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Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs

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Abstract

At sites of inflammation, ligation of leukocyte integrins is critical for the activation of cellular effector functions required for host defense. However, the signaling pathways linking integrin ligation to cellular responses are poorly understood. Here we show that integrin signaling in neutrophils and macrophages requires adaptors containing immunoreceptor tyrosine-based activation motifs (ITAMs). Neutrophils and macrophages lacking two ITAM-containing adaptor proteins, DAP12 and FcRγ, were defective in integrin-mediated responses. Activation of the tyrosine kinase Syk by integrins required that DAP12 and FcRγ were first phosphorylated by Src family kinases. Retroviral transduction of neutrophils and macrophages with wild-type and mutant Syk or DAP12 demonstrated that the Src homology 2 domains of Syk and the ITAM of DAP12 were required for integrin signaling. Our data show that integrin signaling for the activation of cellular responses in neutrophils and macrophages proceeds by an immunoreceptor-like mechanism.

Integrins are transmembrane adhesion receptors that coordinate cellular responses with the extracellular environment. Integrin function is especially important in neutrophils and macrophages, key effector cells that kill or suppress invading microorganisms during the innate immune response. In neutrophils and macrophages, integrin signaling is critical for cellular functions such as firm adhesion, cell spreading, chemotaxis, the production of

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AUTHOR CONTRIBUTIONS

A.M. and C.A.L. initiated the study; A.M., C.L.A., Z.J. and C.A.L. designed and did the experiments; A.M. supervised the work in Budapest; Y.H. did fetal liver stem cell transfers and maintained mouse colonies; L.L.L. provided intellectual guidance and manuscript editing; and A.M., C.L.A. and C.A.L. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

reactive oxygen intermediates and the release of antimicrobial granule proteins or various cytokines[1]. Genetic deficiency in the β_2 integrin chain (CD18) in children, a disease known as type I leukocyte adhesion deficiency, leads to severe bacterial infections because of impaired innate immune function[2,3]. A similar immune defect is also reflected by the spontaneous infections in mice after targeted deletion of the gene encoding CD18 (ref. 4). In contrast, exaggerated inflammatory responses occur when integrins become inappropriately activated, as noted in animals deficient in the C-terminal Src kinase Csk[5]. Those observations demonstrate the fact that tight control over integrin signaling and function is required for appropriate coordination of innate immune and inflammatory responses.

Although several molecules required for relaying signals 'downstream' of leukocyte integrins (often called 'outside-in' signaling) have been identified, the initial steps of β_2 integrin signaling remain poorly understood. Src family kinases are involved in an early step of integrin signaling in neutrophils[6] and macrophages[7,8]. Also, the Syk tyrosine kinase is essential for integrin signaling in neutrophils[9], macrophages[10] and platelets[11]. As Syk is probably involved in a receptor-proximal event during integrin signal transduction, the mechanism of activation of Syk by integrins and its relationship to Src family kinases may be the key to understanding the initiation of integrin signaling. Unfortunately, despite attempts to clarify that issue, the mechanism of activation of Syk by integrins remains poorly understood.

Syk and the related kinase Zap70 are also essential for signaling downstream of immunoreceptors, such as B cell and T cell receptors and Fc receptors. In contrast to integrin signal transduction, the mechanism of Syk activation initiated by ligation of these immunoreceptors is well characterized. Engagement of immunoreceptors leads to Src family kinase–mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on receptor-associated transmembrane adaptor proteins[12]. Those adaptors provide docking sites for the tandem Src homology 2 (SH2) domains of the Syk or Zap70 tyrosine kinases, which leads to kinase activation and initiation of further downstream signaling. Genetic deletion of the ITAM-bearing adaptors (the Fc receptor γ-chain (FcRγ), immunoglobulin α, immunoglobulin β and CD3ζ) or of Syk or Zap70 leads to defective immunoreceptor-mediated responses, such as arrested B cell or T cell development or defective FcRε-mediated allergic responses[12].

In contrast to the understanding of immunoreceptor signaling, the present view is that activation of Syk by integrins does not require the interaction of the Syk SH2 domains with phosphorylated ITAM tyrosines. That conclusion originated from work reporting that Syk activation by the platelet integrin $\alpha_{IIb}\beta_3$, when expressed in Chinese hamster ovary cells, does not require the Syk SH2 domains and cannot be prevented by sequestration of phosphorylated ITAM–containing molecules by overexpression of the tandem SH2 domains of Syk[13]. Subsequent studies with bacterially expressed protein fragments and Chinese hamster ovary transfectants concluded that Syk associates directly with the cytoplasmic tail of various integrin β-subunits in a phosphorylated tyrosine–independent way[14,15]. Those studies established the present view of phosphorylated ITAM–independent activation of Syk by integrins and suggested that immunoreceptors and integrins use two different signaling

mechanisms. Unfortunately, the conclusion of those studies has not been confirmed in primary cells.

Given those uncertainties and the well established involvement of Src family kinases and ITAM-containing adaptors in Syk activation during immunoreceptor signaling, we sought to determine whether an ITAM-based mechanism was also required for integrin signaling in neutrophils and macrophages. Our analyses included various gene-targeted mouse strains combined with retroviral gene transduction of hematopoietic cells *in vivo*. Our results led us to conclude that integrin signaling in neutrophils and macrophages proceeds by an immunoreceptor-like mechanism using the ITAM-containing DAP12 and FcRγ adaptor proteins to couple integrin ligation to Syk activation and downstream signaling events.

RESULTS

Integrin responses in neutrophils lacking DAP12 and FcRγ

Neutrophils express at least two ITAM-containing transmembrane adaptors, DAP12 and FcRγ[12]. We first tested whether those molecules are required for integrin-mediated functional responses of neutrophils. Mice deficient in DAP12 (DAP12-knockout), FcRγ (FcRγ-knockout) or both adaptors (DF double-knockout) produced normal numbers of neutrophils with wild-type expression of various integrin subunits and the mouse granulocytic maturation marker Gr-1 (Supplementary Fig. 1 online), indicating that neutrophil development and integrin expression was unaffected in mice lacking either one adaptor or both adaptors.

Integrin-mediated neutrophil activation can be attained by plating the cells on a surface coated with integrin ligand (such as fibrinogen) in the presence of a proinflammatory stimulus such as tumor necrosis factor (TNF). Responses initiated by such activation require CD18 in humans[16] and mice[9]. DF double-knockout neutrophils, lacking both DAP12 and FcRγ, were defective in integrin-mediated respiratory burst when plated on fibrinogen in the presence of TNF (Fig. 1a). That defect was not specific for TNF stimulation or fibrinogen, as DF double-knockout neutrophils were also impaired when plated onto fibrinogen in the presence of the chemokine MIP-2 (Fig. 1b) or when plated on ICAM-1 in the presence of TNF (Fig. 1c). Although maximum stimulation of neutrophils requires both integrin signals and nonintegrin signals (such as TNF)[17], plating of neutrophils on a surface coated with the ‘engineered’ multivalent integrin ligand peptide poly-RGD leads to superoxide release even in the absence of additional stimuli[9]. The defective responses of DF double-knockout neutrophils when plated on poly-RGD alone (Fig. 1d) suggested that DAP12 and/or FcRγ are required for signaling of integrins and not nonintegrin costimulatory signals. That was also confirmed by the normal responses of DF double-knockout neutrophils to TNF and MIP-2 in suspension (discussed below). The response of DF double-knockout neutrophils plated on poly-RGD in the presence of TNF, the strongest stimulus, was also defective (Fig. 1e).

In addition to stimulation of oxidative burst, engagement of CD18 by surface ligands induces degranulation, neutrophil spreading and cellular adhesion[6,9,18]. Integrin signaling leading to the release of gelatinase granules (Fig. 2a) or of the secondary granule marker

lactoferrin (data not shown) was impaired in the DF double-knockout neutrophils. DF double-knockout neutrophils also failed to spread over fibrinogen in the presence of TNF (Fig. 2b) or when plated on poly-RGD in the absence of any additional stimulus (data not shown). As a result, TNF-stimulated DF double-knockout cells did not adhere to fibrinogen as well as wild-type cells did (Fig. 2c). CD18 is required only partially for the adhesion of unstimulated neutrophils to fibrinogen[9] (probably reflecting experimental background), which may explain the only slightly lower adhesion of unstimulated DF double-knockout cells. In all the functional assays reported above, neutrophils deficient in either DAP12 or FcRγ alone had phenotypes intermediate between those of wild-type and double-knockout responses (Figs. 1 and 2a,c), indicating partially redundant functions for DAP12 and FcRγ during integrin signaling of neutrophils.

