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Etk/BMX, a Btk Family Tyrosine Kinase, and Mal Contribute to the Cross-Talk between MyD88 and FAK Pathways

Noha Semaan,* Ghada Alsaleh,* Jacques-Eric Gottenberg, † Dominique Wachsmann, 2,3* and Jean Sibilia 2‡

MyD88 and focal adhesion kinase (FAK) are key adaptors involved in signaling downstream of TLR2, TLR4, and integrin α5β1, linking pathogen-associated molecule detection to the initiation of proinflammatory response. The MyD88 and integrin pathways are interlinked, but the mechanism of this cross-talk is not yet understood. In this study we addressed the involvement of Etk, which belongs to the Tec family of tyrosine kinases, in the cross-talk between the integrin/FAK and the MyD88 pathways in fibroblast-like synoviocyte s (FLS) and in IL-6 synthesis. Using small interfering RNA blockade, we report that Etk plays a major role in LPS- and protein I/II (a model activator of FAK)-dependent IL-6 release by activated FLS. Etk is associated with MyD88, FAK, and Mal as shown by coimmunoprecipitation. Interestingly, knockdown of Mal appreciably inhibited IL-6 synthesis in response to LPS and protein I/II. Our results also indicate that LPS and protein I/II induced phosphorylation of Etk and Mal in rheumatoid arthritis FLS via a FAK-dependent pathway. In conclusion, our data provide support that, in FLS, Etk and Mal are implicated in the cross-talk between FAK and MyD88 and that their being brought into play is clearly dependent on FAK. The Journal of Immunology, 2008, 180: 3485–3491.

 Toll-like receptors play an important role in activating the innate immune system. These receptors, expressed by a variety of immune and nonimmune cells, recognize microbial components as well as some host-derived molecules (1, 2). Eleven TLRs that activate signal transduction pathways have been identified in mammals, with specificity regarding recruitment of the four proximal adaptor molecules Mal, MyD88, TRIF (Toll/IL-1R domain-containing adaptor inducing IFN-β), and TRAM (TRIF-related adaptor molecule). MyD88 and TRIF control distinct signaling pathways that activate either NF-κB and AP-1 (Mal/MyD88) or IRF transcriptions factors (TRIF/TRAM) (3). Very recent data (4, 5) showed that TIRAP (Toll/IL-1R domain-containing adaptor protein)/Mal, which is never used without MyD88, functions to recruit MyD88 to phosphatidyl inositol 4,5-biphosphate (PIP2)4-rich plasma membrane subdomains to initiate TLR2 and TLR4 signaling. PIP2 production is controlled by many signaling pathways and, among them, integrins are known to regulate PIP2 turnover through activation of the ARF6 (ADP ribosylation factor 6) GTase.

MyD88 is also a key adaptor involved in other signaling pathways. We demonstrated previously (6) that MyD88 plays a major role in protein I/II/integrin-induced cytokine release and that this effect is independent of TLR2, TLR4, and TLR6. We also showed that focal adhesion kinase (FAK), involved in signaling downstream of integrins (7), is critical for signaling by LPS via TLR4 and by Pam3CSK ((S)-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]()-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys2-CH2) via TLR2. The MyD88 and FAK pathways are interlinked, but the mechanism of this cross-talk is not yet understood.

MyD88 is not essential for FAK activation, as this kinase is phosphorylated in MyD88−/− macrophages stimulated with either LPS or protein I/II. Thus, FAK seems to occur upstream of MyD88 recruitment and might be a key proximal effector of MyD88. FAK possesses neither a Toll/IL-1R (TIR) domain nor a death domain for direct interaction with MyD88, but alternatively FAK may participate to the MyD88-induced signaling pathway by interacting with or activating another component.

