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Nonmuscle myosin light-chain kinase mediates neutrophil transmigration in sepsis-induced lung inflammation by activating β_2 integrins

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Abstract

Nonmuscle myosin light-chain kinase (MYLK) mediates increased lung vascular endothelial permeability in lipopolysaccharideinduced lung inflammatory injury, the chief cause of the acute respiratory distress syndrome. In a lung injury model, we demonstrate here that MYLK was also essential for neutrophil transmigration, but that this function was mostly independent of myosin II regulatory light chain, the only known substrate of MYLK. Instead, MYLK in neutrophils was required for the recruitment and activation of the tyrosine kinase Pyk2, which mediated full activation of β_2 integrins. Our results demonstrate that MYLK-mediated activation of β_2 integrins through Pyk2 links β_2 integrin signaling to the actin motile machinery of neutrophils.

Neutrophils are the first line of defense against invading micro-pathogens. To kill pathogens, neutrophils must first attach to the blood vessel wall, then transmigrate into tissue to reach the site of infection and consume pathogens by phagocytosis[1]. Therefore, pathogen-induced adhesion and migration of neutrophils are critical events in the innate immune response[2,3]. In contrast, inappropriate sequestration of neutrophils in tissue coupled with their activation can also cause profound injury, such as that in sepsis-induced acute respiratory distress syndrome[4,5].

Myosin light-chain kinase (MYLK; A000026) is a calcium-calmodulin–dependent kinase that phosphorylates myosin II regulatory light chain (MLC), the substrate for MYLK[6,7]. MYLK has two isoforms: smooth muscle (short form; 108–130 kilodaltons) and nonmuscle (long form; 210 kilodaltons)[8,9]. Mice deficient in nonmuscle MYLK (*Mylk*$^{-/-}$ mice) are protected from endotoxin-induced lung injury and also have much better survival[10]. The protection in *Mylk*$^{-/-}$ mice may result from inhibition of the endothelial hyper-permeability response and impaired neutrophil transmigration and activation due to the loss of nonmuscle MYLK function. Nonmuscle MYLK has been shown to be pivotal in endothelial barrier disruption through the regulation of actomyosin contractility in endothelial cells[11,12]. However, the

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AUTHOR CONTRIBUTIONS

J.X. designed experiments, did research and wrote the paper; A.B.M. designed research and wrote the paper; X.-P.G. designed and did research; and R.R., Y.-Y.Z. and S.M.V. did research.

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function of nonmuscle MYLK in regulating neutrophil transmigration in the innate immune response has not been investigated.

Integrins are a family of transmembrane receptors for extracellular matrix proteins[13]. The β_2 integrins, the main integrins expressed in neutrophils and other leukocytes, are key effectors mediating the adhesion of neutrophils to endothelial cells during inflammation and other immune responses[14]. Integrins function not only as adhesive proteins but also as signaling molecules[13–16]. Intracellular signals such as those stimulated by chemokine receptors activate integrins to an intermediate active state and induce the binding of integrins to their ligands in the extracellular matrix[17]. Integrin-matrix interactions in turn induce 'outside-in' signals that activate the tyrosine kinases c-Src, Syk and Pyk2 (also called Ptk2b; A001952)[18], which promote actin polymerization, thereby reinforcing the integrin-cytoskeleton connection. This interaction leads to the full activation of integrins[18–21]. Therefore, actin dynamics regulated by tyrosine kinases are important for β_2 integrin–mediated adhesion of neutrophils to the endothelium[14,22–24].

MYLK is involved in integrin-mediated attachment of the leading edge to the matrix in T cells and adhesion disassembly in fibroblasts through the regulation of myosin II (refs. 25,26). In addition to its kinase activity, MYLK interacts with cytoskeletal components such as actin, microtubules and cortactin[27–30], which raises the possibility that MYLK can regulate integrin function by organizing the cytoskeletal architecture[9]. In this study, we demonstrate that the nonmuscle MYLK was required for neutrophil adhesion to and migration across the endothelium in a lipopolysaccharide (LPS)-induced model of lung injury. Unexpectedly, this function of MYLK was mostly independent of myosin II activity. Instead, we found that MYLK was required for actin polymerization and the full activation of β_2 integrins and thus mediated neutrophil transendothelial migration. Our results demonstrate that MYLK's binding to and activation of the tyrosine kinases c-Src and Pyk2 was crucial for the recruitment of Pyk2 to β_2 integrins. These observations suggest that MYLK mediates a regulatory pathway in neutrophils during innate immune responses. This pathway may provide a therapeutic target for the prevention and treatment of inappropriate neutrophil migration and sequestration in conditions such as sepsis-induced lung inflammation and injury.