The β_2 integrins are also required for directed migration of neutrophils to the site of inflammation[9]. However, CD18-mediated neutrophil migration can proceed in the absence of signaling molecules critical for most other CD18-dependent neutrophil functions[9,19]. Like cells lacking Src family kinases or Syk[9], DF double-knockout neutrophils showed a partial defect in migration toward submicromolar concentrations of the synthetic peptide fMLP (formyl-Met-Leu-Phe), but their migration was normal at higher concentrations of fMLP (Fig. 2d). DF double-knockout neutrophils also migrated normally toward various concentrations of MIP-2 *in vitro* (data not shown) and showed no substantial defect in migration in an *in vivo* thioglycollate peritonitis model (data not shown). These results indicated that like Src family kinases[9], Syk[9] and members of the guanine nucleotide–exchange factor Vav family[19], the ITAM-bearing molecules DAP12 and FcRγ are not critical for the CD18-dependent migration of neutrophils, despite being required for most other CD18-dependent neutrophil functions.

Integrin ligation initiates tyrosine phosphorylation of various downstream substrates. After integrin ligation, wild-type neutrophils showed increased tyrosine phosphorylation of many proteins (mainly in the 60- to 150-kilodalton range), whereas DF double-knockout neutrophils failed to show any notable changes (Fig. 3a). Specifically, integrin-mediated tyrosine phosphorylation of Vav and the tyrosine kinase Pyk2, as well as phosphorylation of the Erk and p38 mitogenactivated protein kinases was impaired in DF double-knockout neutrophils (Fig. 3b–e).

Nonadherent responses in neutrophils lacking DAP12 and FcRγ

To investigate the response of DF double-knockout neutrophils to nonintegrin stimuli, we assessed many adhesion-independent responses. Upregulation of the CD11b and CD18 integrin chains (Fig. 4a,b) and phosphorylation of p38 as well as phosphorylation and degradation of the inhibitor IκBα (Fig. 4c) all occurred normally in suspended DF double-knockout neutrophils stimulated by TNF. Phosphorylation of p38, as well as phosphorylation and degradation of IκBα stimulated by the Toll-like receptor 2 agonist lipopeptide Pam_3CSK_4, were also similar in wild-type and DF double-knockout neutrophils (Fig. 4d). Similarly, deficiency in DAP12 and FcRγ did not affect actin polymerization responses (Fig. 4e), calcium signaling (Fig. 4f) or the phosphorylation of Erk or p38 (data not shown) after MIP-2 stimulation. Actin polymerization (data not shown), calcium

signaling (Fig. 4g) and respiratory burst (Fig. 4h) induced by fMLP were normal in suspended DF double-knockout neutrophils. The double-mutant cells also responded normally to the nonphysiological neutrophil-activating agent phorbol 12-myristate 13-acetate (Fig. 4i). Thus, neither DAP12 nor FcRγ is required for adhesion-independent neutrophil responses triggered by cytokines, Toll-like receptor ligands, chemokines or bacterial chemoattractants, indicating that the defective adhesion-dependent functions (Figs. 1–3) of the DF double-knockout cells were caused by impaired integrin signaling rather than by alterations in signaling induced by costimulatory agents such as TNF or MIP-2. Our results also demonstrated that DF double-knockout neutrophils do not have any general defects in actin polymerization, degranulation, assembly of the NADPH oxidase or initiation of multiple downstream signaling events.

Integrins signal through Src kinases, DAP12, FcRγ and Syk

The functional and signaling defects as well as the normal migratory and nonintegrin-mediated responses of DF double-knockout neutrophils are similar to those of cells deficient in Syk (Syk-knockout)[9,20]. DAP12 and FcRγ both have functional ITAMs that can recruit and activate Syk in an immunoreceptor-like way. Consequently, we tested whether Syk activation via integrins required DAP12 or FcRγ. We readily detected phosphorylation of Syk in wild-type neutrophils plated on fibrinogen in the presence of TNF or on poly-RGD without a soluble stimulus. Kinetic analysis of the response induced by poly-RGD showed that Syk phosphorylation was detectable in wild-type cells after 5 min of stimulation and reached a maximum between 10 and 20 min (data not shown). In contrast, there was no Syk phosphorylation in DF double-knockout neutrophils plated on fibrinogen in the presence of TNF (Fig. 5a) or on poly-RGD without an additional stimulus (Fig. 5b). Basal phosphorylation of Syk in unstimulated cells is independent of both adhesion and β_2 integrins[9].

Integrin-mediated phosphorylation of Syk was also defective in neutrophils lacking the Src family kinases Hck, Fgr and Lyn[9] (Fig. 5c,d), as well as in human neutrophils pretreated with the Src family kinase inhibitor PP2 (Supplementary Fig. 2 online). Such data are consistent with the fact that neutrophils deficient in Src family kinases have integrin signaling defects similar to those of Syk-knockout or DF double-knockout cells[6,9,18,21]. Src family kinases mediate ITAM phosphorylation, resulting in recruitment of Syk during immunoreceptor signaling[12]. We speculated that Src family kinases might phosphorylate DAP12 and/or FcRγ after integrin engagement in neutrophils. Plating wild-type neutrophils on poly-RGD induced DAP12 phosphorylation (Fig. 5e), which was absent in neutrophils lacking Src family kinases (Fig. 5f). To confirm that result and to assess adhesion-induced phosphorylation of the FcRγ chain, we used a glutathione *S*-transferase (GST) fusion protein of the tandem SH2 domains of Syk (GST-Syk-$(SH2)_2$) as a ‘probe’ for phosphorylated tyrosine. The GST-Syk-$(SH2)_2$ probe precipitated tyrosine-phosphorylated proteins of about 30 kilodaltons (corresponding to the molecular weight of DAP12 and FcRγ in nonreducing conditions) from adherent wild-type but not DF double-knockout neutrophils (Fig. 5g) with kinetics similar to those of Syk phosphorylation (data not shown). A similar phosphorylation signal was lacking in mouse neutrophils deficient in Src family kinases (Fig. 5h) and PP2-treated human neutrophils (Supplementary Fig. 2). Similar experiments with DAP12-

knockout or FcRγ-knockout single-mutant cells in reducing conditions showed that the upper and lower bands of the immunoblot corresponded to DAP12 and FcRγ, respectively (Fig. 5i,j). Notably, those phosphorylated proteins were not precipitated with a GST-Syk-$(SH2)_2$ fusion protein containing substitutions (R41A and R194A) known to impair the phosphorylated tyrosine–binding capacity of both SH2 domains (Fig. 5k). Although we were not able to detect immunoprecipitation of Syk together with DAP12 or FcRγ by immunoblot, we did note Syk autophosphorylation in FcRγ immunoprecipitates from adherent wild-type neutrophils but not from cells deficient in either FcRγ or Syk, which was consistent with integrin-stimulated recruitment of Syk to phosphorylated FcRγ (Fig. 5l and Supplementary Fig. 3 and Supplementary Note online). However, we were unable to reproducibly use a similar approach to demonstrate association of Syk with DAP12 because of high signal background obtained with the antisera.

Our results presented above indicated that ligation of neutrophil integrins leads to phosphorylation of DAP12 and FcRγ by members of the Src kinase family. Such phosphorylation allows the adaptors to associate with the tandem SH2 domains of Syk by a phosphorylated ITAM-SH2 interaction, leading to Syk activation (Supplementary Fig. 4 online). That pathway of integrin signaling to Syk is analogous to the ITAM-based signaling by classical immunoreceptors in lymphocytes.

