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Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase involved in signaling downstream of integrins, linking bacterial recognition, cell entry, and initiation of proinflammatory response through MAPKs and NF-κB activation. In this study, using protein I/II from Streptococcus mutans as a model activator of FAK, we investigated the potential link between FAK and TLR pathways. Using macrophages from TLR- or MyD88-deficient mice, we report that MyD88 plays a major role in FAK-dependent protein I/II-induced cytokine release. However, response to protein I/II stimulation was independent of TLR4, TLR2, and TLR6. The data suggest that there is a cross talk between FAK and MyD88 signaling pathways. Moreover, MyD88-dependent, LPS-induced IL-6 secretion by human and murine fibroblasts required the presence of FAK, confirming that MyD88 and FAK pathways are interlinked. The Journal of Immunology, 2005, 174: 7393–7397.

Bacterial recognition implicates a wide variety of pattern recognition receptors specific for pathogen-associated microbial patterns (PAMPs), including TLRs, lectins, scavenger receptors, and integrins. Among them, TLRs are critical for the development of innate immune responses (1). TLR signaling is mediated by cytoplasmic Toll/IL-1R (TIR) domain homo- or heteromeric associations with TIR-containing adaptors (2). MyD88, an adaptor protein used by all TLRs except TLR3, activates MAPKs and NF-κB, leading to proinflammatory cytokine expression. MyD88-independent pathways, involving the TIR domain-containing adaptor inducing IFN-β (TRIF) are used by TLR4 and TLR3, together with TRIF-related adaptor molecule in the case of TLR4. TRIF pathway activates IFN regulatory factor 3, leading to the synthesis of IFN-α and -β. Moreover, receptor interacting protein 1, a component of the TNFR signaling pathway, associates with the C-terminal region of TRIF and mediates TLR4 and TLR3-NF-κB late activation (3). Thus, various PAMP/pattern recognition receptor interactions result in MAPK and NF-κB activation and consequent expression of proinflammatory cytokines, and there is now strong evidence that many intracellular signaling molecules are shared among the activated signaling pathways. However, the kinases involved in these pathways are not yet fully elucidated.

Focal adhesion kinase (FAK), a nonreceptor protein tyrosine kinase involved in signaling downstream of integrins (4), was shown recently to trigger inflammatory responses. Protein I/II, a cell wall component from oral streptococci, binding to integrin α5β1 induces the production of inflammatory mediators such as IL-6 and IL-8 by human monocytes, epithelial cells, endothelial cells, and fibroblasts (5, 6). The signaling events leading to this cytokine release involve FAK, ERK1/2, and JNKs as well as AP-1-binding activity and nuclear translocation of NF-κB (6, 7). FAK is an interesting candidate to link bacterial detection, initiation of proinflammatory responses, and cell entry, because FAK is involved in invasin-mediated bacterial uptake (8). Based on these observations, we investigated the possibility of a cross talk between the integrin/FAK and TLR pathways.

Using protein I/II from Streptococcus mutans as a model activator of FAK, together with macrophages from TLR- or MyD88-deficient mice, we show that MyD88 and FAK, but neither TLR2, TLR4, nor TLR6, are involved in the response to protein I/II that leads to cytokine release. This cross talk between FAK and MyD88 seems to be a general phenomenon in proinflammatory cytokine response, because LPS-induced cytokine release also depends on the presence of both FAK and MyD88, as evidenced in primary human and murine cells.

Materials and Methods

Mice

Six- to 12-wk-old mice deficient for TLR4 (from S. Akira (Department of Host Defense, Research Institute for Microbial Disease, Osaka University, Osaka, Japan); Ref. 9), for TLR2 (from C. Kirschning (Institute of Medical Microbiology, Immunology and Hygiene, Technical University Munich, Munich, Germany); Ref. 10), for TLR6 (11), or for MyD88 (12), and their control littermates were bred under specific pathogen-free conditions in the Transgenese Institute animal breeding facility (Orleans, France).

Cell cultures

Human fibroblast-like synoviocytes (FLSs) were isolated from rheumatoid arthritis synovial tissues at the time of knee joint arthroscopic synovectomy and cultured as previously described (7). FLSs (5 × 10⁴ cells per well) were grown to confluence in 96-well plates and serum-starved for 24 h before activation experiments. FAK⁺/⁺ and FAK⁻/⁻ primary mouse embryonic fibroblasts (American Type Culture Collection; CRL-2644 and -2645) were cultured as previously described (6). Cells were plated in 96-well plates (5 × 10⁴ cells per well) and serum-starved 24 h before activation experiments.