RESULTS

MYLK is required for neutrophil transmigration

Mylk$^{-/-}$ mice are less susceptible to LPS-induced acute lung injury[10]. We found that after injection of LPS, *Mylk*$^{-/-}$ mice had a smaller increase in lung vascular permeability and edema formation than did wild-type mice (Supplementary Fig. 1 online). As nonmuscle MYLK is expressed both in endothelial cells and neutrophils (Supplementary Fig. 2 online), the protective effect in *Mylk*$^{-/-}$ mice could have been the result of either less permeability of the endothelial barrier or impaired migratory ability of neutrophils. To distinguish the function of nonmuscle MYLK in neutrophil transmigration from that in endothelial barrier regulation, we used an *ex vivo* lung injury assay, perfusing isolated wild-type mouse lungs with neutrophils from *Mylk*$^{-/-}$ or wild-type mice[31]. We measured the lung microvessel filtration coefficient and gain of lung wet weight to assess the vascular permeability of lungs and edema formation, respectively. The group with *Mylk*$^{-/-}$ neutrophil perfusion had significantly lower microvessel filtration coefficient values and wet weight gain (by about 50% in each case) than did the group perfused with wild-type neutrophils in the presence of LPS (Fig. 1a,b). As we used wild-type lungs as recipients in both groups, the difference represents the impaired ability of *Mylk*$^{-/-}$ neutrophils to cause lung injury. These functional studies demonstrate that MYLK in neutrophils is a critical factor mediating LPS-induced lung injury.

To examine lung neutrophil sequestration in these studies, we perfused wild-type lungs with ^{111}In-oxine–labeled neutrophils along with LPS, the bacterial chemoattractant fMLP (*N*-formyl-methionylleucyl-phenylalanine) or saline (control). After washing out unbound neutrophils from vessels, we measured the γ-radioactivity of lung tissue, which represented the resident neutrophils. Although both LPS and fMLP stimulated the uptake of wild-type neutrophils in lungs, the uptake of *Mylk*$^{-/-}$ neutrophils was significantly lower (by about 40% and 90% in response to each stimulus, respectively; Fig. 1c).

Neutrophil adhesion and migration are two critical events during the transendothelial migration of neutrophils; thus, we addressed by *in vitro* assay whether either step was affected for *Mylk*$^{-/-}$ neutrophils. We purified wild-type and *Mylk*$^{-/-}$ neutrophils and added them to cultured wild-type mouse lung vascular endothelial cells. After washing away nonadherent cells, we counted residual neutrophils to determine adhesive activity. With LPS stimulation, the number of adherent wild-type neutrophils was sixfold higher than without (Fig. 1d), whereas the number of adherent *Mylk*$^{-/-}$ neutrophils was about 50% less than wild type (Fig. 1_d). We analyzed neutrophil migration by Transwell assay with two chambers separated by a monolayer of endothelial cells. We applied LPS-primed wild-type or *Mylk*$^{-/-}$ neutrophils to the upper chamber and the chemoattractant fMLP to the lower chamber. We assessed cells that had migrated into the lower chamber and attached to the basal side of the filter after 4 h of incubation. The transendothelial migration of LPS-primed wild-type neutrophils was sixfold higher after fMLP stimulation relative to that with LPS only. However, the fMLP-induced migration of LPS-primed *Mylk*$^{-/-}$ neutrophils was about 55% that of wild-type neutrophils (Fig. 1e). We also consistently noted less LPS-induced transalveolar migration of neutrophils in *Mylk*$^{-/-}$ mice than in wildtype mice (Fig. 1f). These data demonstrate that both adhesion to endothelial cells and migration across the pulmonary endothelium are substantially impaired in *Mylk*$^{-/-}$ neutrophils.

We then compared the migratory activity of wild-type and *Mylk*$^{-/-}$ neutrophils after adherence to a fibrinogen-coated surface *in vitro*. Wild-type neutrophils after fMLP stimulation migrated in a more linear way with fewer turns. *Mylk*$^{-/-}$ neutrophils were still able to polarize and migrate, but they frequently changed direction (Supplementary Fig. 3a,b online), which suggested involvement of MYLK in stabilizing the neutrophil leading edge. The MYLK-deficient neutrophils also had migrated faster (Supplementary Fig. 3c). These data collectively show that MYLK is an important determinant of neutrophil migration both *in vivo* and *in vitro*.

Myosin II is activated in the absence of MYLK

As MLC is the only known substrate for MYLK (MYLK phosphorylates MLC at Thr18 and Ser19)[6,7], we next determined whether MYLK regulates the adhesion and migration of neutrophils secondary to activation of MLC. We assessed MLC activation with an antibody that recognizes the phosphorylated subunit of MLC (p19-MLC). In wild-type lungs and neutrophils, p19-MLC was 1.5-fold higher after LPS stimulation (Fig. 2a,b). Although basal p19-MLC was slightly lower in *Mylk*$^{-/-}$ lungs (Fig. 2a) and neutrophils (Fig. 2b), MLC was activated after LPS stimulation to amounts similar to those of wildtype. We obtained similar results with fMLP stimulation (Supplementary Fig. 4 online). The question arose of whether MLC is activated by the smooth-muscle MYLK isoform, which is also present in *Mylk*$^{-/-}$ mice. However, we noted that *Mylk*$^{-/-}$ neutrophils showed little MYLK activity after LPS stimulation (Supplementary Fig. 5 online). Thus, MYLK was not required for LPS- or fMLP-induced MLC phosphorylation, and the phenotype resulting from MYLK deletion was not mediated by MLC signaling. These results indicate alternative functions for MYLK in mediating neutrophil adhesion and migration.