Integrin signaling requires Syk SH2 domains and DAP12 ITAM

To confirm the functional involvement of an immunoreceptor-like, ITAM-based mechanism during integrin signaling in neutrophils, we did structure-function studies by expressing wild-type and mutant versions of Syk or DAP12 in Syk-knockout or DF double-knockout neutrophils *in vivo*. As neutrophils are short-lived, terminally differentiated cells in which expression of exogenously added gene products is very limited, these experiments required the transduction of hematopoietic stem cells, which we then injected into lethally irradiated recipient mice to allow *in vivo* generation of transduced neutrophils. That approach allowed us to use primary neutrophils for these studies and hence was much more physiologically relevant than previously published approaches. We transduced hematopoietic stem cells using an mouse stem cell virus–based bicistronic retroviral vector expressing green fluorescent protein (GFP) from a downstream internal ribosome entry site, thereby allowing the identification of transduced cells by flow cytometry[22]. Retroviral expression of wild-type Syk in Syk-knockout neutrophils restored the respiratory-burst response of cells stimulated by adhesion to poly-RGD in the presence of TNF (Fig. 6a). In contrast, an SH2-defective point mutant of Syk that no longer bound phosphorylated ITAMs (R194A)[22] did not restore a functional response, although a similar percentage of cells expressed GFP and an equivalent amount of Syk protein was expressed (Fig. 6b). We obtained similar results when we restored Syk expression by retroviral introduction of wild-type or SH2-defective versions of a Syk-GFP fusion protein (data not shown). Retroviral expression of a kinase-inactive Syk mutant also failed to correct the integrin-mediated functional defect of Syk-knockout neutrophils (data not shown). Our data suggested that functional phosphorylated tyrosine–binding SH2 domains of Syk (as well as Syk kinase activity) are required for the integrin-mediated activation of neutrophils.

We also tested whether expression of DAP12 or of an ITAM-defective mutant of DAP12 in which the two ITAM tyrosine residues were replaced by phenylalanine residues (Y65F and Y76F)[22] was able to restore the adhesion-dependent functional responses of DF double-knockout neutrophils. Because cell surface expression of DAP12 is required for its function, we tested effective reconstitution by both immunoblot and flow cytometry analysis for the Flag epitope tag attached to the N terminus of recombinant DAP12. *In vivo* expression of wild-type DAP12 in DF double-knockout neutrophils restored the integrin-mediated respiratory burst (Fig. 6c) to a burst equivalent to that of single-mutant FcRγ-knockout cells. In contrast, the ITAM-defective DAP12 mutant was unable to restore superoxide release in DF double-knockout neutrophils, despite having similar expression (Fig. 6d,e). These results suggested that the ITAM tyrosines of DAP12 are required for the DAP12-dependent component of integrin-mediated neutrophil activation.

Integrin responses in macrophages lacking DAP12 and FcRγ

Like neutrophils, integrin-mediated activation of macrophages also requires Syk[10], which prompted us to investigate whether DAP12 and FcRγ are involved in integrin signaling of macrophages. We plated bone marrow–derived macrophages on Valmark plastic Petri dishes, which engages CD18 integrins[23]. As a 'readout' of integrin-mediated macrophage activation, we used biochemical analysis of Erk phosphorylation, because that response (unlike most functional responses, such as the release of proinflammatory cytokines) could be readily induced by cellular adhesion without a soluble stimulus and, as predicted from published studies[10,23], required both the CD18 integrin chain (Fig. 7a) and the Syk tyrosine kinase (Fig. 7b). Adhesion-dependent Erk phosphorylation was absent from DF double-knockout macrophages, with predominant involvement of DAP12 in this response (Fig. 7c). The defective Erk activation in DF double-knockout macrophages was not due to the lack of β_2 integrins or altered macrophage development, because wild-type and double-mutant cells had equal expression of CD18 as well as other integrins and macrophage markers (Supplementary Fig. 5 online and data not shown). Our data demonstrated that like neutrophils, macrophages lacking the ITAM-containing adaptors have defective integrin signaling responses, and although neutrophils can use both DAP12 and FcRγ, loss of DAP12 alone is sufficient to impair integrin signaling in macrophages. Adhesion-dependent Syk phosphorylation was also defective in DF double-knockout macrophages (Fig. 7d), consistent with a requirement for ITAM-containing adaptors in Syk activation.

Immunoreceptor-like signaling by macrophage integrins

To further confirm the ITAM model for integrin signaling in macrophages, we did a series of retroviral reconstitution experiments. Retroviral expression of wild-type Syk in Syk-knockout macrophages restored adhesion-dependent Erk phosphorylation, whereas comparable expression of the SH2-defective Syk mutant (R194A) did not (Fig. 8a). Similarly, reconstitution of DF double-knockout macrophages with wild-type DAP12 restored adhesion-dependent Erk phosphorylation, but reconstitution with the ITAM-defective DAP12 mutant did not (Fig. 8b). We confirmed equivalent expression of wild-type or mutant DAP12 by immunoblot and found equivalent surface expression of exogenous DAP12 by flow cytometry (Fig. 8b,c). These results indicated that an interaction between

the Syk SH2 domains and the ITAM of DAP12 is required for signaling downstream of integrins in macrophages.

DISCUSSION

Many studies have demonstrated the dependence on Src family and Syk tyrosine kinases in both integrin and immunoreceptor signaling pathways. With those observations in mind, we tested the hypothesis that similar signaling adaptors are needed to initiate both pathways. We found that the ITAM-containing molecules DAP12 and FcRγ were indispensable for β_2 integrin–mediated Syk activation and cellular responses in neutrophils and macrophages. Integrin-dependent functions that do not require Src family kinases or Syk, such as neutrophil migration, also did not require DAP12 or FcRγ. After integrin ligation, the ITAM-bearing adaptor molecules became phosphorylated by Src family kinases and associated with the SH2 domains of Syk. Structure-function analysis of neutrophils and macrophages suggested that integrin signaling requires the SH2 domains of Syk and the ITAM tyrosine residues of DAP12 (and probably FcRγ). These data have demonstrated that signal transduction by β_2 integrins follows the ITAM-based paradigm of immunoreceptor signaling. The unexpected similarity between proximal signaling by adhesion receptors and receptors of the adaptive immune system can be extended further downstream, as members of the Vav and SLP-76 families, originally thought to be involved mainly in lymphocyte antigen receptor signaling[24,25], have been shown to be required for integrin signaling in neutrophils[19,26], macrophages[27], osteoclasts[28] and platelets[29].

Our findings challenge the present view of integrin signal transduction, which is proposed to be unrelated to immunoreceptor-like signaling mechanisms. In particular, it has been concluded that the SH2 domains of Syk are not required for its activation by the platelet integrin $\alpha_{IIb}\beta_3$ heterologously expressed in Chinese hamster ovary cells[13]. Although the reason for that apparent contradiction is unclear, it may result from differential signaling by β_2 and β_3 integrins or alternative signaling pathways used by the $\alpha_{IIb}\beta_3$ heterodimers when expressed in nonhematopoietic cells that do not express ITAM-containing adaptors. Related to that issue is the reported direct binding of Syk to the cytoplasmic tails of multiple integrin β-chains in a phosphorylated ITAM–independent way when expressed together in nonhematopoietic cells[14,15]. However, those observations do not exclude the possibility of a phosphorylated ITAM–dependent step in the integrin-mediated activation of Syk.

Both DAP12 and FcRγ associate with many cell surface receptors in various hematopoietic cell types[12]. The molecular basis of that association is the presence of opposing charged residues in the transmembrane segments of the molecules involved, allowing intra-membrane salt bridges to be formed between DAP12 or FcRγ and their associated receptors. Integrins lack such charged residues in their transmembrane segments. Furthermore, the surface expression of integrins was normal in DF double-knockout leukocytes, unlike that of most DAP12- or FcRγ-associated receptors. In addition, we were unable to demonstrate immunoprecipitation of DAP12 or FcRγ together with CD18 in adherent neutrophils. Therefore, integrins are unlikely to associate directly with DAP12 or FcRγ.

A more likely scenario is that DAP12- or FcRγ-associated receptors are functionally linked to integrins and participate in their signal transduction in an ITAM-dependent way. That hypothesis is also supported by preliminary experiments in which a DAP12 transmembrane 'charge mutant' unable to associate with known DAP12-associated receptors failed to restore integrin-dependent signaling to DAP12-deficient macrophages (C.L.A. and C.A.L., unpublished observations). Although reports have suggested that ITAM-containing Fc receptors and integrins may function cooperatively[17,30], it is unlikely that the Fc receptors themselves couple ITAM-containing adaptors to the integrin signaling pathway, for several reasons. First, DF double-knockout cells have functional and signaling defects even in the absence of Fc receptor–ligating immunoglobulins; second, DAP12 is important in integrin-mediated responses even though it does not associate with Fc receptors; and third, TNF-induced responses of neutrophils plated on fibrinogen were normal in neutrophils lacking the FcRγ-associated receptors FcγRI and FcγRIII (Z.J. and A.M., unpublished observations). Given the many non–Fc receptor molecules identified that associate with and signal through DAP12 and FcRγ[12], it is likely that DAP12- or FcRγ-associated molecules other than Fc receptors might be involved in integrin signaling of neutrophils and macrophages.

