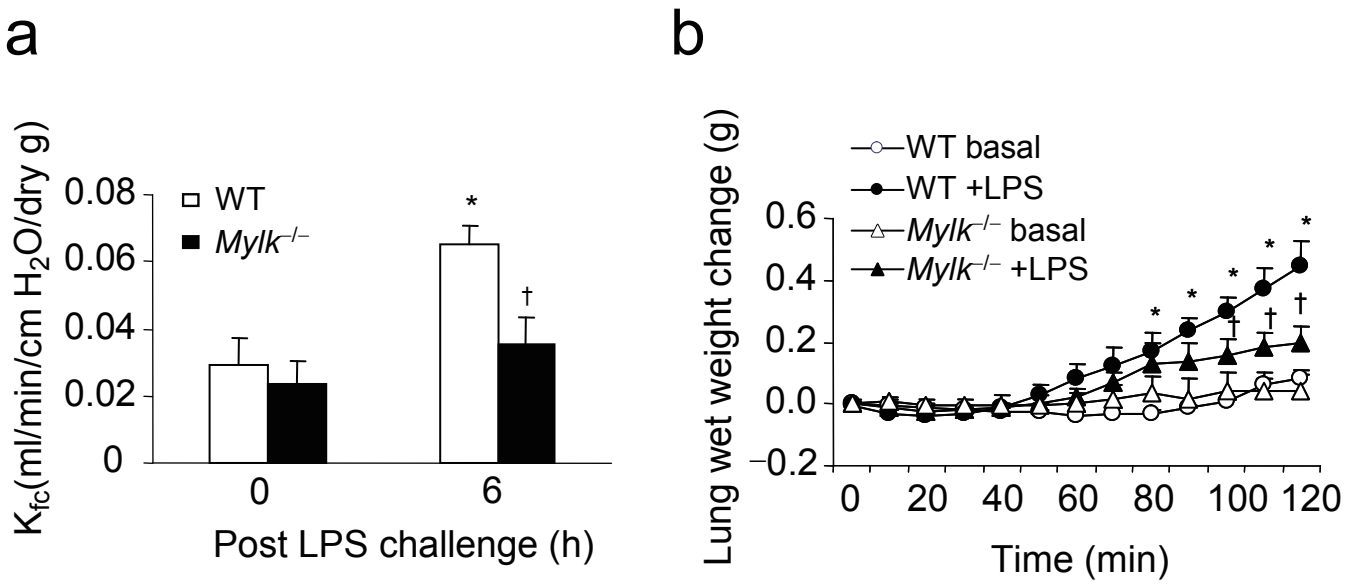
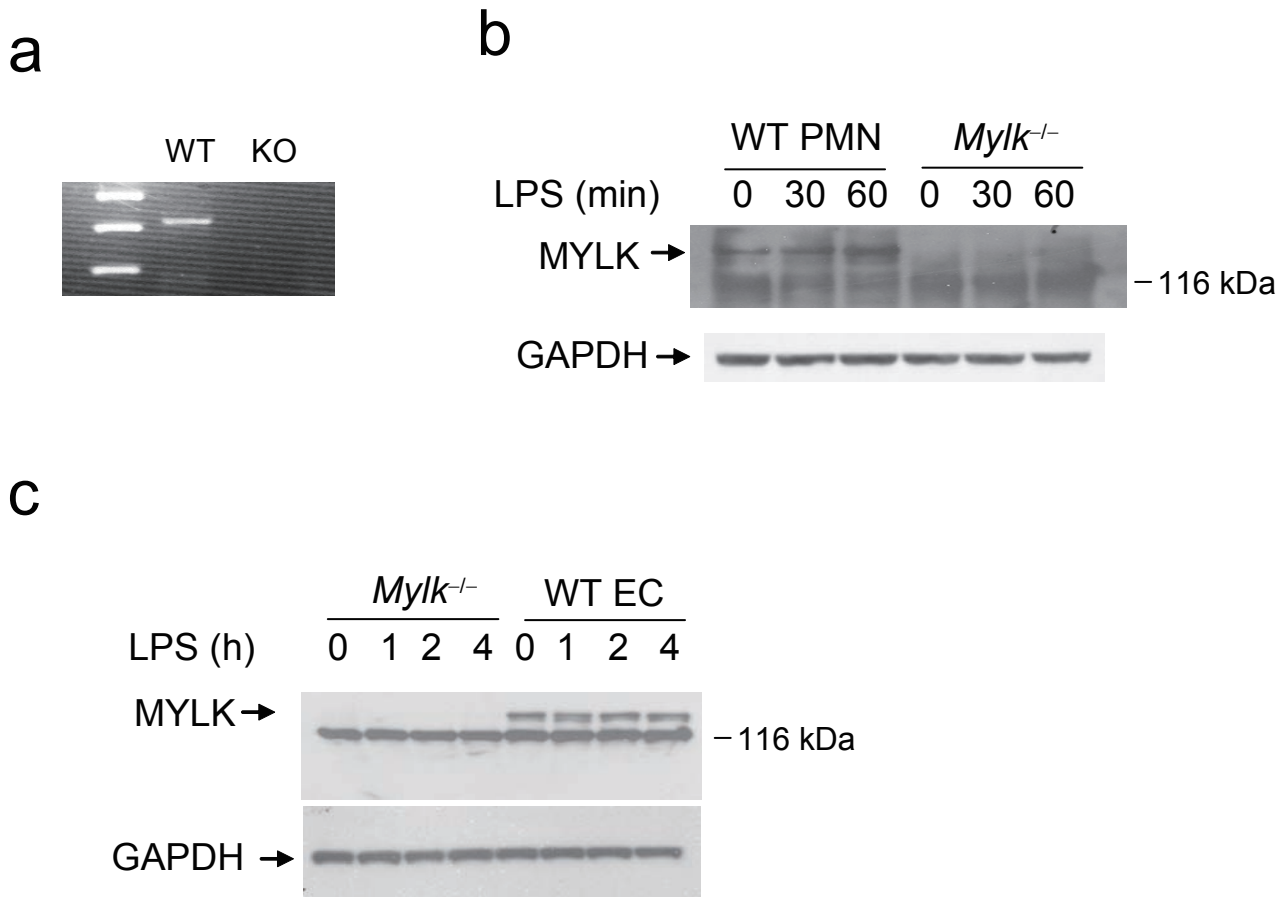
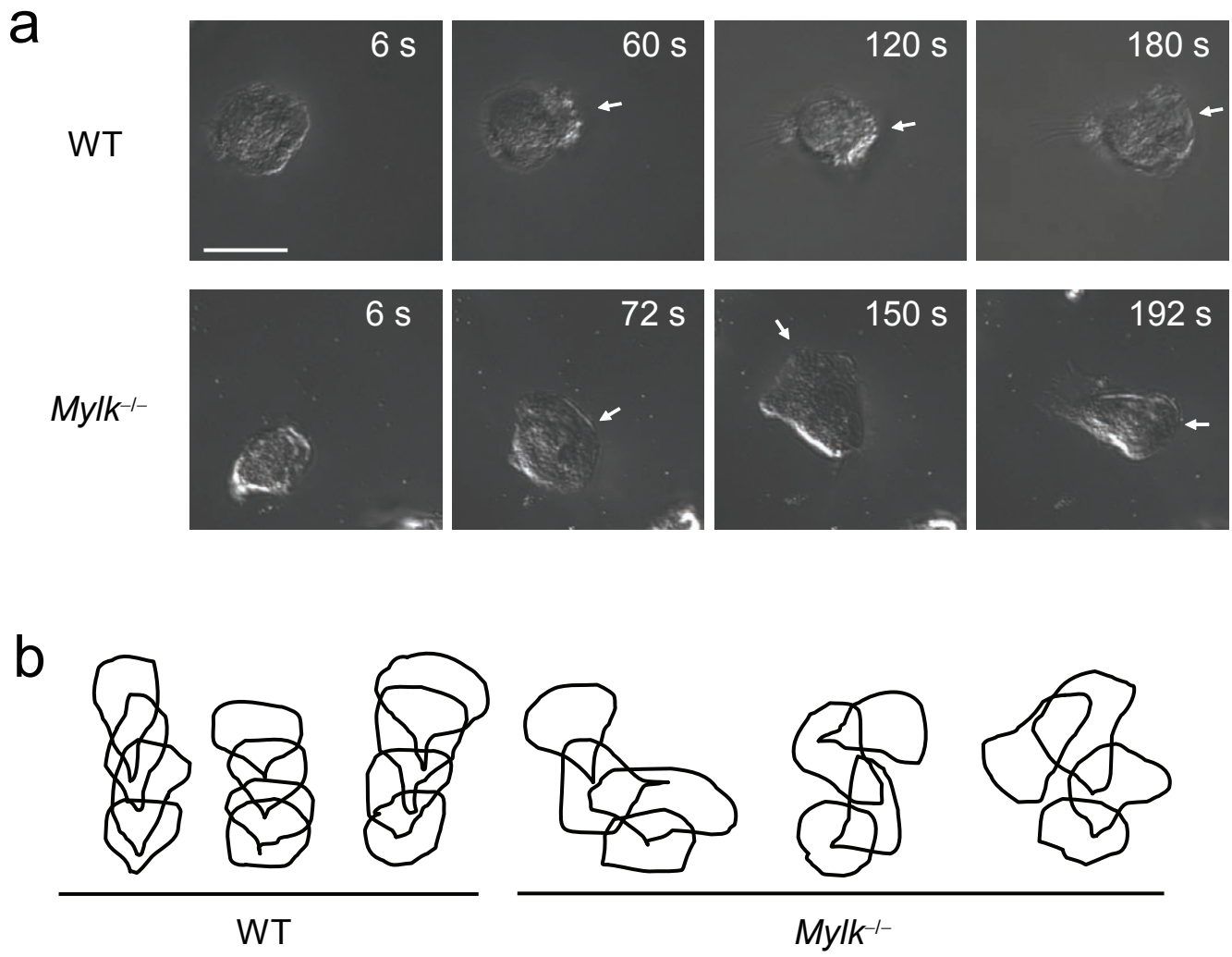