That hypothesis was supported by morphological and functional studies of myosin II and MYLK. In polarized neutrophils, active myosin II (as visualized by staining with antibody to p19-MLC (anti-p19-MLC)) accumulated in the rear of cells[32] (Fig. 2c). In contrast, MYLK was located mainly at the leading edge of polarized neutrophils (Fig. 2c). In addition to the distinct subcellular distribution of MYLK, loss of function of myosin II or MYLK resulted in different cell migration. Blocking myosin II activity with the inhibitor blebbistatin[33] impaired the neutrophil rear contraction, as shown by noncontracted long tails[32]. However, *Mylk*$^{-/-}$ neutrophils had normal rear contraction with accumulation of MLC at the trailing edges during migration (Supplementary Figs. 3 and 6 online). Moreover, both *Mylk*$^{-/-}$ neutrophils and wild-type neutrophils treated with the MYLK inhibitor ML-7 showed less adhesion to the endothelium (Fig. 1d and Fig. 2d). In contrast, the myosin II inhibitor blebbistatin and the Rho kinase inhibitor Y27632 (which blocks myosin II activation[34,35]) resulted in enhanced neutrophil adhesion (Fig. 2d).

Myosin II is known to mediate the 'backness' signal regulated by the RhoA and Rho kinase pathway, which prevents lateral pesudopod formation during neutrophil migration[32]. Inhibition of the 'backness' signal can enhance the 'frontness' signal, such as F-actin formation[32,36]. Wild-type neutrophils treated with blebbistatin or Y27632 had much more actin polymerization before and after LPS stimulation (Supplementary Fig. 7a online). In contrast, F-actin content was lower in *Mylk*$^{-/-}$ neutrophils than in wild-type neutrophils (40% less; Supplementary Fig. 7b) and was lower in wild-type neutrophils after ML-7 treatment (40% less than no treatment; Supplementary Fig. 7a), which suggested that MYLK does not act as a 'backness' signal and has a function opposite to that of myosin II in cytoskeleton assembly. Thus, MYLK and myosin II have distinct subcellular localizations and different functions during neutrophil migration, which indicates an important function for MYLK in regulating neutrophil migration by a pathway other than through MLC phosphorylation.

MYLK is required for β_2 integrin activation in neutrophils

As MYLK regulation of neutrophil adhesion and migration is independent of MLC phosphorylation, we next explored a previously unknown function for MYLK. The β_2 integrins are the main cell surface adhesion molecules required for the adhesion of neutrophils to the endothelium[14]. Because loss MYLK function led to impaired adhesive ability and migration as well as less tissue sequestration of neutrophils (Fig. 1c–e), we examined the possibility that β_2 integrin function was disrupted in *Mylk*$^{-/-}$ neutrophils. On surfaces coated with ICAM-1 (a ligand for β_2 integrins), wild-type neutrophils adhered and spread with clustering of β_2 integrin, as detected with antibody to α_M, an α-subunit of β_2 integrins (Fig. 3a,b). In contrast, 50% fewer *Mylk*$^{-/-}$ neutrophils adhered to ICAM-1 in the presence of LPS with much less spreading (Fig. 3a,b), which indicated that β_2 integrin function was impaired in *Mylk*$^{-/-}$ neutrophils. To assess the activation of β_2 integrins, we used a soluble ICAM-1 binding assay. We incubated freshly isolated wild-type and *Mylk*$^{-/-}$ neutrophils with Fc-tagged ICAM-1 and analyzed them by flow cytometry. LPS-induced ICAM-1 binding in the presence of Mn^{2+} was significantly lower in *Mylk*$^{-/-}$ neutrophils than in wild-type neutrophils (Fig. 3c), which indicated that MYLK is required for the activation of β_2 integrin. To determine whether the lower integrin activation was due to less surface expression of integrin, we assessed LPS-induced cell surface expression of α_L, α_M and β_2 on wild-type and *Mylk*$^{-/-}$ neutrophils. LPS increased the expression of all three integrin molecules at the surface of *Mylk*$^{-/-}$ neutrophils; such expression was similar to that of wild-type neutrophils (Fig. 3d).

MYLK is required for activation of Src and Pyk2

Integrin activation can be divided into two stages[18–21]. 'Inside-out' signals activate integrins to an intermediate active state that promotes integrin-ligand binding. After integrin-ligand binding, 'outside-in' signals then activate tyrosine kinases such as c-Src, Syk and Pyk2, thereby

fully activating integrins, possibly through additional actin polymerization and reinforced integrin-cytoskeleton interaction[18–21]. To explore how MYLK functions in the mechanism of integrin activation, we first studied whether crosstalk of MYLK with these tyrosine kinases regulated actin polymerization. We assessed activation of c-Src, Syk and Pyk2 with phosphorylation-specific antibodies in wild-type and *Mylk*$^{-/-}$ neutrophils. All three tyrosine kinases were phosphorylated in wild-type neutrophils after LPS challenge (Fig. 4a). However, phosphorylation of c-Src and Pyk2 was about 80% and 60% lower, respectively, in *Mylk*$^{-/-}$ neutrophils, whereas phosphorylation of Syk was only slightly lower (Fig. 4a), which indicated that MYLK is required for the activation of c-Src and Pyk2. Furthermore, our functional data showed that blocking c-Src activity with its inhibitor, PP2, resulted in less adhesion of neutrophils to the endothelium (Fig. 2d) and less F-actin formation (Supplementary Fig. 7a), consistent with the results obtained with *Mylk*$^{-/-}$ neutrophils (Fig. 1d and Supplementary Fig. 7b).