The association of integrins and ITAM-containing adaptors could be through interactions between amino acids outside the transmembrane domain, through interactions with other cell surface molecules, such as tetraspanins, which seem to regulate integrin signals in many cell types[31], or through the association with lipid microdomains, although the last possibility has not been fully investigated in neutrophil signal transduction. Determining the molecular details of how β_2 integrins couple to DAP12 or FcRγ is an area for further research. Furthermore, it is unclear whether integrins discriminate between different ITAM-containing adaptors for signaling, as do immunoreceptors.

We have shown here that integrin signaling in neutrophils and macrophages uses an ITAM-based, immunoreceptor-like mechanism acting via the DAP12 and FcRγ adaptor molecules. Our findings fundamentally alter the present view of integrin 'outside-in' signal transduction in hematopoietic cells. They may also provide new insights into the pathogenesis of, and possible targets for the therapy of, infectious and inflammatory diseases.

Note added in proof: It has been independently confirmed that the SH2 domains of Syk are required for integrin signaling in neutrophils[32].

METHODS

Animals

Tyrobp$^{-/-}$ (DAP12-knockout) mice[33] were crossed with *Fcer1g*$^{-/-}$ (FcRγ-knockout) mice[34] (Taconic) to generate *Tyrobp*$^{-/-}$*Fcer1g*$^{-/-}$ (DF double-knockout) mice. Single- and double-knockout mice as well as wild-type control mice were identified by PCR-based genotyping. *Syk*$^{-/-}$ (Syk-knockout) mice have been described[35]. Syk-knockout and wild-type control cells were obtained from bone marrow chimeras generated by using fetal liver cells to reconstitute lethally irradiated mice as described[9]. *Itgb2*$^{-/-}$ (CD18-knockout) mice have been described[4]. Mice lacking the Src family kinases Hck, Fgr and Lyn have also been described[9]. All mice were backcrossed to the C57BL/6 background for six or more

generations. Mice were kept in a specific pathogen–free facility (University of California, San Francisco) or in individually sterile ventilated cages in a conventional facility (Semmelweis University School of Medicine). All experiments were approved by the University of California, San Francisco Institutional Animal Care and Use Committee and by the Committee on Animal Experimentation of Semmelweis University.

Reagents

Antibodies were from the sources described[9,20,22] except antibody to IκBα (anti-IκBα) (Cell Signaling Technologies), biotin-conjugated anti-Flag (Sigma), Alexa Fluor 680–conjugated goat anti-rabbit (Invitrogen) and IRDye 800–conjugated goat anti-mouse (Rockland Immunochemicals), and anti-Syk and anti-DAP12. The SH2-defective version of the GST-Syk-$(SH2)_2$ fusion protein was generated by the introduction of R41A and R194A substitutions with the QuikChange Site-Directed Mutagenesis kit (Stratagene). Wild-type and mutant Syk and DAP12 constructs have been described[22].

Neutrophil assays

Neutrophil isolation, flow cytometry and functional assays were done as described[9,20]. For gelatinase zymography, sample buffer (10% (volume/volume) glycerol, 2% (weight/volume) SDS, 5 mg/ml of bromophenol blue and 62.5 mM Tris, pH 6.8) was added to cell supernatants, which were separated by zymogram gel electrophoresis (Invitrogen), were renatured in 2.5% (volume/volume) Triton X-100 and were developed overnight at 37 °C (in 200 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris, pH 7.4, and 2.5 μg/ml of NaN_3) followed by Coomassie blue staining. An Alpha Innotech Alphaimager was used for quantification. Migration experiments were done with Transwell inserts with an FCS-coated, 3-μm polycarbonate membrane (Corning) essentially as described[9]. Measurement of actin polymerization after chemokine or fMLP stimulation was done by a flow cytometry–based phalloidin-binding assay as described[20].

Immunoblot and immunoprecipitation

These experiments were done as described[9,20,22]. Some immunoblots used fluorescence-labeled secondary antibodies followed by detection with the Odyssey Infrared Imaging System (LI-COR Biosciences). The GST-Syk-$(SH2)_2$ fusion protein was coupled to glutathione-Sepharose beads (GE Healthcare) and the result was incubated with cell lysates prepared with Triton X-100 (ref. 36) or radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% (volume/volume) Triton X-100 buffer, 1% (weight/volume) sodium deoxycholate, 0.1% (weight/volume) SDS, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EDTA, 1 mM Pefabloc, 10 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 mM dithiothreitol, 1 μg/ml of pepstatin and 1 mM di-isopropyl fluorophosphate). Syk kinase autophosphorylation assays were done on anti-FcRγ immunoprecipitates ‘captured’ with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) from Nonidet-P40 lysates (20 mM HEPES, pH 7.4, 150 mM NaCl and 1% (volume/volume) Nonidet-P40, plus the inhibitors listed above). Beads were washed once with kinase assay buffer (20 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 2 mM $MgCl_2$ and 1 mM dithiothreitol), then were incubated for 20 min at 25 °C in kinase assay buffer containing 10 μCi [γ-^{32}P]ATP. The reaction was stopped by the addition of

sample buffer, then samples were boiled and separated by SDS-PAGE. Gels were stained with Coomassie blue, were destained and dried and were exposed to X-ray film.

Retroviral gene transduction

Retroviral supernatants were generated by transient cotransfection of 293T cells with the pMIG-W vector containing wild-type and mutant Syk or DAP12 and plasmids containing MoMuLV gagpol and VSVg envelope with Lipofectamine 2000 (Invitrogen). For neutrophil reconstitution, fetal liver cells from day-16 Syk-knockout or DF double-knockout embryos were cultured for 24 h in DMEM with 10% (volume/volume) FCS containing penicillin and streptomycin, 20 ng/ml of mouse IL-3, 10 ng/ml of mouse IL-6 and 100 ng/ml of mouse SCF (R&D Systems). Retroviral supernatants supplemented with cytokines and 8 μg/ml of polybrene (Sigma) were added to fetal liver cells, which were then centrifuged for 1 h at 800*g* and 25 °C, followed by incubation for 8–12 h at 37 °C in 5% CO_2. A second infection was done, and 48–72 h after being collected, the infected fetal liver cells were injected into lethally irradiated 6- to 8-week-old male Ly5.1 congenic recipients (Taconic). Peripheral blood from the chimeras was analyzed by flow cytometry 6 weeks after transfer (to assess chimerism by GFP expression and staining for the Ly5.1 and Ly5.2 markers) before characterization of purified neutrophils.