Bruton’s tyrosine kinase (Btk), the prototype member of the Tec family of tyrosine kinases, is an early component of the TLR signaling pathway (8–10). Btk is activated following stimulation with LPS. Its activation fits into a two-step model requiring first the disruption of the intracellular interaction between the SH3 (Src homology 3) domain and the proline-rich regions of this kinase by binding the pleckstrin homology (PH) domain to phosphoinositides or βγ subunits of G protein or the FERM (protein 4.1 ezrin/radixin/moesin) domain of FAK. Btk is then translocated to the membrane, where its kinase activity is induced by Src kinases. Recently, Mal was identified as one substrate that is phosphorylated by Btk during TLR2 and TLR4 signal transduction (11). Btk expression is restricted to myeloid cells. Conversely, Etk, another member of the Tec family of tyrosine kinases, is present in various cell types such as epithelial and endothelial cells and fibroblasts (12, 13). Activation of Etk is also regulated by FAK.

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4 Abbreviations used in this paper: PIP2, phosphatidyl inositol 4,5-biphosphate; Btk, Bruton’s tyrosine kinase; FAK, focal adhesion kinase; FLS, fibroblast-like synoviocytes; PH, pleckstrin homology; RA, rheumatoid arthritis; siRNA, small interfering RNA.
through interaction between the PH domain of Etk and the FERM domain of FAK (14). Based on these observations, we investigated the possible role of Etk in the cross-talk between the integrin/FAK and MyD88 pathways in fibroblast-like synoviocytes (FLS) and in IL-6 synthesis.

Our data indicate that Etk and Mal are the main components of the cross-talk activated by TLR4 or integrin α5β1 ligands and that FAK plays an essential role in their recruitment and activation.

### Materials and Methods

#### Purification of protein I/II

Recombinant protein I/II of Streptococcus mutans OMZ 175 was purified from pHBox-l-transformed Escherichia coli cell extract by gel filtration and immunodiffusion chromatography as described previously (15). Potential endotoxin in protein I/II preparation was removed by using polymyxin B-agarose (Detoxi-Gel) according to the manufacturer’s recommendation (Pierce). Protein I/II had an endotoxin content of <0.01 ng/125 mM protein I/II as tested by the Limulus chromogenic assay (Charles River Laboratories). Throughout this study, buffers were prepared with pyrogenic water obtained from Braun Medical.

#### Cell culture

Human FLS were isolated from rheumatoid arthritis (RA) synovial tissues at the time of knee joint arthroscopic synovectomy and cultured as previously described (16). FLS (5 × 10^5 cells per well) were grown to confluence in 96-well plates and serum starved for 24 h before the activation experiments. FAK −/− and FAK +/− primary mouse embryo fibroblasts (CRL-2644 and CRL-2645; American Type Culture Collection) were cultured as previously described (17). Cells were plated in 96-well plates (5 × 10^5 cells/well) and serum starved 24 h before activation experiments.

Mice deficient for Btk and their control littermates were obtained from V. Quesniaux (The Transgenose Institute, Centre National de la Recherche Scientifique, Orlean, France). Murine bone marrow cells were isolated from the femurs of Btk-deficient and control mice and cultured as previously described (18). The bone marrow-derived macrophages were plated in 96-well plates (10^5 cells/well) before the activation experiments.

#### siRNA duplexes and transfections

The small interfering RNA (siRNA) duplexes used in our study were designed to target sequences for human Etk/BMX (GenBank accession no. NM_001721) and Mal/TIRAP (GenBank accession no. NM_148910) genes. Four selected siRNA oligonucleotides consisting of sequences of 21 nucleotides were supplied by Dharmacon (Perbio Science).

The four Etk/BMX siRNA sequences are as follows: 1. GUACAGGU CGACCAGAUAAU (sense sequence) and 5'-P UAUUGGCGCUAG ACUGUGAUCAAUGCUU (antisense sequence); 2. GAAGAUCAGCUAGCGGCUAGCUU (sense sequence) and 5'-P AACUGAGCGCCAGAUAAU (antisense sequence); 3. GAAGAGCCGAAGUACUU (sense sequence) and 5'-P ACUGA CUCCGGCUCUUCCUU (antisense sequence); 4. GAAGAAUAUAG GUUAGAAAU (sense sequence) and 5'-P UUUUCAACCAUAAUGCUU (antisense sequence).