MYLK has been shown to bind to c-Src[37], and here we detected that MYLK was also associated with Pyk2 in both coimmunostaining and coimmunoprecipitation assays (Fig. 4b). Notably, although both c-Src and Pyk2 bound to β_2 integrins in wild-type neutrophils (Fig. 4c), the Pyk2–β_2 integrin interaction but not the c-Src–β_2 integrin interaction was disrupted in *Mylk*$^{-/-}$ neutrophils (Fig. 4c). We further explored how MYLK regulates Pyk2 activation by *in vitro* assay. We found that MYLK interacted with Pyk2 directly and phosphorylated Pyk2 *in vitro* (Supplementary Fig. 8a,b online) and that ML-7 partially blocked Pyk2 activation (Supplementary Fig. 8c), which indicated that the kinase activity of MYLK and the MYLK-Pyk2 interaction may both be required for Pyk2 activation. These results collectively demonstrate that MYLK is critical for the activation both c-Src and Pyk2 and is required for the recruitment of Pyk2 to the β_2 integrin complex.

MYLK regulates the β_2 integrin–cytoskeleton interaction

The interaction of integrin with F-actin through actin-associated proteins such as talin is critical for strong integrin-cytoskeleton connections and integrin activation[18]. Pyk2 has been shown to activate the small Rho GTPase Cdc42 by inhibiting PSGAP (a Rho GTPase–activating protein), thus promoting actin polymerization[38]. We found less F-actin formation in *Mylk*$^{-/-}$ neutrophils (Supplementary Fig. 7b), which suggested that Pyk2-mediated actin polymerization and β_2 integrin–cytoskeleton interactions could be a chief signaling mechanism ‘downstream’ of MYLK responsible for neutrophil transmigration.

To test that hypothesis, we determined whether MYLK participates in mediating β_2 integrin–cytoskeleton assembly. Although β_2 integrins bound to talin in wild-type neutrophils[15], this binding was much lower in *Mylk*$^{-/-}$ neutrophils (Fig. 5a). We obtained similar results for the interaction of β_2 integrin with another actin-associated protein, α-actinin (Fig. 5b). However, we did not detect any interaction of MYLK with β_2 integrins, talin or α-actinin in wild-type neutrophils (data not shown). Thus, MYLK may indirectly regulate the interaction of β_2 integrins and actin-associated proteins, possibly through Pyk2-mediated actin polymerization. Our results have collectively shown that MYLK is required for the activation of c-Src and Pyk2 during the transendothelial migration of neutrophils and for the recruitment of Pyk2 to the β_2 integrin complex. The activation of tyrosine kinases in turn is essential in the mechanism of actin polymerization; it reinforces the β_2 integrin–cytoskeleton connection and fully activates β_2 integrins, a crucial requirement for the adhesion and transmigration of neutrophils (Supplementary Fig. 9 online).

DISCUSSION

The nonmuscle isoform of MYLK is fundamental in the regulation of endothelial barrier function[11,12]. Studies have also shown that *Mylk*$^{-/-}$ mice are less susceptible to endotoxin-

induced lung injury[10]. However, such studies have not distinguished the function of MYLK in neutrophils from that in endothelial cells. In our study here, the use of an *ex vivo* sepsis model and isolated neutrophils allowed us to address the function of MYLK in the mechanism of neutrophil transmigration in lungs. We noted that loss of MYLK function inhibited neutrophil sequestration and transalveolar migration in wild-type lungs and, moreover, the lower sequestration of *Mylk*$^{-/-}$ neutrophils resulted in less of a neutrophil-mediated increase in pulmonary vascular permeability and lung edema formation. Our findings have shown that activation of MYLK in neutrophils resulting in the adhesion of neutrophils to pulmonary vascular endothelium is essential in neutrophil-mediated inflammatory lung injury.

Although MLC is the only known substrate of MYLK, our findings have indicated that the function of MYLK in regulating neutrophil adhesion and migration is not mediated by MLC phosphorylation.We found that MLC could still be phosphorylated in *Mylk*$^{-/-}$ neutrophils. Although the smooth-muscle isoform of MYLK is also present in *Mylk*$^{-/-}$ mice, we found that *Mylk*$^{-/-}$ neutrophils showed little MYLK activity after LPS stimulation, which suggests that activation of MLC in *Mylk*$^{-/-}$ mice is not mediated by the smooth-muscle isoform MYLK. It is possible, however, that MLC phosphorylation in neutrophils is regulated by other kinases such as Rho kinase and Zip kinase[34,35,39]. MLC and MYLK also showed distinct subcellular localizations and different functions during neutrophil transmigration, consistent with the biochemical data. MYLK was localized to the leading edge of polarized neutrophils, whereas active MLC accumulated at the trailing edge. Furthermore, blocking myosin II and MYLK produced opposite effects on both neutrophil adhesion and actin polymerization. These data collectively indicate that MYLK has a MLC phosphorylation–independent function that mediates neutrophil adhesion and migration during LPS-induced lung injury.