Xu et al. “Non-muscle myosin light chain kinase mediates neutrophil transmigration in sepsis-induced lung inflammation by the activation of β2 integrins”



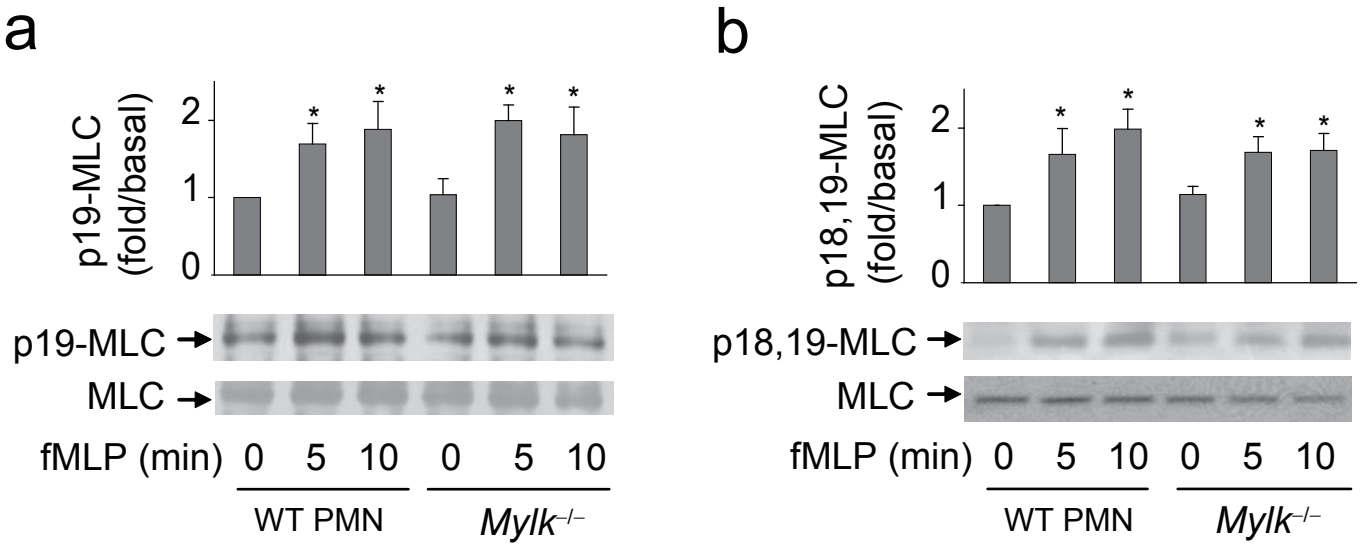
Supplementary figure 1. LPS-induced lung flammatory injury in vivo.