The four Mal/TIRAP siRNA sequences are as follows: 1. GACGGAGCACUAAGGCUUU (sense sequence) and 5'-P GAGGCUAAGU CGUUUCAUU (antisense sequence); 2. GAAGAUCUGCAAGCUAU (sense sequence) and 5'-P CAUCCGCUGAGGCCGUUUU (antisense sequence); 3. AACTAGGCGCAGCAUGGUU (sense sequence) and 5'-P ACGAGCGGCGGU (antisense sequence); 4. CAGCCAUCAUCAAGGCUUU (sense sequence) and 5'-P UGUCUGAGGCGGU (antisense sequence).

Transient transfection of FLS with siRNA (100 nM) was performed using the Human Dermal Fibroblast Nucleofector kit from Maxima as previously described (4). FLS were then plated in 12-well plates (2 × 10^5 cells per well). All assays were performed 48 h after transfection. The control was conducted with the DHARMA siControl nontargeting siRNA consisting of a four-oligonucleotides pool. Transfection efficiency was evaluated with the pmxGFP control vector. Cell numbers and cell viability were assessed using the MTT test as described elsewhere (19).

#### Cell activation

Cells were stimulated with protein I/II (125 pM) diluted in serum-free RPMI 1640 with antibiotics or LPS (1 μg/ml) (Salmonella abortus-equi; Sigma-Aldrich) diluted in medium containing 5% heat-inactivated FCS. After stimulation, supernatants were harvested and assayed for cytokine content by using commercially available ELISA reagents for human (DuoSet; R&D Systems) and mouse (OptEIA kits, BD Pharmingen) IL-6.

#### Western blot detection of tyrosine phosphorylation

FLS (10^6 cells) were incubated for various times with LPS (1 μg/ml) or protein I/II (125 pM). Controls were performed with cells maintained in serum-free medium (protein I/II) or medium with 5% FCS (LPS) for 15 min. After stimulation, cells were centrifuged and the pellets were suspended 20 min on ice in 100 μl of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitors). Lysates were centrifuged for 10 min at 14,000 × g at 4°C and supernatants were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked using 1% BSA in TBS (20 mM Tris (pH 7.5) and 150 mM NaCl) for 1 h at 25°C. The blots were incubated with anti-phospho-Etk (Y40) rabbit polyclonal Abs (US Biotechnological) for 2 h at 25°C followed by incubation with HRP-conjugated goat anti-rabbit IgG polyclonal Abs (1 h at 25°C) and detected by ECL (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce) according to the manufacturer’s instructions. To confirm the presence of equal amounts of Etk, bound Abs were removed from the membrane by incubation in 62.5 mM Tris (pH 6.7), 100 mM 2-ME, and 2% SDS for 30 min at 95°C and reprobed again with anti-Etk (clone 40) mouse mAbs (BD Transduction Laboratories).

Cell lysates from LPS and protein I/II stimulated FAK +/+ and FAK −/− cells were centrifuged (14,000 × g for 10 min at 4°C) and supernatants (0.5–1 mg/ml protein) were precleared by mixing with protein G-Sepharose 4 Fast Flow (Sigma-Aldrich) in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA for 2 h at 4°C. Immunoprecipitation was performed with anti-phosphotyrosine mAbs (yP20)/protein G-Sepharose 4 Fast Flow (Sigma-Aldrich) or anti-Mal rabbit polyclonal Abs (FL-235, Santa-Cruz Biotechnology)/protein A-Sepharose 4 Fast Flow for 1 h at 4°C. Samples were washed with the lysis buffer (12,000 × g for 20 s) and the complexes were eluted with Laemml buffer (Bio-Rad) for 10 min at 95°C and centrifuged at 12,000 × g for 20 s. Immunoprecipitates were separated by SDS-PAGE and further analyzed by Western blotting using anti-FAK mouse mAbs/anti-Etk mouse mAbs and anti-phosphotyrosine mAbs (yP20). Blots were then incubated with HRP-conjugated anti-rabbit and anti-mouse IgG polyclonal Abs (1 h at 25°C) and detected by ECL (Super Signal West Femto Maximum Sensitivity Substrate; Pierce) according to the manufacturer’s instructions.