For macrophage reconstitution, bone marrow was collected from Syk-knockout fetal liver chimeras or DF double-knockout mice. Cells were cultured for 24 h in α-MEM with 10% (volume/volume) FCS containing penicillin, streptomycin and conditioned medium from 3T3 cells expressing mouse monocyte colony-stimulating factor. Suspended cells were incubated overnight at 37 °C in 5%CO_2 with retroviral supernatants supplemented with 8 μg/ml of polybrene and medium conditioned with monocyte colony-stimulating factor, followed by a repeat overnight infection. The viral supernatant was replaced with culture medium and cells were differentiated for 6–9 d. Identical results were obtained with a published macrophage infection protocol[37]. Infected macrophages were analyzed by flow cytometry for GFP expression and expression of the macrophage markers F4/80 and CD11b. Infected macrophages were detached from plates with cell-dissociation buffer (Invitrogen), were resuspended in DMEM with 2% (volume/volume) FCS and were allowed to ‘rest’ for 2–4 h at 37 °C on a rocking platform. Cells were collected in suspension or were plated onto Valmark plastic Petri dishes for 45 min at 37 °C before being lysed in radioimmunoprecipitation buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Berton G, Lowell CA. Integrin signalling in neutrophils and macrophages. Cell. Signal. 1999; 11:621–635. [PubMed: 10530871]
2. Bunting M, Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving β_2 integrins and selectin ligands. Curr. Opin. Hematol. 2002; 9:30–35. [PubMed: 11753075]
3. Rosenzweig SD, Holland SM. Phagocyte immunodeficiencies and their infections. J. Allergy Clin. Immunol. 2004; 113:620–626. [PubMed: 15100664]
4. Scharffetter-Kochanek K, et al. Spontaneous skin ulceration and defective T cell function in CD18 null mice. J. Exp. Med. 1998; 188:119–131. [PubMed: 9653089]
5. Thomas RM, et al. C-terminal SRC kinase controls acute inflammation and granulocyte adhesion. Immunity. 2004; 20:181–191. [PubMed: 14975240]
6. Lowell CA, Fumagalli L, Berton G. Deficiency of Src family kinases p59/61^{hck} and p58$^{c\text{-}fgr}$ results in defective adhesion-dependent neutrophil functions. J. Cell Biol. 1996; 133:895–910. [PubMed: 8666673]
7. Suen PW, et al. Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages. J. Cell Sci. 1999; 112:4067–4078. [PubMed: 10547366]
8. Meng F, Lowell CA. A β_1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J. 1998; 17:4391–4403. [PubMed: 9687507]
9. Mócsai A, Zhou M, Meng F, Tybulewicz VL, Lowell CA. Syk is required for integrin signaling in neutrophils. Immunity. 2002; 16:547–558. [PubMed: 11970878]
10. Vines CM, et al. Inhibition of β_2 integrin receptor and Syk kinase signaling in monocytes by the Src family kinase Fgr. Immunity. 2001; 15:507–519. [PubMed: 11672534]
11. Obergfell A, et al. Coordinate interactions of Csk, Src, and Syk kinases with $\alpha_{IIb}\beta_3$ initiate integrin signaling to the cytoskeleton. J. Cell Biol. 2002; 157:265–275. [PubMed: 11940607]
12. Fodor S, Jakus Z, Mócsai A. ITAM-based signaling beyond the adaptive immune response. Immunol. Lett. 2006; 104:29–37. [PubMed: 16332394]
13. Gao J, Zoller KE, Ginsberg MH, Brugge JS, Shattil SJ. Regulation of the pp72^{syk} protein tyrosine kinase by platelet integrin $\alpha_{IIb}\beta_3$. EMBO J. 1997; 16:6414–6425. [PubMed: 9351824]
14. Woodside DG, et al. Activation of Syk protein tyrosine kinase through interaction with integrin β cytoplasmic domains. Curr. Biol. 2001; 11:1799–1804. [PubMed: 11719224]
15. Woodside DG, et al. The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin β cytoplasmic domains. J. Biol. Chem. 2002; 277:39401–39408. [PubMed: 12171941]
16. Nathan C, et al. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. J. Cell Biol. 1989; 109:1341–1349. [PubMed: 2475511]
17. Jakus Z, Berton G, Ligeti E, Lowell CA, Mócsai A. Responses of neutrophils to anti-integrin antibodies depends on costimulation through low affinity FcγRs: full activation requires both integrin and nonintegrin signals. J. Immunol. 2004; 173:2068–2077. [PubMed: 15265942]
18. Mócsai A, Ligeti E, Lowell CA, Berton G. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. J. Immunol. 1999; 162:1120–1126. [PubMed: 9916742]
19. Gakidis MA, et al. Vav GEFs are required for β_2 integrin-dependent functions of neutrophils. J. Cell Biol. 2004; 166:273–282. [PubMed: 15249579]
20. Mócsai A, et al. G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. Blood. 2003; 101:4155–4163. [PubMed: 12531806]

21. Pereira S, Zhou M, Mócsai A, Lowell C. Resting murine neutrophils express functional α_4 integrins that signal through Src family kinases. J. Immunol. 2001; 166:4115–4123. [PubMed: 11238661]
22. Mócsai A, et al. The immunomodulatory adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc. Natl. Acad. Sci. USA. 2004; 101:6158–6163. [PubMed: 15073337]
23. Roach T, et al. CD45 regulates Src family member kinase activity associated with macrophage integrin-mediated adhesion. Curr. Biol. 1997; 7:408–417. [PubMed: 9197241]
24. Turner M, Billadeau DD. Vav proteins as signal integrators for multi-subunit immune-recognition receptors. Nat. Rev. Immunol. 2002; 2:476–486. [PubMed: 12094222]
25. Koretzky GA, Abtahian F, Silverman MA. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. Nat. Rev. Immunol. 2006; 6:67–78. [PubMed: 16493428]
26. Newbrough SA, et al. SLP-76 regulates Fcγ receptor and integrin signaling in neutrophils. Immunity. 2003; 19:761–769. [PubMed: 14614862]
27. Hall AB, et al. Requirements for Vav guanine nucleotide exchange factors and Rho GTPases in FcγR- and complement-mediated phagocytosis. Immunity. 2006; 24:305–316. [PubMed: 16546099]
28. Faccio R, et al. Vav3 regulates osteoclast function and bone mass. Nat. Med. 2005; 11:284–290. [PubMed: 15711558]
29. Obergfell A, et al. The molecular adapter SLP-76 relays signals from platelet integrin $\alpha_{IIb}\beta_3$ to the actin cytoskeleton. J. Biol. Chem. 2001; 276:5916–5923. [PubMed: 11113155]
30. Zhou MJ, Brown EJ. CR3 (Mac-1, $\alpha_M\beta_2$, CD11b/CD18) and FcγRIII cooperate in generation of a neutrophil respiratory burst: requirement for FcγRIII and tyrosine phosphorylation. J. Cell Biol. 1994; 125:1407–1416. [PubMed: 7515890]
31. Tarrant JM, Robb L, van Spriel AB, Wright MD. Tetraspanins: molecular organisers of the leukocyte surface. Trends Immunol. 2003; 24:610–617. [PubMed: 14596886]
32. Abtahian F, et al. Mol. Cell. Biol. 2006; 26:6936–6949. [PubMed: 16943434]
33. Bakker AB, et al. DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. Immunity. 2000; 13:345–353. [PubMed: 11021532]
34. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR γ chain deletion results in pleiotrophic effector cell defects. Cell. 1994; 76:519–529. [PubMed: 8313472]
35. Turner M, et al. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature. 1995; 378:298–302. [PubMed: 7477352]
36. Mócsai A, et al. Differential effects of tyrosine kinase inhibitors and an inhibitor of the mitogen-activated protein kinase cascade on degranulation and superoxide production of human neutrophil granulocytes. Biochem. Pharmacol. 1997; 54:781–789. [PubMed: 9353132]
37. Nishiya T, DeFranco AL. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. J. Biol. Chem. 2004; 279:19008–19017. [PubMed: 14976215]

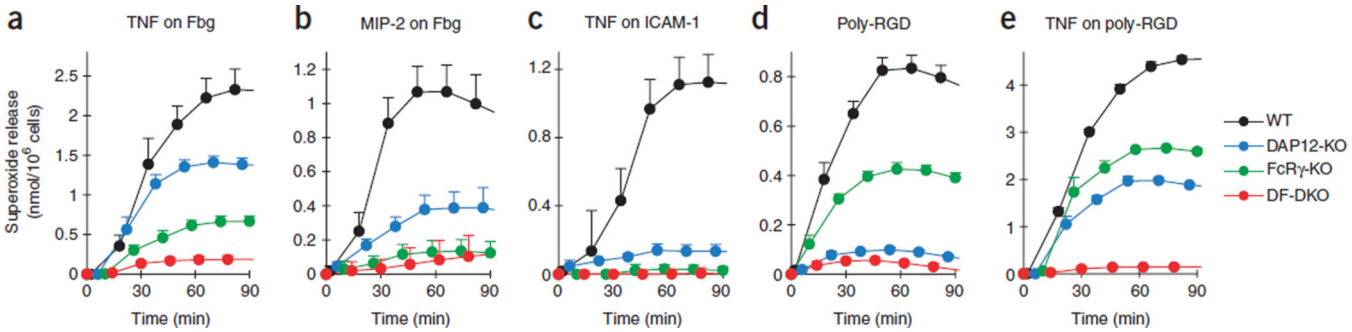

Figure 1.

Defective integrin-mediated respiratory burst in DF double-knockout neutrophils. Superoxide release of wild-type neutrophils (WT), DAP12-knockout neutrophils (DAP12-KO), FcRγ-knockout neutrophils (FcRγ-KO) and DF double-knockout neutrophils (DF-DKO) plated on surfaces coated with integrin ligand (fibrinogen (Fbg; **a,b**), ICAM-1 (**c**) or poly-RGD (**d,e**)) in the presence or absence of an additional stimulus (50 ng/ml of mouse TNF (**a,c,e**), 100 ng/ml of mouse MIP-2 (**b**) or none (**d**)). Unstimulated values (no TNF or MIP-2) were subtracted from stimulated values in **a–c,e**; error bars, s.d. of triplicate measurements. Data are representative of a minimum of three independent experiments each.