MYLK, in addition to having a kinase domain, contains sites that bind to cytoskeletal proteins and tyrosine kinases[27,29,37], which suggests that MYLK may function as an adaptor protein through these interactions. We have provided evidence here that in addition to binding to the tyrosine kinase c-Src, MYLK also bound to Pyk2. MYLK interacted with Pyk2 directly and phosphorylated Pyk2 *in vitro*, and ML-7 partially blocked Pyk2 activation, which indicates that the kinase activity of MYLK and the MYLK-Pyk2 interaction may be both required for Pyk2 activation. Nevertheless, it remains unclear whether MYLK can directly phosphorylate Pyk2 *in vivo*. It has been reported that activation of Pyk2 activates Cdc42 and subsequently induces actin polymerization[38]. We noted that loss of MYLK also abolished Cdc42 activation (data not shown) as well as actin polymerization stimulated by LPS. Thus, MYLK may be required for the activation of a tyrosine kinase–Cdc42–actin signaling pathway mediating neutrophil adhesion and migration.

The β_2 integrins are the main adhesion molecules expressed in neutrophils and are responsible for mediating the adhesion of neutrophils to the endothelium. Our data showed lower activation of β_2 integrins in *Mylk*$^{-/-}$ neutrophils. In the two-step model of integrin activation[18–21], both c-Src and Pyk2 are the ‘outside-in’ signals activated after integrin-matrix binding and thereby promote actin polymerization[24,38,40]. The additional actin polymerization reinforces the integrin-cytoskeleton interaction, which sustains β_2 integrins in the high-affinity and high-avidity state, reflecting the full activation of integrins[18–21]. Here we have shown that in addition to being required for integrin-matrix binding, MYLK is also required for the activation of c-Src and Pyk2, and it thereby contributes to a later stage of β_2 integrin activation through the recruitment and activation of Pyk2 to the integrin complex. In this model, it remains unclear how MYLK regulates Src, although our data have shown that Src activation depended on the presence of MYLK. Src is not a substrate for MYLK, as ML-7 did not inhibit Src activation (data not shown). Src has been shown to directly phosphorylate MYLK[37], but it is also possible, as evident from our results, that MYLK can itself regulate Src through a feedback mechanism.

Although MYLK is known as myosin II regulatory light-chain kinase, we have identified here an MLC phosphorylation–independent function for MYLK in complex with c-Src and Pyk2. This function of MYLK was essential for neutrophil transmigration during sepsis-induced lung injury. In contrast to myosin II, which is known as a 'backness' molecule responsible for rear contraction, MYLK functions as a 'frontness' molecule that accumulates at the front of neutrophils, where it is responsible for actin polymerization and the full activation of β_2 integrins.

METHODS

Mice, cell cultures and reagents

Wild-type C57BL/6 mice were from Charles River Laboratories. Mice deficient in MYLK were gifts from D.M. Watterson[10]. All mice were bred and housed in pathogen-free conditions with free access to food and water in the Animal Care Facility. All experimental procedures complied with institutional and National Institutes of Health guidelines for animal use. Mouse neutrophils were purified from mouse bone marrow and peripheral venous blood with a discontinuous Percol gradient as described with some modifications[41]. This procedure yielded polymorphonuclear neutrophil (PMN) purity of over 95% and viability of over 95%, as assessed by Trypan blue exclusion and analysis of cytospin preparations (Shandon) stained with the Hema 3 system (Fisher)[31]. Culture of neutrophil-like dHL60 cells and transfection were done as described[32]. MYLK cDNA was from Open Biosystems. Pyk2 cDNA was a gift from X. Zhu. Antibodies to MYLK were from Sigma (M7905) and Abgent (AP79669). Antibodies to p19-MLC (3671) and to phosphorylated c-Src (2113), Pyk2 (3291) and Syk (2715), as well as purified glutathione *S*-transferase–MYLK (Gly1425–Ser1776), were from Cell Signaling. Pyk2 protein was from Millipore.

Lung injury and neutrophil sequestration *ex vivo*

The pulmonary microvessel filtration coefficient and increases in lung wet weight were assessed as described[31]. For analysis of neutrophil sequestration, isolated bone marrow neutrophils were labeled with ^{111}In-labeled oxine (Amersham) as described[42]. Neutrophils (1×10^6) were infused over 5 min with a syringe pump into the pulmonary artery line of the lung preparation, followed by a 30-minute washout period. Venous effluent samples were collected at 5-minute intervals and radioactivity was measured. The number of accumulating radiolabeled neutrophils per gram of dry lung was calculated at the end of the washout period.

MYLK activity assay

The activation of MYLKwas measured in total cell lysates as described[43]. Cells were suspended in ice-cold kinase buffer (40 mM HEPES, pH 7.0, 5 mM Mg acetate, 0.55 mM $CaCl_2$ and 0.1% (vol/vol) Tween-80) containing freshly added 1 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethyl-sulfonyl fluoride, 10 μg/ml of pepstatin, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin and 100 μg/ml of soybean trypsin inhibitor, then were disrupted by a cycle of freezing and thawing. Samples were sonicated with a probe twice for 12 s each on ice and were centrifuged with a microfuge. MYLK activity was assessed in supernatants of cell lysates in the presence or absence of substrate. A saturating concentration of 300 μM substrate was used for the MYLK activity assay. Reaction mixture containing substrate peptide or buffer plus 5 μCi [γ-^{32}P]ATP and 0.5 mM unlabeled ATP was added to each tube and samples were incubated for 10 min at 30 °C. Reactions were stopped by filtration through Whatman P81 paper. After being washed, filters were added to scintillation fluid and radioactivity was measured in a scintillation counter (Wallac). 'Blanks' were assay samples without substrate.