#### Immunoprecipitation analysis

FLS (4 × 10^6 cells) were incubated for 10 min with LPS (1 μg/ml) or protein I/II (125 pM). Controls were performed with serum-free medium (protein I/II) or medium with 5% FCS. After centrifugation, cells were suspended in ice-cold PBS containing 1 mM sodium orthovanadate and protease inhibitors and then disrupted by sonication. Cell lysates were centrifuged (14,000 × g for 10 min at 4°C) and supernatants (0.5–1 mg/ml protein) were precleared by mixing with protein G-Sepharose 4 Fast Flow in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA for 2 h at 4°C. Immunoprecipitation complexes were immunoprecipitated from supernatants using anti-Mal mouse Abs/protein G-Sepharose 4 Fast Flow or anti-FAK mouse Abs/protein G-Sepharose 4 Fast Flow for 18 h at 4°C. Equal amounts of mouse IgG were used as negative controls (Alpha Diagnostic International). Samples were washed with the lysis buffer (12,000 × g for 20 s) and the complexes were eluted with Laemmli buffer (Bio-Rad) for 10 min at 95°C and centrifuged at 12,000 × g for 20 s. Immunoprecipitates were separated by SDS-PAGE and further analyzed by Western blotting using anti-FAK rabbit polyclonal Abs/anti-Mal rabbit polyclonal Abs or anti-phosphotyrosine mAbs (yP20). Blots were then incubated with HRP-conjugated anti-rabbit and anti-mouse IgG polyclonal Abs (1 h at 25°C) and detected by ECL (Super Signal West Femto Maximum Sensitivity Substrate; Pierce) according to the manufacturer’s instructions.

### Statistical methods

Values are represented as the mean ± SEM. The significance of the results was analyzed by ANOVA with pairwise comparison by Scheffe’s method using Stat View software. Values of p < 0.05 were considered significant.

### Results

LPS and protein I/II induce phosphorylation of Etk in RA FLS

Etk, which belongs to the Tec family of tyrosine kinases, is the most ubiquitously expressed member of this group. Etk is present in various cell types such as epithelial and endothelial cells and fibroblasts but is absent in myeloid cells. In this study, we first verified that Etk is expressed in human FLS isolated from four RA patients. Cell lysates were analyzed directly by Western blotting...
Etk is detectable in the FLS of all RA patients. Etk is involved in integrin signaling and promotes cell migration. To determine whether the stimulation of FLS with protein I/II had any effect on Etk activation, we analyzed the tyrosine phosphorylation of Etk in response to protein I/II. Cell lysates were analyzed by blotting with specific anti-phospho-Etk Abs (Y40). Stimulation of FLS with protein I/II (125 pM) for various times (5, 10, 15, and 30 min) resulted in an increased amount of phosphorylated Etk which was detectable within 5 min and remained elevated for at least 15 min (Fig. 1B). We also analyzed the phosphorylation state of Etk in response to LPS (1 μg/ml). Similar results were obtained as shown in Fig. 1C.

**LPS and protein I/II-induced IL-6 release is dependent on Etk**

We then tested the role of Etk in IL-6 release from FLS activated with LPS or protein I/II. Doubled-strand siRNA have emerged as powerful tools to examine the function of specific gene products. We used a combination of four siRNA duplexes targeting Etk. FLS were transfected with Etk siRNA duplexes at a concentration of 100 nM. GFP was used to evaluate transfection efficiency. As shown in Fig. 2A, transfection of Etk siRNA impaired endogenous Etk protein expression as compared with Etk expression in cells transfected with a nontargeting siRNA (control).

**siRNA-mediated inhibition of Etk impaired IL-6 release by LPS- and protein I/II-activated FLS.**

A. Effect of Etk siRNA on FLS endogenous Etk expression. The expression of Etk was analyzed by Western blotting 48 h posttransfection of the siRNA duplexes added in combination (final concentration: 100 nM). As control, FLS were transfected with nontargeting siRNA (Cont). IB, Immunoblotting.