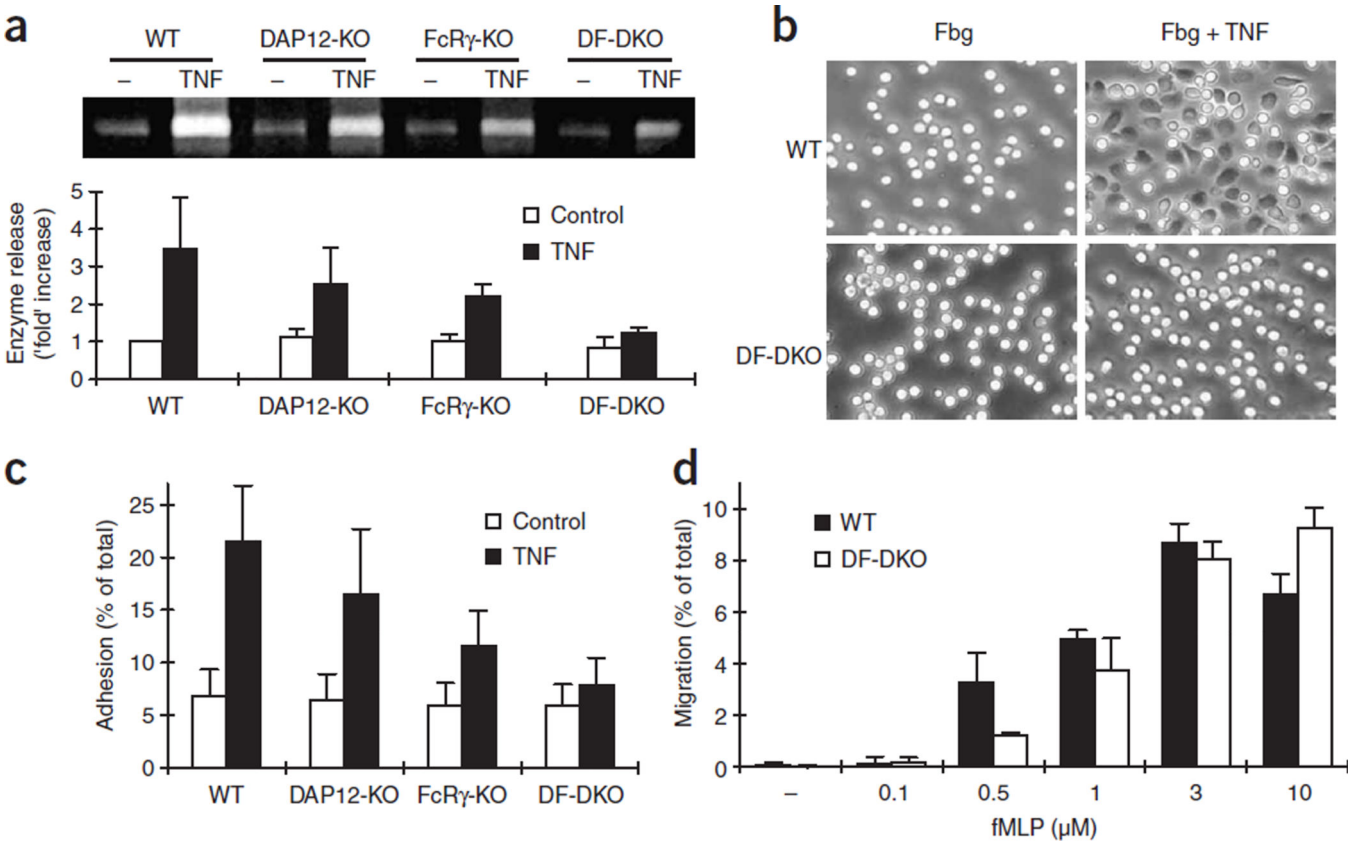

Figure 2.

Defective integrin-mediated nonoxidative responses in DF double-knockout neutrophils. (**a**) Release of gelatinase granules by wild-type, DAP12-knockout, FcRγ-knockout and DF double-knockout neutrophils plated for 60 min on fibrinogen in the presence (TNF) or absence (− or Control) of 50 ng/ml of mouse TNF and analyzed by gelatinase zymogram. (**b**) Phase-contrast microscopy of wild-type and DF double-knockout neutrophils plated for 30 min on fibrinogen in the presence (right) or absence (left) of 50 ng/ml of mouse TNF. Original magnification, ×40. (**c**) Firm adherence of neutrophils treated as described in **a**. (**d**) Migration of wild-type and DF double-knockout neutrophils through FCS-coated Transwells in response to increasing concentrations of fMLP, assessed after 60 min. Data are representative of a minimum of three independent experiments each (error bars, s.d. of triplicate measurements).

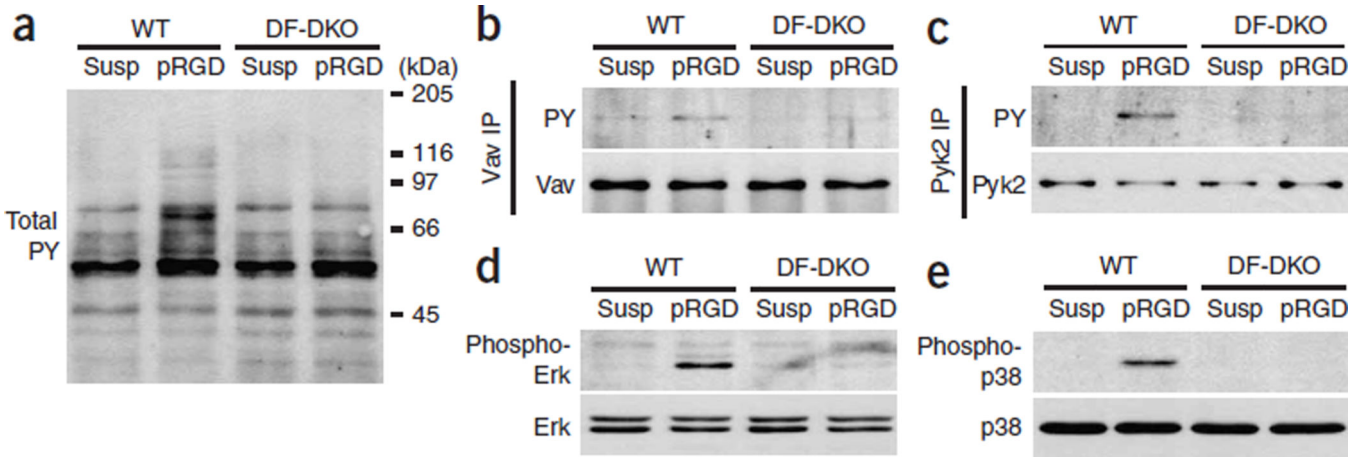

Figure 3.

Defective integrin-mediated signaling in DF double-knockout neutrophils. Immunoassays of lysates of wild-type and DF double-knockout neutrophils kept in suspension (Susp) or plated for 15 min on a surface coated with poly-RGD (pRGD). (**a**) Immunoblot analysis of total lysates with antibody to phosphorylated tyrosine (PY). (**b,c**) Immunoprecipitation (IP) with anti-Vav (**b**) or anti-Pyk2 (**c**) followed by immunoblot analysis (antibodies, left margin). (**d,e**) Immunoblot analysis of total lysates with antibody to phosphorylated Erk (Phospho-Erk) or to total Erk (Erk; **d**) or with antibody to phosphorylated p38 (Phospho-p38) or to total p38 (p38; **e**). All data are representative of three or more independent experiments.