(**a**) Changes of WT and *Mylk*$^{-/-}$ mice in pulmonary microvessel permeability (measured as capillary filtration coefficient ($K_{f,c}$) in lungs isolated after LPS challenge.
(**b**) The changes of WT and *Mylk*$^{-/-}$ mice in formation of pulmonary edema (measured by increase in wet weight) of lungs isolated after LPS challenge. *, increase ($P < 0.05$) in $K_{f,c}$ and lung wet weight from basal value; †, denotes decreased ($P < 0.05$) $K_{f,c}$ and lung wet weight in *Mylk*$^{-/-}$ group compared to WT group. Results are mean of 5 independent experiments; bars indicate SEM.



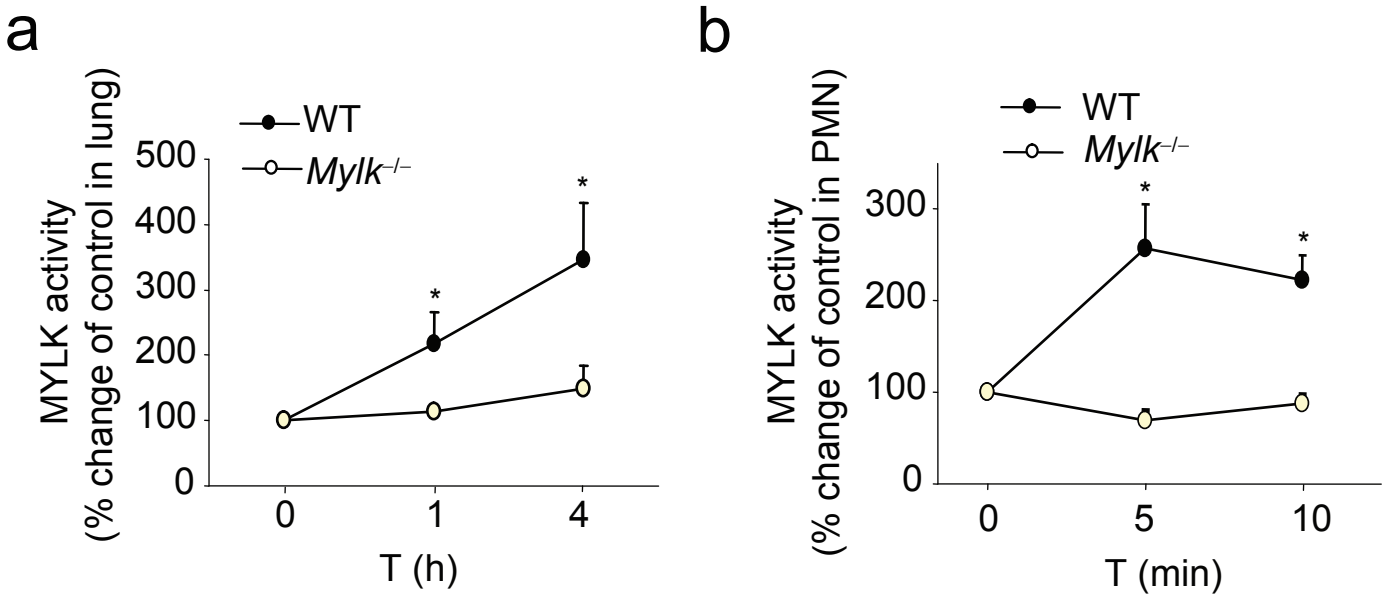
Supplementary Figure 2. Expression of the long isoform MYLK in WT and *Mylk*$^{-/-}$ mice. (**a**) MYLK transcript expression in WT and Mylk$^{-/-}$ mice. RT-PCR profile was compared with Gapdh. The experiment was repeated 3 times.
(**b, c**) Immunoblot analysis of PMNs and endothelial cells probed with anti-MYLK (Sigma). Equal protein loading was verified by membrane probing with GAPDH antibodies. Shown are results representative of 3-4 independent experiments.