B. LPS- and protein I/II-induced IL-6 release is dependent on Etk. Macrophages from wild-type mice or mice deficient for Btk were stimulated with protein I/II (PI/II)(125 pM) or LPS (1 μg/ml) for 20 h. IL-6 levels were quantified by ELISA. Data are expressed as mean values ± SD and are representative of two experiments with n = 2 mice per genotype. ***, p < 0.02.
nontargeting siRNAs (cont). IB, Immunoblotting. Western blotting 48 h posttransfection of siRNA duplexes added in combination (final concentration: 100 nM). As control, FLS were transfected with Etk siRNA for 48 h and cells were then stimulated with LPS (1 μg/ml) or protein I/II (125 pM) (Fig. 2). The expression of transfection on cell viability was also determined by the MTT test with no significant difference observed for cells transfected with targeting and nontargeting siRNA. Taken together, these data demonstrate that Etk is involved in α5β1 integrin and TLR4 signaling leading to IL-6 release by activated FLS.

We also evaluated the response of Btk−/− macrophages stimulated with either LPS or protein I/II. As shown in Fig. 2D, IL-6 release was significantly reduced in Btk−/− macrophages as compared with IL-6 release by wild-type macrophages. These results indicate that in LPS or protein I/II-stimulated macrophages, IL-6 release is also in part Btk dependent.

**Etk associates with FAK, MyD88, and Mal**

In our first analysis of the cross-talk between FAK and MyD88, we had observed that MyD88 is not essential for FAK activation because this kinase is phosphorylated in MyD88−/− macrophages activated with either LPS or protein I/II. These results indicated that FAK occurs upstream of MyD88 recruitment. FAK is also an activator of Etk. Thus, it is conceivable that FAK might lead to the recruitment of MyD88 via Etk because this kinase is implicated in both signaling pathways. To test this hypothesis, coimmunoprecipitation experiments were conducted in FLS activated for 10 min with LPS or protein I/II. Cell lysates were immunoprecipitated with anti-FAK Abs and analyzed by blotting using anti-MyD88, anti-Etk, and anti-Mal Abs. As shown in Fig. 3A, Etk was detected by Western blot analysis via anti-Etk immunoblotting in FAK immune complexes from LPS-activated FLS. Moreover, MyD88 and Mal were also detected in FAK immune complexes by anti-MyD88 and anti-Mal immunoblotting. A light constitutive association of Etk, MyD88, and Mal with FAK was observed in unstimulated cells. Likewise, similar results were obtained when FLS were stimulated with protein I/II for 10 min (Fig. 3B).

We therefore postulated that Etk formed a complex with FAK, MyD88, and Mal in LPS and protein I/II-activated FLS.
Mal is implicated in IL-6 release from LPS and protein I/II stimulated FLS

It was recently demonstrated that Mal is recruited to the plasma membrane by its PIP2 binding domain and then functions to recruit MyD88 to activated TLR4 to initiate signal transduction. To further define the role of Mal in the cross-talk between FAK and MyD88 in FLS, we analyzed the requirement for Mal in IL-6 release from activated FLS. As previously described for Etk, FLS were transfected with targeting and nontargeting Mal siRNA duplexes for 48 h and cells were then stimulated with LPS (1 mg/ml) or protein I/II (125 pM) for 20 h at 37°C. Supernatants were tested for levels of IL-6 using ELISA. As illustrated in Fig. 4, impaired expression of Mal (Fig. 4A) significantly reduced IL-6 release by protein I/II (Fig. 4B) and LPS-stimulated FLS (Fig. 4C) (11 ± 0.05 vs 19 ± 0.02 ng/ml (p < 0.02) and 6 ± 0.04 vs 13 ± 0.03 ng/ml (p < 0.001), respectively). These data indicate that IL-6 release in response to LPS or protein I/II is dependent on Mal.

LPS and protein I/II induced phosphorylation of Etk and Mal in RA FLS via a FAK-dependent pathway

Previous results have shown that activation of Etk by extracellular matrix proteins is regulated by FAK through an interaction between the PH domain of Etk and the FERM domain of FAK. Y40 is a phosphorylation site for FAK and its phosphorylation is required for Etk activation. We next asked whether FAK could be implicated in Etk phosphorylation in response to LPS (1 mg/ml) or protein I/II (125 pM). To test this hypothesis, we used FAK+/+ and FAK−/− primary mouse embryo fibroblasts. FAK+/+ and FAK−/− cells were incubated with LPS or protein I/II for various times and immunoblotting experiments were performed. As shown in Fig. 5, stimulation of FAK+/+ cells with either LPS (Fig. 5A) or protein I/II (Fig. 5B) increased Etk phosphorylation; however, both stimuli failed to stimulate tyrosine phosphorylation of Etk in FAK−/− cells. Taken together, these results indicate that Etk phosphorylation is modulated by FAK in protein I/II- and LPS-stimulated cells and suggest that Etk could serve as a common signal transducer in response to integrin α5β3 and TLR4 ligands.