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Abbreviations used in this paper: PAMP, pathogen-associated microbial pattern; TIR, Toll/IL-1R; TRIF, TIR domain-containing adaptor inducing IFN-β; FAK, focal adhesion kinase; FLS, fibroblast-like synoviocyte; Pamp,CSK₅₋₋; [2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys₂OH, trihydrochloride; BLF, bacterial lipoprotein; FRNK, FAK-related nonkinase.
Transfections

FAK+/− cells were transfected with FRNK-YCam (a generous gift from K. Takeda) by the calcium phosphate method as previously described (6). Following transfection, cells were rinsed and then cultured in complete DMEM medium containing genetin (1.5 ng/ml) for 2 wk. The antibiotic-resistant cells were then pooled and used for further analysis. GFP was used to determine the transfection efficiency. Transient transfection of FLSs was performed using the Nucleofector kit as previously described (6). Cells were then plated in 96-well plates (5 × 10^4 cells per well) before activation experiments.

Protein I/II purification

Recombinant protein I/II of S. mutans OMZ 175 was purified from pHBr-1-transformed Escherichia coli cell extract by gel filtration and immuno-affinity chromatography as previously described (7). Potential endotoxin in protein I/II preparation was removed using polymyxin B-agarose (Detoxigel), according to the manufacturer’s recommendation (Pierce). Protein I/II had an endotoxin content <0.01 ng/125 pM protein I/II, as tested by the Limulus chromogenic assay (Charles River Laboratories). Throughout this study, buffers were prepared with apyrogenic water obtained from Braun Medical.

Cell activation

Murine bone marrow cells were isolated from femurs of TLR- and MyD88-deficient and control mice and cultivated as previously described (13). The bone marrow-derived macrophages were plated in 96-well plates (10^5 cells per well) before activation experiments. Appropriate stimuli were diluted in serum-free RPMI 1640 or DMEM with antibiotics, except for LPS activation of fibroblasts, which was performed in medium containing 5% heat-inactivated FCS. Cells were stimulated with 0.1 μg/ml LPS (E. coli, serotype O111:B4; Sigma-Aldrich), 0.5 μg/ml synthetic bacterial lipopeptide S-[2,3-bis(palmitoyl oxy)-5(R)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH, trihydrochloride (Pam3CSK4; EMC Microcollections), or protein I/II (at the indicated concentrations). After stimulation, the supernatants were harvested and analyzed immediately or stored at −20°C until further use. Cell number and cell viability were examined by the 3-(4,5-dimethylthiazol-2-hydroxyl)-2,3-diphenyl-2H-tetrazolium bromide test. Alternatively, stimulated cells were used for Western blotting experiments.

Western blot

A total of 10^5 cells was incubated for various times in 100 μl of medium with or without protein I/II (125 pM) or LPS (0.1 μg/ml). Proteins from cell lysates were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. FAK was detected using anti-FAK (pY397) or anti-FAK Abs. Stimulation with protein I/II resulted in phosphorylation of FAK in murine bone marrow-derived macrophages. Cell lysates were analyzed directly by blotting with specific anti-FAK (pY397) Abs. Stimulation with protein I/II induced a strong release of TNF-α and NO by activated macrophages, reaching levels similar to those obtained after endotoxin stimulation (Fig. 1, B and C, wild-type).

Results

Protein I/II, a ligand of integrin αβ1, induces FAK phosphorylation and TNF-α and NO production

We showed previously that protein I/II, a ligand of integrin αβ1, induces cytokine production by human endothelial and epithelial cells and FLSs and that this effect is FAK dependent (5, 6). In this study, we first verified the capacity of protein I/II to stimulate phosphorylation of FAK in murine bone marrow-derived macrophages. Cell lysates were analyzed directly by blotting with specific anti-FAK (pY397) Abs. Stimulation with protein I/II resulted in an increasing amount of phosphorylated FAK (Fig. 1A), which was detectable within 5 min and remained elevated for at least 30 min. In addition, protein I/II induced a strong release of TNF-α and NO by activated macrophages, reaching levels similar to those obtained after endotoxin stimulation (Fig. 1, B and C, wild-type).