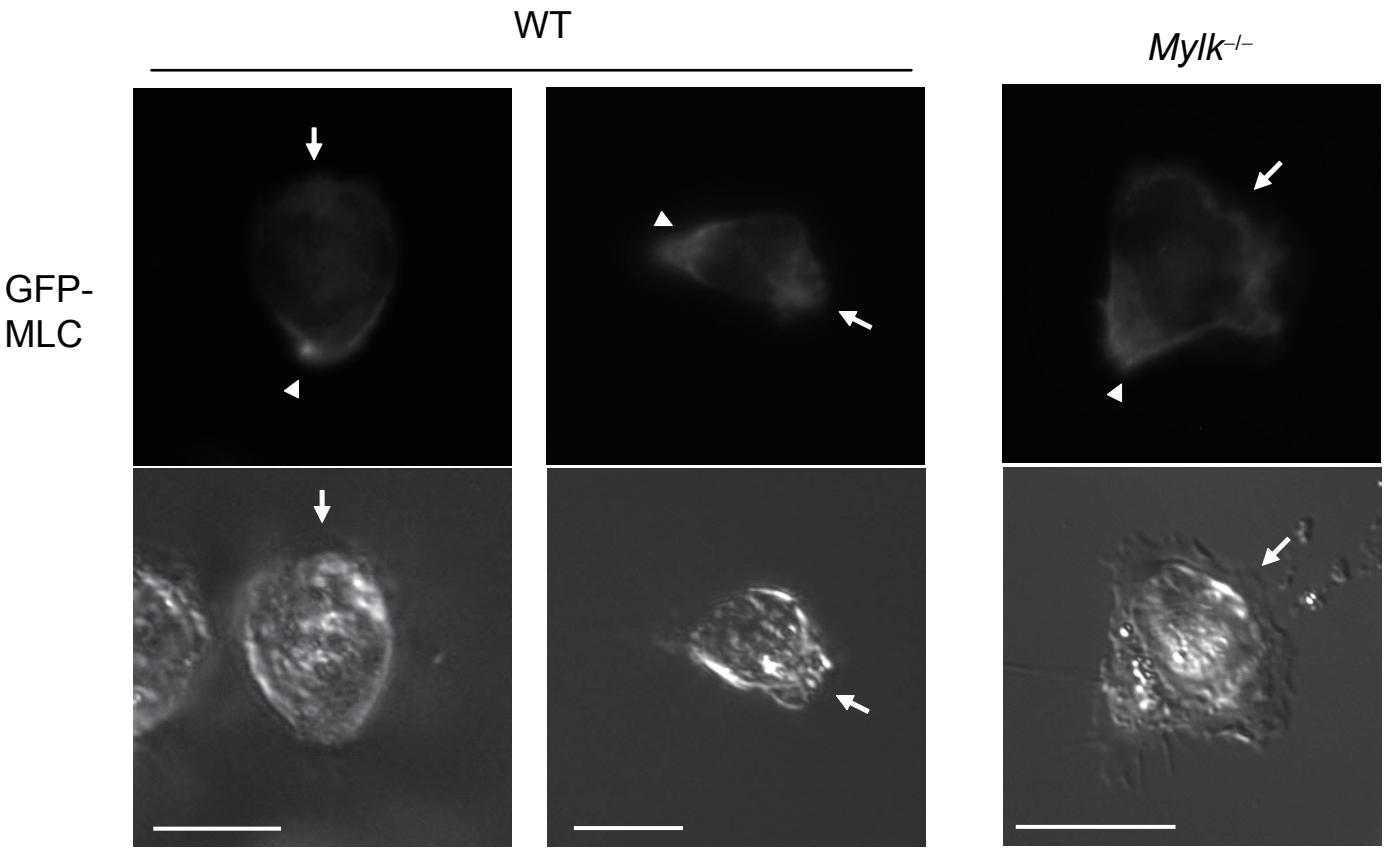
Supplementary Figure 3. (**a**) Pattern of migration of a single WT mouse PMN (top) and a single *Mylk*$^{-/-}$ PMN (bottom) stimulated with 100 nM fMLP. Arrows point to the leading edges. (**b**) Migration of WT and *Mylk*$^{-/-}$ PMNs in presence of 100 nM fMLP. Outlines indicate shape and location of the cell periphery at 1 min intervals; Bars, 10 μm. (**c**) Migration speed of WT and *Mylk*$^{-/-}$ PMNs on fibrinogen-coated coverslips in presence of 100nM fMLP. *, $P < 0.05$ compared to WT; results are mean of 3 independent experiments; bars indicate SEM.