Recent evidence also indicated that in THP1 monocytic cells, Mal is phosphorylated during TLR2 and TLR4 signal transduction. To establish whether or not the phosphorylation of Mal is dependent on FAK, we analyzed tyrosine phosphorylation of Mal. Cells were incubated with LPS or protein I/II for various times and immunoblotting experiments were performed. Stimulation of FAK+/+ cells with either LPS or protein I/II increased Mal phosphorylation (Fig. 6), whereas both stimuli failed to stimulate tyrosine phosphorylation of Mal in FAK−/− cells. Taken together, these results indicate that Mal phosphorylation is modulated by FAK in protein I/II- and LPS-stimulated FLS. These results provided further support that Etk and Mal are main components of the cross-talk between FAK and MyD88 and that FAK plays an essential role in their recruitment and activation.

Discussion

In the present study we first demonstrated that Etk plays an important role in protein I/II-integrin-induced cytokine release. Several lines of evidence are presented to support this notion. First, we found that activation of FLS with protein I/II resulted in increased phosphorylation of Etk on Y40, which is required for Etk activation. Secondly, in FLS transfected with siRNA targeting Etk there is a failure to respond to protein I/II treatment as determined by their reduced IL-6 production after stimulation. Activation of Etk by integrins in response to extracellular matrix proteins has been
documented in epithelial and endothelial cells where Etk is known to promote cell migration (14). But this is the first study to demonstrate that Etk is implicated in signaling pathways leading to proinflammatory cytokine release in response to integrin αβ1 stimulation, suggesting that integrin function is regulated by Etk at multiple steps.

Moreover, we also showed that Etk is involved in TLR4 signaling in FLS stimulated with LPS because Etk is phosphorylated in response to LPS and, furthermore, IL-6 response to LPS is severely impaired in FLS transfected with targeting Etk siRNA. Our findings extend to Etk the observation that Btk, another member of the Tec tyrosine kinase family, is involved in TLR signaling. In XLA monocytes that lack functional Btk there is a failure to respond to LPS as determined by their reduced TNF-α production after stimulation (20). Activation of Btk was also found to be required for the TLR2-induced expression of TNF-α and IL-1β in primary human monocytes/macrophages (21, 22). Btk is implicated in TLR-induced IL-10 production (23). But the findings of Horwood et al. (22) revealed an unexpected level of complexity insofar as they showed that in primary human macrophages Btk is not required for TLR2- and TLR4-induced IL-6 and IL-8 release from X-linked agammaglobulinemia PBMC stimulated with LPS. These data are, however not in agreement with two of our observations: 1) that Btk−/− macrophages stimulated with either LPS or protein I/II showed an impaired release of IL-6 as compared with wild-type cells; and 2) that in FLS Etk is necessary for IL-6 production in response to LPS and protein I/II. The reason for this discrepancy is not clear at present. IL-6 and TNF-α synthesis is highly dependent on NF-κB, and Btk is known to phosphorylate the NF-κB p65 unit that is critical for the activation of NF-κB. But these cytokines are differentially regulated and the most likely explanation is that selective regulation pathways may be used by various cells. In fact, FLS stimulated by TLR ligands never produce TNF-α and IL-1β.