F-actin content of neutrophils

F-actin was measured in neutrophils as described with some modifications[44]. Cells were stimulated for various times at 37 °C with LPS (*Escherichia coli* 0111:B4; EMD Chemicals) or fMLP (Sigma). Cells were immediately fixed for 30 min at 25 °C with 3.7% (vol/vol) paraformaldehyde in PBS and were washed with PBS. Cells were simultaneously made permeable and stained for 30 min at 37 °C in the dark with a fresh mixture of L-α-lysophosphatidylcholine palmitoyl (0.5 mg/ml; Sigma) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin (0.1 μg/ml; Molecular Probes). After being stained, samples were washed and resuspended in PBS. F-actin was measured with fluorescein phalloidin (Invitrogen) with a Triad LT plate reader at excitation of 495 nm and an emission of 520 nm (Dynex Technologies).

Immunostaining and confocal microscopy

Freshly isolated neutrophils (2×10^6) added to coverslips coated with fibrinogen (10 μg/ml) were challenged for various times at 37 °C with LPS (1 μg/ml; *E. coli* 0111:B4; EMD Chemicals). Cells were then fixed and made permeable and were stained sequentially with the appropriate antibodies. Images for confocal microscopy were obtained with a Zeiss LSM 510 microscope with excitation laser lines of 488 nm and 543 nm.

Neutrophil adhesion to endothelial cells and transendothelial migration

Mouse lung vascular endothelial cells were isolated[45] and grown to confluence in 96-well gelatin-coated plates. Neutrophils loaded with calcein (acetoxy-methyl ester) were added to mouse lung vascular endothelial cells and PMN adhesion to and migration on endothelial cells were assayed as described[45].

Immunoprecipitation and immunoblot analysis

For immunoblot analysis, lysates were prepared as described[45]. Phosphorylation of MLC was measured as described[46]. For densitometry, the optical density of bands on autoradiograms was assessed with scanned autoradiograph films and the National Institutes of Health Image program.

Statistical analysis

Statistical comparisons were made with the two-tailed Student's *t*-test. Differences in mean values were considered significant at a *P* value of less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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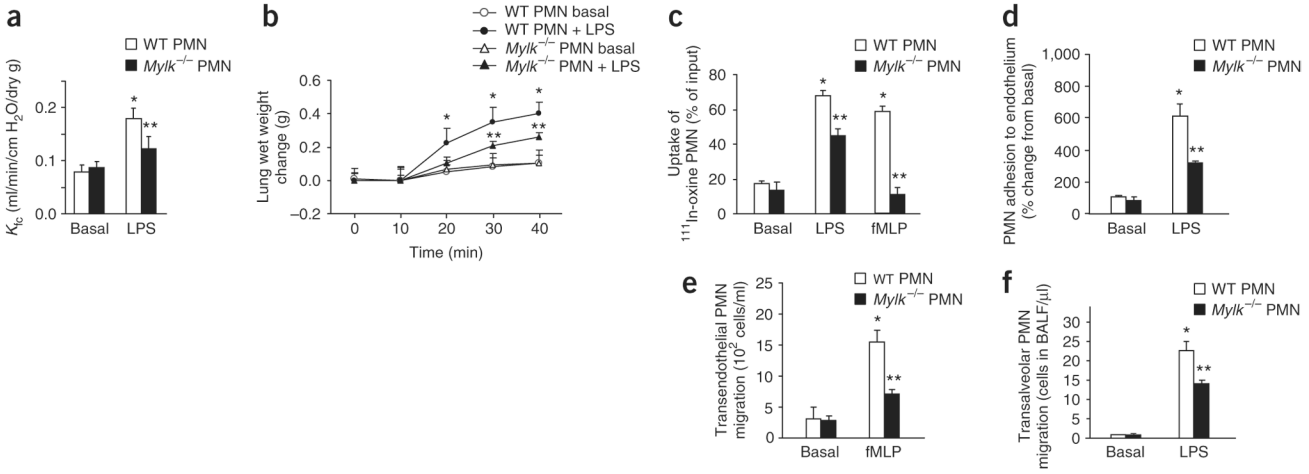

Figure 1. *Ex vivo* LPS-induced lung injury and edema formation

(**a**) Change in microvessel permeability in wild-type lungs after perfusion of wild-type (WT) or *Mylk*$^{-/-}$ neutrophils (PMN), followed by challenge for 16 h with LPS (10 mg/kg given intraperitoneally), assessed as the pulmonary microvessel filtration coefficient (K_{fc}) and presented as per gram of dry lung weight (dry g). (**b**) Time-dependent change in pulmonary edema formation, as assessed by the increase in wet weight of lungs treated as described in **a**. (**c**) Sequestration of ^{111}In-oxine–labeled PMNs in lungs after ^{111}In-labeled PMNs were stimulated with LPS or fMLP and then added to perfusates of lung preparations. (**d**) Adhesion of wild-type and *Mylk*$^{-/-}$ PMNs to cultured mouse lung vascular endothelial cells. (**e**) Transmigration of LPS-primed wild-type and *Mylk*$^{-/-}$ PMNs across endothelial cells toward fMLP in the lower chamber of a Transwell. (**f**) Migration of PMNs into the airspace of wild-type and *Mylk*$^{-/-}$ mice challenged with LPS. *, $P < 0.05$, compared with basal; **, $P < 0.05$, compared with wild type after LPS stimulation. Data are the mean of five (**a,b**), four (**c–e**) or six (**f**) independent experiments (error bars, s.e.m.).