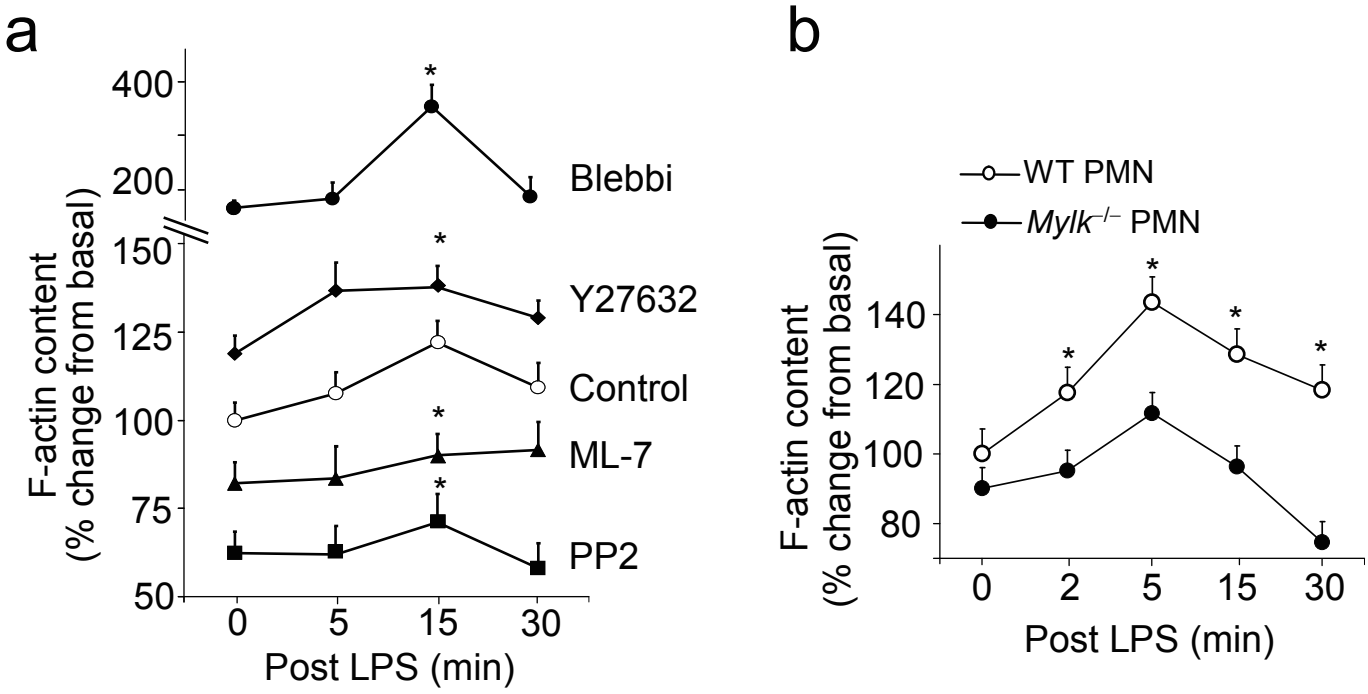
Supplementary Figure 4. MLC phosphorylation, p19-MLC (**a**) and p18,19-MLC (**b**) in WT and *Mylk*$^{-/-}$ PMNs. PMNs were stimulated with fMLP (100 nM) at 37 ^{o}C for indicated times. Equal protein loading was verified by membrane probing with MLC antibodies. *****, compared to no fMLP stimulation (basal value, $P < 0.05$). $n = 3$; results of one representative experiments are shown.