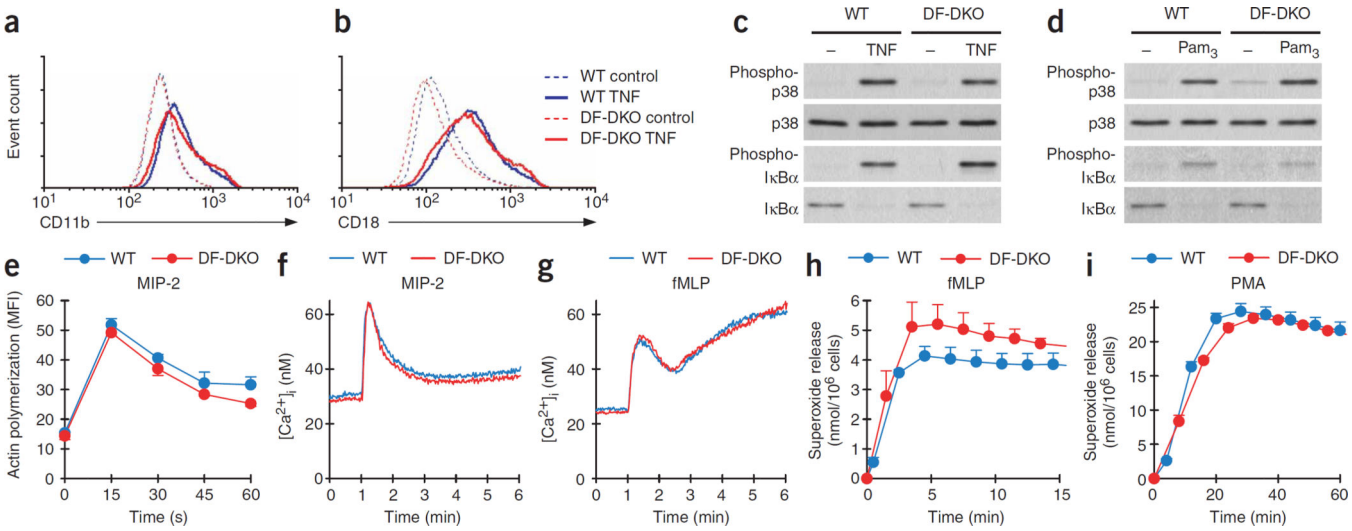

Figure 4.

Normal adhesion-independent responses of DF double-knockout neutrophils. (**a,b**) Flow cytometry of surface expression of CD11b (**a**) and CD18 (**b**) by wild-type or DF double-knockout neutrophils stimulated in suspension for 30 min with 50 ng/ml of mouse TNF or left unstimulated (Control). (**c,d**) Immunoblot of lysates of suspended wild-type or DF double-knockout neutrophils left unstimulated (−) or stimulated for 5 min with 50 ng/ml of mouse TNF (**c**) or for 10 min with 1 μg/ml of Pam_3CSK_4 (Pam_3; **d**). Antibodies, left margin (Phospho-, phosphorylated). (**e**) Actin polymerization by suspended wild-type or DF double-knockout neutrophils stimulated with 10 ng/ml of mouse MIP-2. MFI, mean fluorescent intensity. (**f,g**) Release of intracellular calcium ($[Ca^{2+}]_i$) from suspended wild-type and DF double-knockout neutrophils stimulated with 100 ng/ml of mouse MIP-2 (**f**) or 3 μM fMLP (**g**). (**h,i**) Superoxide release from suspended wild-type and DF double-knockout neutrophils pretreated with 10 μM cytochalasin B and stimulated with 3 μM fMLP (**h**) or stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA; **i**). Unstimulated values are subtracted in **h** and **i**. Data are representative of a minimum of three independent experiments each (error bars, s.d. of triplicate measurements).

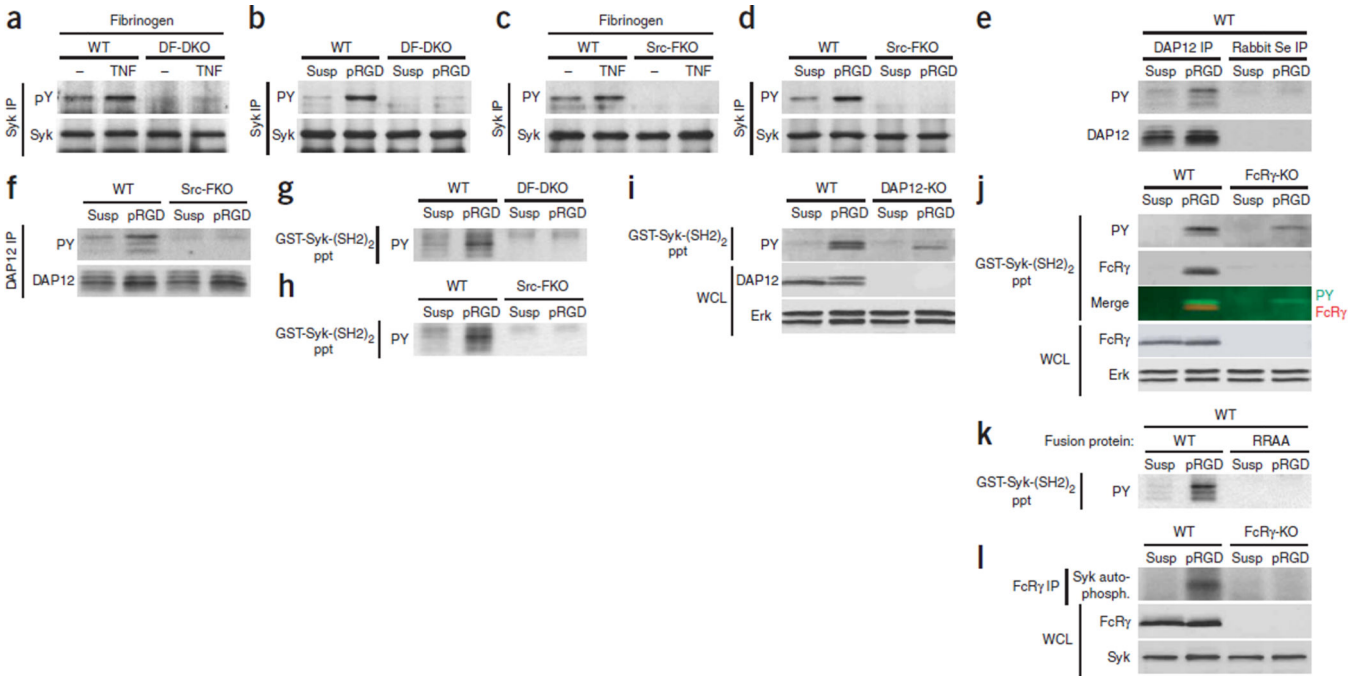

Figure 5.

Evidence of a Src family kinase–DAP12 or FcRγ–Syk pathway during integrin signaling of neutrophils. (**a–d**) Immunoassay of lysates of neutrophils plated on fibrinogen in the presence (TNF) or absence (−) of 50 ng/ml of mouse TNF (**a,c**) or on a surface coated with poly-RGD (**b,d**). Lysates prepared after 15 min of stimulation were immunoprecipitated with anti-Syk. Control, unstimulated cells in suspension (Susp; **b,d**). (**e–l**) Immunoassay of lysates of neutrophils plated on a surface coated with poly-RGD with (**i,j,l**) or without (**e–h,k**) 50 ng/ml of mouse TNF. Lysates prepared after 15 min of stimulation were immunoprecipitated with anti-DAP12 (**e,f**) or anti-FcRγ (**l**) or were precipitated (ppt) by incubation with GST-Syk-$(SH2)_2$ (**g–k**). Control, unstimulated cells in suspension (Susp). (**j**) Merge, overlay of phosphorylated-tyrosine and FcRγ blots above. (**l**) Top, autophosphorylation of Syk (Syk auto-phosph.) in anti-FcRγ immunoprecipitates. Immunoblots are in reducing conditions except for **e–h,k**. Src-FKO, ‘Src family knockout’ (lacking Hck, Fgr and Lyn); Rabbit Se, normal rabbit serum; WCL, whole-cell lysate; RRAA, GST-Syk-$(SH2)_2$ with R41A and R194A substitutions. All data are representative of three or more independent experiments.