Protein I/II-induced TNF-α and NO release is dependent on MyD88

In this study, we hypothesized that FAK might be one of the tyrosine kinases in cross talk with MyD88. To test this hypothesis, we first asked whether MyD88 is involved in the TNF-α and NO responses of macrophages activated with protein I/II. Bone-marrow-derived macrophages from MyD88-deficient mice failed to release significant TNF-α and NO concentrations in response to protein I/II (Fig. 1, B and C). Similar results were obtained with MyD88-deficient bone-marrow-derived dendritic cells (data not shown). Thus, protein I/II-induced activation of cytokine and NO secretion is mediated through MyD88.

Protein I/II-induced TNF-α release is independent of TLR2, -4, -6

It was suggested that TLRs could colocalize with other receptors and that integrins may be part of this complex (15, 16). We next asked whether TLRs might also play a role in macrophage activation by protein I/II. To test this hypothesis, we used TLR2 and TLR4 knockout mice as sources of macrophages. We first focused
on TLR2 and TLR4, because they recognize bacterial ligands expressed by most Gram-positive and Gram-negative bacteria: peptidoglycan, lipoteichoic acids, lipoproteins, and endotoxins. We examined TNF-α and NO production by TLR2 -/-, TLR4 -/-, and wild-type macrophages in response to protein I/II and used synthetic bacterial lipopeptide Pam3CSK4 (bacterial lipoprotein (BLP)) and LPS as reference TLR2 and TLR4 agonists, respectively. As shown in Fig. 2, protein I/II induced a dose-dependent release of TNF-α and NO in wild-type cells, and this release was not significantly different in TLR2- or TLR4-deficient cells, whereas these cells did not respond to BLP and LPS, respectively. These results indicate that neither TLR2 nor TLR4 are essential for protein I/II-induced TNF-α and NO release by activated macrophages. Because TLR6 is known to constitute heterodimers with TLR2, we also tested the stimulation of TLR6-independent of TLR2 and TLR4. Macrophages from wild-type mice or mice deficient for TLR2 or TLR4 were stimulated with protein I/II, BLP, or LPS at the indicated concentrations for 20 h. TNF-α-deficient for TLR2 or TLR4 were stimulated with protein I/II and found unimpaired TNF-α and NO production compared with wild-type macrophages (data not shown).

The TLR2 and TLR4 independence of protein I/II activation was then confirmed in human FLSs, a cell population known to contribute to joint inflammation in rheumatoid arthritis patients. Pretreatment with Abs directed against TLR4 or TLR2 had essentially no effect on protein I/II-induced IL-8 release by FLSs (data not shown).

Taken together, these data demonstrate that TLR2, TLR4, and TLR6 are not involved in protein I/II-induced TNF-α and NO release by activated murine macrophages and in protein I/II-induced IL-6 and IL-8 release by activated human FLSs.

**Protein I/II-induced FAK phosphorylation is independent of MyD88**

We then tested the role of MyD88 in FAK phosphorylation. Stimulation of MyD88 -/- macrophages with protein I/II resulted in an increasing amount of phosphorylated FAK (Fig. 3), which was detectable within 5 min and maximal after 15 min of stimulation, comparable to what was shown in wild-type cells (Fig. 1A). Therefore, although MyD88 is involved in the response to protein I/II that leads to cytokine release, FAK phosphorylation seems to be largely independent of the presence of MyD88, suggesting that phosphorylation of FAK occurs upstream of MyD88 or that both pathways are parallel.

**LPS stimulation induces FAK phosphorylation in macrophages and FLSs**

Our data suggesting that MyD88 and FAK are both necessary in the signaling pathway induced by protein I/II interaction with integrin α<sub>i</sub>β<sub>i</sub>, we next investigated whether this cross talk between FAK and MyD88 was a general phenomenon by assessing the role of FAK in signaling pathways known to involve MyD88. Cytokine response to LPS is largely MyD88 dependent, and we examined the capacity of LPS to induce FAK phosphorylation in murine macrophages. After stimulation, cell lysates were analyzed directly by blotting with specific anti-FAK (pY397) Abs as above. Stimulation by LPS resulted in an increasing amount of phosphorylated FAK (Fig. 4A), which was detectable within 5 min and remained elevated for at least 30 min. Similar FAK phosphorylation was obtained after LPS stimulation of human FLSs (Fig. 4B). Thus, LPS induced FAK phosphorylation in murine and human primary cells.