It remained, however, undetermined whether Etk was implicated in the cross-talk between FAK and MyD88. To this end, we performed coimmunoprecipitation with anti-FAK Abs. Etk was found to form a complex with FAK and MyD88 in lysates from FLS activated with either LPS or protein I/II. These results support the conclusion that Etk contributes to the cross-talk between MyD88 and FAK. We also identified Mal in the complex. Mal is a Toll/IL-1R (TIR)-containing adaptor implicated in MyD88-dependent signaling (24, 25). We further defined the role of Mal by using siRNA targeting Mal. As a result, IL-6 release from LPS-and protein I/II-activated FLS was impaired. Consequently, our results supported a role for Mal in the cross-talk leading to IL-6 release in response to TLR4 and integrin αβ1 stimulation. These data are in agreement with previous results (26) demonstrating that Mal is implicated in LPS-induced cytokine production in synovial fibroblasts but not in macrophages. Recently the exact mechanism of Mal engagement by TLR4 was demonstrated (4): Mal contains a PIP2-binding domain that mediates its recruitment to the membrane and facilitates the delivery of MyD88 to activated TLR4. However, the role of Mal as a sorting adaptor that is required for the efficient recruitment of MyD88 to a subset of TLR is quite difficult to imagine in the protein I/II/integrin αβ1 pathway. Indeed, we demonstrated previously using macrophages isolated from TLR4−/−, TLR2−/−, and TLR6−/− mice that protein I/II-induced IL-6 release is completely independent of these TLRs (6). One might speculate that Mal contributes to the recruitment of MyD88, which was demonstrated to be essential in IL-6 release in response to protein I/II, and that the recruitment of MyD88 is sufficient to activate downstream signaling pathways. But how Mal is recruited to the membrane in our model remained unclear.

We also demonstrated that Etk phosphorylation is dependent on FAK because Etk phosphorylation is inhibited in FAK−/− fibroblasts stimulated with either LPS or protein I/II. These results are not entirely unexpected, as it was demonstrated previously that the activation of Etk by the extracellular matrix is regulated by FAK through the interaction between the PH domain of Etk and the FERM domain of FAK (14). Our results strongly argue that FAK is an upstream kinase that is required for Etk activation even when integrins are activated by other ligands such as protein I/II. These findings correlate well with our previous data showing that FAK is probably an upstream activator of this pathway because FAK remained phosphorylated in MyD88−/− macrophages (6). The relatively equivalent activation of Etk and FAK suggest that the activation of Etk might be a one-step process; nevertheless, it cannot be excluded that additional components might be required to activate Etk kinases. Src kinase could be one of these components. Indeed, activation of the Btk family kinases via direct phosphorylation by Src family kinases was demonstrated (27, 28); the association of Etk with FAK opens the close conformation of Etk, which becomes accessible to FAK-associated Src kinases. This is probably not the case in our model as in previous experiments; having addressed the involvement of Src kinases in protein I/II signaling we have found that protein I/II did not activate Src (L. A. Neff, unpublished data). Accordingly, recent data (29) demonstrated that TNF-α activates FAK but that FAK-associated signaling promoting IL-6 expression and release does not require Src.

Insights in the potential molecular mechanisms implicated in FAK/Etk/Mal signaling came from results showing that Mal is phosphorylated in FAK−/− cells in response to LPS and protein I/II and that this phosphorylation is completely abolished in FAK−/− cells. These results support the conclusion that FAK expression is necessary for efficient phosphorylation of Mal after stimulation with LPS and protein I/II. The role of FAK seems specific to this pathway because we demonstrated the involvement of FAK in the TLR2-dependent secretion of IL-6 (6), whereas FAK does not play any role in the TLR3-dependent secretion of IL-6 in which neither Mal nor MyD88 are involved (data not shown). Phosphorylation of Mal is critical in TLR signaling as was recently shown by Gray et al. who demonstrated that following activation of TLR2 and TLR4 in the monocytic THP-1 cell line, Mal is tyrosine phosphorylated and that this phosphorylation is required for Mal to signal through transactivation of NF-κB subunit p65 (11). As it has also been demonstrated that in combination with signaling, phosphorylation of Mal allows its degradation by SOCS-1 (suppressor of cytokine signaling 1), ending TLR signaling (30), we can therefore postulate that FAK may contribute to Mal behavior and play an important role in TLR signaling pathway regulation.

In conclusion, our data provide support that in FLS, Etk and Mal are implicated in the cross-talk between FAK and MyD88 and that their being brought into play is clearly dependent of FAK. Overall, these data indicate that the stimulation of innate immunity leading to cytokine synthesis is regulated by a complex network that can be shared by pattern recognition receptors belonging to different families. The understanding of these complex interactions is important for therapeutic applications.

Disclosures
We have no financial conflict of interest.

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