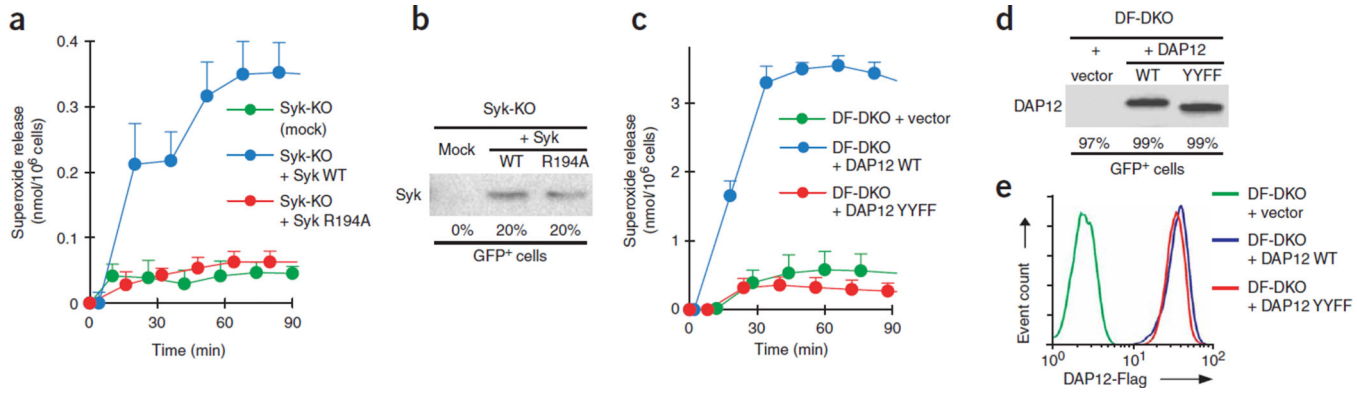

Figure 6.

Critical function for the Syk SH2 domains and the ITAM tyrosine residues of DAP12 for integrin-mediated responses of neutrophils. Retrovirus-transduced Syk-knockout (**a,b**) or DF double-knockout (**c–e**) fetal liver hematopoietic stem cells were injected into lethally irradiated recipient mice and, after reconstitution of the hematopoietic system, neutrophils were isolated for functional and gene expression studies. (**a,c**) Respiratory burst of neutrophils (purified from reconstituted mice) stimulated with 50 ng/ml of mouse TNF on a surface coated with poly-RGD (unstimulated values are subtracted; error bars, s.d. of triplicate measurements). (**b,d**) Immunoblot of protein expression and flow cytometry for percent GFP^+ cells (below lanes). Control, neutrophils purified from mice reconstituted with stem cells that were 'mock infected' (Mock) or were infected with vector only (+ vector). (**e**) Flow cytometry for surface expression of exogenous DAP12 on transduced DF double-knockout neutrophils, detected by anti-Flag. YYFF, DAP12 Y65F and Y76F mutant. Data in **a,b** and in **c–e** are from the same experiment and are representative of three to five independent experiments.

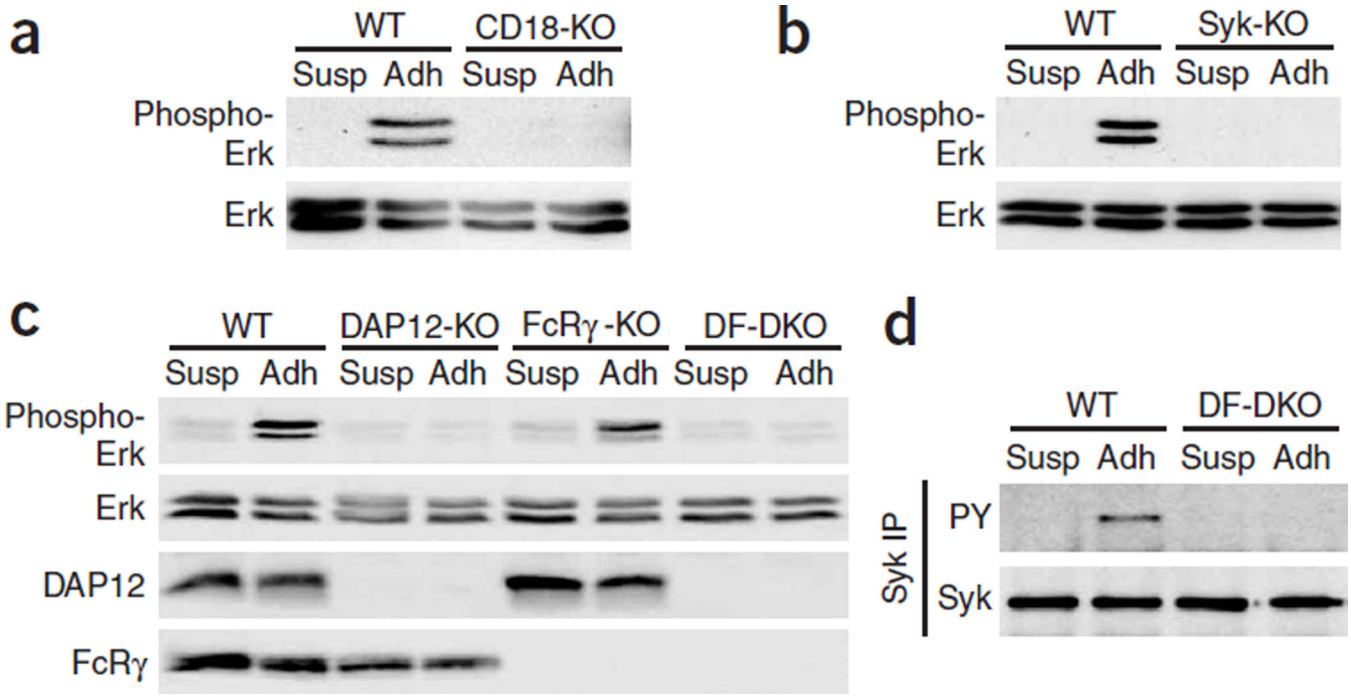

Figure 7.

Defective integrin-mediated responses in DF double-knockout macrophages. Bone marrow–derived macrophages were plated on Valmark dishes for 30–45 min (Adh) or were kept in suspension (Susp), then lysates were prepared. (**a–c**) Immunoblot analysis of Erk phosphorylation. (**d**) Immunoprecipitation with anti-Syk, followed by immunoblot analysis of tyrosine phosphorylation. All data are representative of three or more independent experiments.

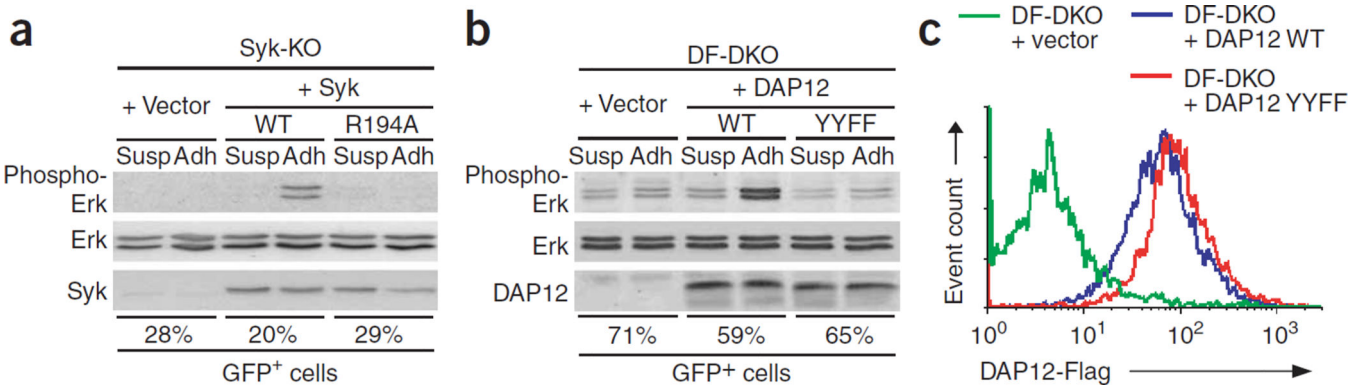

Figure 8.

Immunoreceptor-like signaling by macrophage integrins. (**a,b**) Immunoblot analysis of Erk phosphorylation and flow cytometry of percent GFP$^+$ cells (below lanes) for Syk-knockout (**a**) or DF double-knockout (**b**) bone marrow–derived macrophages infected with retrovirus containing wild-type or mutant Syk or DAP12, respectively, then plated on Valmark dishes for 30–45 min; cell lysates were prepared and analyzed. (**c**) Flow cytometry for surface expression of exogenous DAP12 on GFP$^+$ macrophages from **a,b**, detected by anti-Flag staining. Data are representative of at least four independent experiments.