**LPS-induced IL-6 release occurs via a FAK-dependent pathway**

We next asked whether FAK could contribute to the signaling events leading to cytokine release from LPS-activated cells. First, using FAK<sup>+/+</sup> and FAK<sup>−/−</sup> primary mouse embryo fibroblasts, we showed that LPS induced IL-6 release by FAK<sup>+/+</sup> fibroblasts, but there was essentially no IL-6 production by FAK<sup>−/−</sup> fibroblasts (p < 0.01). This suggested that FAK is involved in LPS-induced release of IL-6. To rule out the possibility that the lack of
IL-6 release could be due to a nonspecific inhibition of other cellular functions, FAK+/+ fibroblasts were transfected with FAK-related nonkinase (FRNK), the C-terminal region of FAK, which is known to block FAK activation when overexpressed. FAK+/+ fibroblasts transfected with a GFP-expressing vector were used as a control. As shown in Fig. 5A, overexpression of FRNK strongly inhibited IL-6 release from LPS-activated FAK+/+ cells (p < 0.01), confirming the involvement of FAK in LPS-induced IL-6 secretion.

Second, to further demonstrate the requirement of FAK in LPS-induced cytokine release, we analyzed the cytokine response of human FLSs transiently transfected with FRNK. FLSs transfected with a GFP-expressing vector were used as a control. Fig. 5, B and C, shows that overexpression of FRNK significantly inhibited IL-6 and IL-8 release from LPS-activated FLSs. Thus, FAK is an essential component of the LPS-induced signaling pathways leading to cytokine stimulation, both in murine and human fibroblastic cells.

LPS-dependent FAK phosphorylation is independent of MyD88
To further characterize whether FAK phosphorylation was downstream of LPS-induced MyD88 recruitment, we tested the ability of LPS to phosphorylate FAK in MyD88−/− cells. FAK phosphorylation was not inhibited in MyD88−/− cells stimulated with LPS (Fig. 6), indicating that phosphorylation of FAK occurred independently of MyD88.

Taken together, our results show that MyD88 is involved in the response to protein I/II-integrin αβ1 interaction that leads to cytokine release through FAK phosphorylation, and this cross talk between the MyD88 and FAK pathways seems to be a general phenomenon in proinflammatory cytokine response, because MyD88-dependent, LPS-induced IL-6 secretion also depends on the presence of FAK.

Discussion
In the present study, we first demonstrated that absence of MyD88 abrogates or severely impairs TNF-α and NO production by protein I/II-activated macrophages. These data suggest that MyD88, a key downstream adaptor of the TLR pathway, plays a major role in protein I/II-integrin-induced cytokine release. We showed previously, using FAK−/− and FRNK-transfected cells, that FAK is essential in IL-6 and IL-8 release by protein I/II-activated FLSs (6). Thus, we examined next how FAK and MyD88 pathways may interact. Two hypotheses were formulated, namely, protein I/II may activate MyD88 by interacting with a member of the TLR family, or alternatively, protein I/II might trigger a signaling pathway involving an intracellular cross talk between FAK and MyD88. To address these questions, we first used macrophages isolated from TLR2−/− and TLR6−/− mice insofar as these TLRs recognize various components from Gram-positive bacteria. TLR4, the receptor of LPS from Gram-negative bacteria that also interacts with fibronectin, a ligand of integrin αβ1, was also tested. Our results clearly show that TLR2, TLR4, and TLR6 are not essential for the stimulation of macrophages by protein I/II. Also, Abs to TLR4 and TLR2 failed to inhibit protein I/II-induced IL-8 release by human FLSs (data not shown). These data are in agreement with the observation that macrophage activation by group B streptococci requires MyD88 but is independent of TLR1, -2, -4, and -6 (17). Thus, we excluded TLR2, TLR4, and TLR6 as potential receptors for protein I/II.