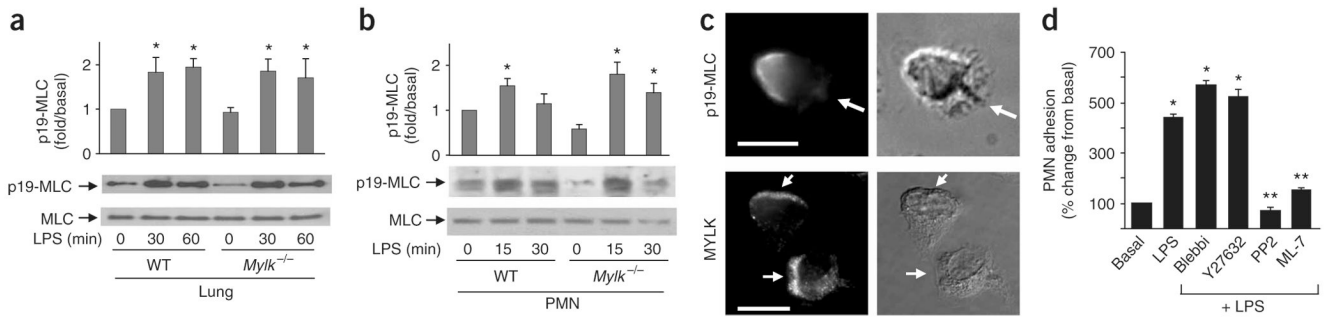

Figure 2. Loss of MYLK function fails to prevent myosin II activation

(**a,b**) MLC phosphorylation in wild-type and *Mylk*$^{-/-}$ lungs (**a**) or PMNs (**b**). Bottom blots, confirmation of equal protein loading by analysis with anti-MLC. Above, densitometry analysis. *, $P < 0.05$, compared with basal (no LPS stimulation). Data are from one experiment representative of three (error bars, s.e.m.). (**c**) Subcellular distribution of activated myosin II (top; anti-p19-MLC) and MYLK (bottom; anti-MYLK) in polarized PMNs. Arrows indicate leading edges. Scale bars, 10 μm. Results are representative of three experiments. (**d**) Adhesion of wild-type PMNs preincubated with LPS (1 μg/ml), then cultured for 30 min at 37 °C together with mouse lung vascular endothelial cells with or without inhibitors (horizontal axis). Blebbi, blebbistatin. *, $P < 0.05$, compared with basal; **, $P < 0.05$, treatment with inhibitor plus LPS compared with LPS alone. Data are the mean of four independent experiments (error bars, s.e.m.).

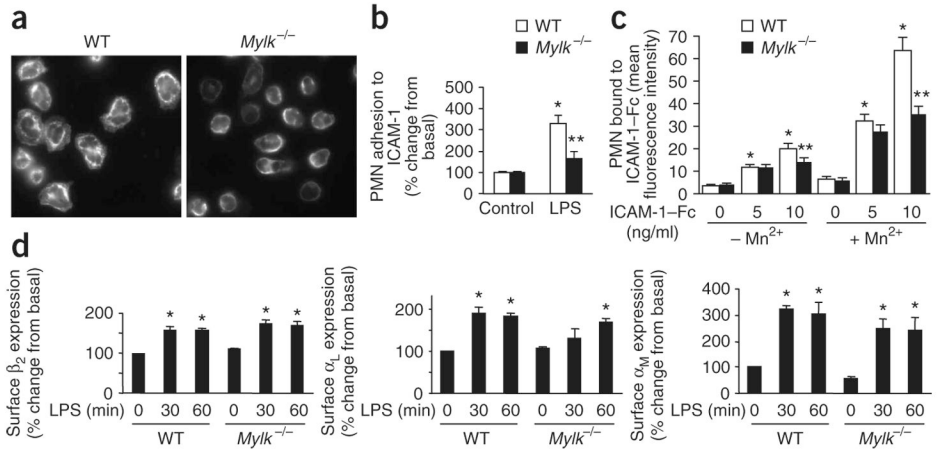

Figure 3. Activation of β_2 integrin in neutrophils

(**a**) Microscopy of wild-type and *Mylk*$^{-/-}$ PMNs spread on ICAM-1–coated surfaces, then fixed with methanol and stained with anti-aM. Original magnification, ×60. (**b**) Adhesion to ICAM-1–coated surfaces of wild-type and *Mylk*$^{-/-}$ PMNs challenged with LPS or not. *, $P < 0.05$, compared with no LPS; **, $P < 0.05$, compared with wild type after LPS stimulation. (**c**) Analysis of β_2 integrin activation by soluble ICAM-1 binding assay with (+) or without (−) 10 mM Mn^{2+}. ICAM-1–Fc, Fc-tagged ICAM-1. *, $P < 0.05$, compared with no stimulation; **, $P < 0.05$, compared with wild type after Mn^{2+} stimulation. (**d**) Flow cytometry of the surface expression of β_2 integrins on circulating wild-type and *Mylk*$^{-/-}$ PMNs. *, $P < 0.05$, compared with no LPS. Data are from one experiment representative of three (**a**) or are the mean of four independent experiments (**b–d**; error bars, s.e.m.).

