neutrophil integrin that mediates adhesion to the extracellular matrix. CR3-mediated adhesion of neutrophils to extracellular matrix proteins, such as fibronectin, is stimulated by TNF-α (Thompson and Matsushima, 1992). To study the role of actin polymerization in neutrophil adhesion, we investigated the effect of jasplakinolide on spontaneous and TNF-α-induced adhesion. TNF-α induces adhesion of neutrophils to fibronectin-coated wells in a time-dependent fashion (Fig.5A). Jasplakinolide
Figure 4.
Role of protein kinases in jasplakinolide-induced (A) and PMA-induced (B) FcyRIIIb shedding from neutrophils. After 10 min of preincubation with staurosporine (100 nM) or Ro31-8220 (10 μM), neutrophils were treated with DMSO (1% v/v, open bars), jasplakinolide (10 μM, filled bars) for 60 minutes, or PMA (200 ng/ml, filled bars) for 10 minutes at 37°C. (A) After 70 minutes or (B) 20 minutes, supernatants were collected and soluble FcyRIIIb was measured by ELISA. The amount of jasplakinolide-induced FcyRIIIb shedding after 60 min (A) (3.6±2.6 pmol/ml; n=4) was taken as 100% or the amount of PMA-induced FcyRIIIb shedding after 10 min (B) (3.1±1.2 pmol/ml; n=4) was taken as 100%.

and cytochalasin D (cyto D), an actin-filament disrupting agent, did not induce spontaneous adhesion (Fig.5A). After pretreatment of the neutrophils with jasplakinolide, TNF-α-induced adhesion was lost (Fig.5B). These results show that increased actin polymerization induced by jasplakinolide does not induce adhesion to fibronectin, indicating that FcγRIIIb shedding is independent from activation of CR3.

Figure 5.
Effects of actin rearrangements on neutrophil adhesion to fibronectin. (A) Human neutrophils (2x10⁶/ml) were put into fibronectin-coated wells at 37°C and were treated after 5 minutes with DMSO (1% v/v, open bars), TNF-α (10 ng/ml, hatched bars), jasplakinolide (10 μM, filled bars) or cyto D (0.5 μg/ml, crossed bars). At various times, adherent cells were lysed and analyzed for LDH content. The amount of LDH in a neutrophil lysate made from 2x10⁶ cells was taken as 100%. The results shown represent the mean ± SD of three independent experiments. (B) Neutrophils (2x10⁶/ml) were preincubated with DMSO (1% v/v, open bars), jasplakinolide (10 μM, filled bars) or cyto D (0.5 μg/ml, crossed bars) for 60 min and put into fibronectin-coated wells at 37°C. The cells were then stimulated with TNF-α (10 ng/ml). At various time points, adherent cells were lysed and analyzed for LDH content. The amount of LDH in a neutrophil lysate made from 2x10⁶ cells was taken as 100%. The results shown represent the mean ± SD of three independent experiments.
DISCUSSION

In this study we show that actin polymerization induced by jasplakinolide results in FcγRIIIB shedding from human neutrophils. This effect represents a general phenomenon, because other membrane-bound molecules, such as L-selectin, CD43 and CD44, were also downregulated following induction of actin polymerization. These membrane-bound molecules are all linked to the actin cytoskeleton (Yonemura et al., 1993; Tsukita et al., 1994; Pavalko et al., 1995), suggesting a common cytoskeleton-dependent mechanism in the process of shedding. Cytoskeletal rearrangements may change the three-dimensional structure of membrane-bound molecules, and these conformational changes may in turn result in enhanced susceptibility to proteinases (Hooper et al., 1997). Because FcγRIIIB is a glycosyl phosphatidylinositol-linked molecule, it is not directly associated with the actin cytoskeleton. Possible effects of actin rearrangements on FcγRIIIB must therefore be due to interactions with transmembrane molecules, such as CR3 (Zhou et al., 1993; Todd III and Petty, 1997). It has been shown that disruption of such an interaction leads to enhanced release of FcγRIIIB upon PI-PLC treatment (Stöckl et al., 1995). Dissociation of the FcγRIIIB-CR3 complex may occur upon neutrophil adhesion and reorganization of the actin cytoskeleton. In line with this, we and others have observed shedding of FcγRIIIB and CD43 upon neutrophil adhesion and spreading to extracellular matrix proteins (manuscript in preparation and Lopez et al., 1998). Similarly, chemotaxis of neutrophils is accompanied by a decreased expression of membrane-bound molecules, including L-selectin and CD43 (Kuijpers et al., 1992; Lopez et al., 1998). However, increased actin polymerization and FcγRIIIB shedding, induced by jasplakinolide, was not accompanied by spontaneous adhesion of neutrophils. Rather, jasplakinolide blocked TNF-α-induced adhesion, suggesting that it prevents CR3-activation by TNF-α, possibly by reducing the lateral mobility of the (cytoskeleton-associated) integrin. Together, these data show that although shedding of FcγRIIIB can be induced by an actin-promoting agent, it is independent from the activation of CR3.

Another explanation for our observations could be that a general ‘sheddase’, such as TNF-α converting enzyme (TACE), might be linked to the actin cytoskeleton (Werb and Yan, 1998) and be activated by cytoskeletal rearrangements. Shedding of various surface molecules is disrupted in cells deficient for TACE (Peschon et al., 1998). However, in human neutrophils, it is unlikely that shedding of FcγRIIIB, L-selectin, CD43 and CD44 is mediated by one single proteinase. This can be concluded from studies that show inhibition of PMA-induced shedding with inhibitors against either serine- or metalloproteinases (Bazil and Strominger, 1994). Our own recent work has shown that at least two different proteinases are involved in FcγRIIIB shedding from human neutrophils (manuscript in preparation). The proteinase involved in jasplakinolide-induced FcγRIIIB shedding is not located in specific granules. This conclusion is based on the observation that treatment of neutrophils with jasplakinolide neither upregulates CD66b nor induces release of lactoferrin, which are markers for specific granules. It is therefore more likely that the proteinase is present at the cell surface.

Earlier studies that addressed the effects of protein kinase C (PKC) inhibitors on actin-filament assembly following PMA activation of neutrophils have shown that Ro31-8220 is able to inhibit this process and that staurosporine has hardly any inhibiting effect (Downey et al., 1992; Keller and Niggli, 1993). Indeed, we found that PMA-induced FcγRIIIB shedding was completely inhibited by Ro31-8220, indicating that PMA-induced shedding may also
involve actin filament assembly. In our experiments, jasplakinolide-induced FcγRIIIb shedding could not be inhibited by Ro31-8220 but was blocked by staurosporine. The kinase involved is probably not a tyrosine kinase, because we failed to inhibit the jasplakinolide-induced FcγRIIIb shedding with herbimycin A or PP1.

Our data show that actin polymerization is sufficient to induce proteinase-mediated release of cell surface molecules. Further studies are required to identify the signalling pathway, activated by actin polymerization, that results in FcγRIIIb shedding.

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