We next showed that FAK plays an important role downstream of TLRs, because cytokine response to LPS is totally abrogated in FAK−/− cells. Furthermore, FAK-expressing cells transfected with the FAK inhibitor FRNK produced significantly lower amounts of IL-6 upon LPS challenge. This is in line with previous results showing that LPS induced FAK phosphorylation in a murine monocytic cell line (18). We further demonstrated that other TLR ligands may also induce FAK phosphorylation at Tyr397 and require FAK for cytokine release because comparable results have been obtained with the TLR2 ligand Pam3CSK4 (data not shown). Also, our findings extend to FAK the observation that TLR2 and TLR4 agonists induced tyrosine phosphorylation of the proline-rich tyrosine kinase 2 (Pyk2), which in turn increases tyrosine phosphorylation of paxillin, an adaptor protein involved in integrin

**FIGURE 5.** FAK is necessary for LPS-induced cytokines release. FAK−/−, FAK+/+ primary mouse embryonic fibroblasts (A) and human FLSs (B and C) transfected or not with FRNK were stimulated with LPS (0.1 μg/ml) for 20 h. IL-6 and IL-8 levels in culture supernatants were determined by ELISA. Results are expressed as mean values ± SD for triplicate determinations and are representative of three experiments.

**FIGURE 6.** LPS-induced FAK phosphorylation is independent of MyD88. Macrophages from MyD88−/− deficient mice were stimulated with LPS (0.1 μg/ml) for the indicated times. Cell lysates were analyzed by Western blotting with anti-FAK pY397 or anti-FAK Abs. The results shown are representative of two experiments.
signaling and binding to Pyk2, vinculin, and FAK, through MyD88-dependent and -independent pathways (19). However, in our experiments, MyD88 does not seem to be essential for FAK autophosphorylation because this kinase is phosphorylated in MyD88−/− macrophages activated with either LPS or protein I/II. Thus, FAK phosphorylation seems to occur upstream of MyD88, or in parallel to MyD88 recruitment, and FAK might be a key proximal effector of MyD88.

Recent studies have shown that PAMP stimulation leads to TLR recruitment to cholesterol-rich regions of the cell membrane such as lipid rafts (16). These specialized domains may contribute to the assembly of a receptor complex involving transmembrane proteins such as TLRs and integrins as well as intracellular proteins. Preliminary experiments suggest that LPS and protein I/II-induced cell activation is dependent on such lipid rafts. Indeed, disruption of raft-like domains with the cholesterol-binding drugs filipin and nystatin inhibits LPS and protein I/II-induced cytokine release, suggesting that receptor complex assembly in microdomains is essential for downstream signaling triggered by these two PAMPs (data not shown). Therefore, MyD88 and FAK may contribute to the intracytoplasmic signaling of this receptor complex.

MyD88 may be activated in at least two different ways. Ligand engagement of members of the IL-1R/TLR family induces the recruitment and phosphorylation of MyD88, but it has also been demonstrated that Fas ligation on macrophages enhances TLR4 signaling by suppressing the inhibitory interaction of Fas-associated death domain protein with MyD88 (20). One might speculate that FAK contributes to MyD88 phosphorylation, but FAK possesses neither a death domain nor a TIR domain for direct interaction with MyD88. Alternatively, FAK may participate in MyD88 activation by recruiting another kinase. In previous experiments, we addressed the involvement of Src kinases in protein I/II-induced signaling. Protein I/II did not induce phosphorylation of Src at Tyr418 (activated Src) nor mediate phosphorylation of FAK at Tyr592, a Src phosphorylation site (L. A. Neff, M. B. Zeisel, V. A. Druet, J. P. Klein, J. Sibilia, and D. Wachsmann, unpublished results). Thus, Src does not seem to play a role in protein I/II-induced signaling. It has long been known that protein tyrosine kinases are required for the induction of proinflammatory cytokines in response to LPS in macrophages. Jefferies et al. (21, 22) demonstrated that Btk is a critical tyrosine kinase in LPS signaling in myeloid cells. Btk interacts with the TIR domain of TLRs as well as with downstream molecules such as MyD88, MAL, and IL-1R-associated kinase-1, but the precise nature of these interactions is not well defined. Btk is probably activated through Src kinases although other tyrosine kinases such as PI3K and Jak1 have also been shown to phosphorylate members of the Btk family (23). Because FAK has been demonstrated to be an upstream activator of Bmx/Etk in endothelial cells (24), it is conceivable that FAK might lead to the recruitment of MyD88 via the activation of a member of the Btk family of kinases and further downstream signaling to MAPKs, AP-1, and NF-κB. Further studies of the involvement of additional kinase players in FAK and MyD88 pathway cross talk will be needed to complete our understanding of the molecular mechanisms linking bacterial detection, cell invasion, and proinflammatory response.

Disclosures

The authors have no financial conflict of interest.

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