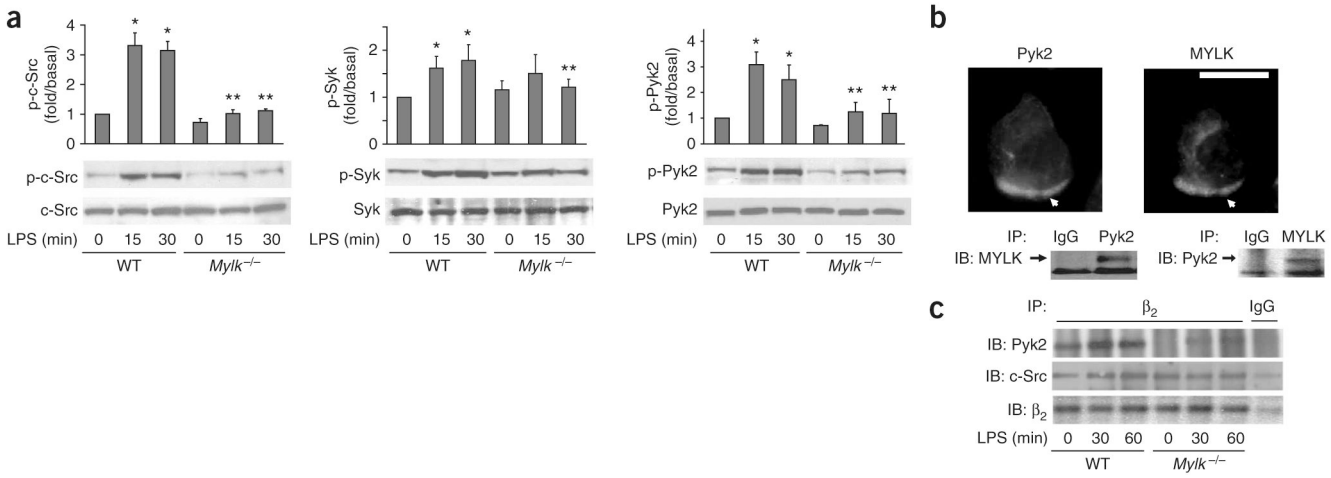

Figure 4. Tyrosine kinase activation and interaction with β_2 integrin

(**a**) Phosphorylation (p-) of c-Src, Syk and Pyk2 in wild-type and *Mylk*$^{-/-}$ PMNs. Bottom blots, confirmation of equal protein loading by analysis with anti-c-Src, anti-Pyk2 and anti-Syk. Above, densitometry analysis. *, $P < 0.05$, compared with no LPS; **, $P < 0.05$, compared with other groups after LPS stimulation. Data are the mean of four independent experiments (error bars, s.e.m.). (**b**) Colocalization of MYLK and Pyk2 in wild-type PMNs stimulated with fMLP and stained with anti-MYLK and anti-Pyk2 (above). Arrows indicate leading edges. Scale bar, 10 μm. Below, coimmunoprecipitation of MYLK and Pyk2 in wild-type PMNs. IP, immunoprecipitation; IB, immunoblot. Data are from one experiment representative of three. (**c**) Association of β_2 integrin with c-Src and Pyk2 in wild-type and *Mylk*$^{-/-}$ PMNs stimulated for 0, 30 or 60 min with LPS (1 μg/ml); equal amounts of protein immunoprecipitated from lysates with anti–β_2 integrin are analyzed by immunoblot with anti-Pyk2, anti-c-Src or anti–β_2 integrin. IgG, immunoglobulin G (control antibody). Data are representative of three independent experiments.

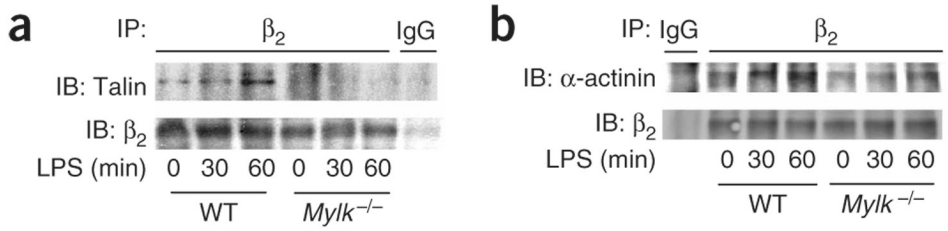

Figure 5. The interaction of β_2 integrin and the cytoskeleton in neutrophils

Association of β_2 integrin with the actin-associated proteins talin (**a**) and α-actinin (**b**) in wild-type and *Mylk*$^{-/-}$ PMNs stimulated for 0, 30 or 60 min with LPS (1 μg/ml); equal amounts of protein immunoprecipitated from lysates with anti–β_2 integrin are analyzed by immunoblot with anti-talin or anti-α-actinin. IgG, control antibody. Data are representative of three independent experiments.