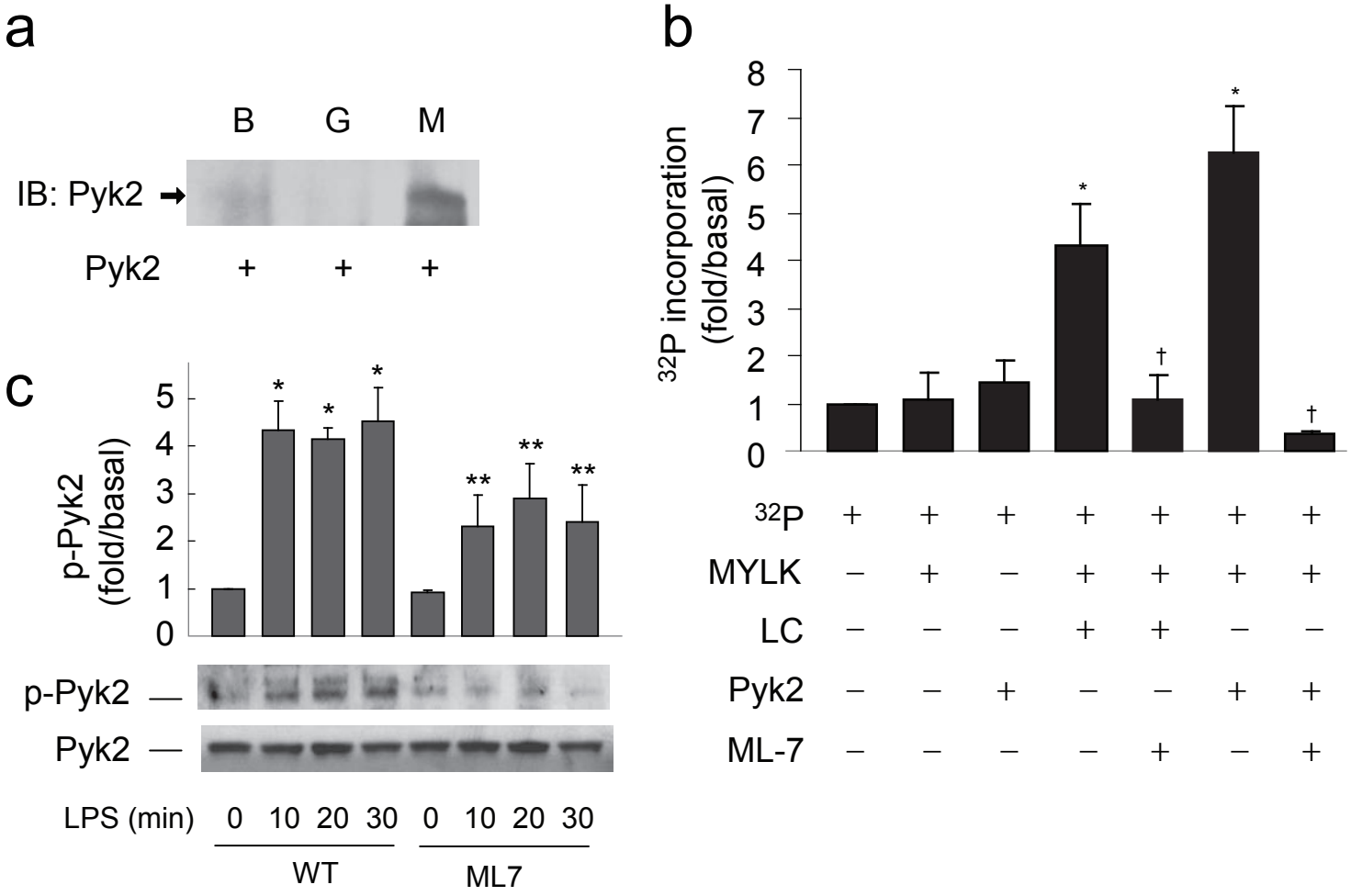
Supplementary Figure 5. MYLK kinase activity in lungs (**a**) and PMNs (**b**). Lungs isolated from WT or *Mylk*$^{-/-}$ mice treated with LPS (10 mg/kg, i.p.) or without (saline) for the indicated times. PMNs (2×10^6/ml) isolated from WT or *Mylk*$^{-/-}$ mice were challenged with LPS (1 μg/ml) or without (saline) at 37 °C for the indicated times. MYLK kinase activity was assayed in lysates by phosphorylation of substrate peptide (MLC, amino acids 11-23) in the presence of [γ-^{32}P] ATP for 10 min at 30° C. Results shown are mean ± SEM of triplicate measurements and of three separate experiments. *****, different from time 0; $P < 0.05$.



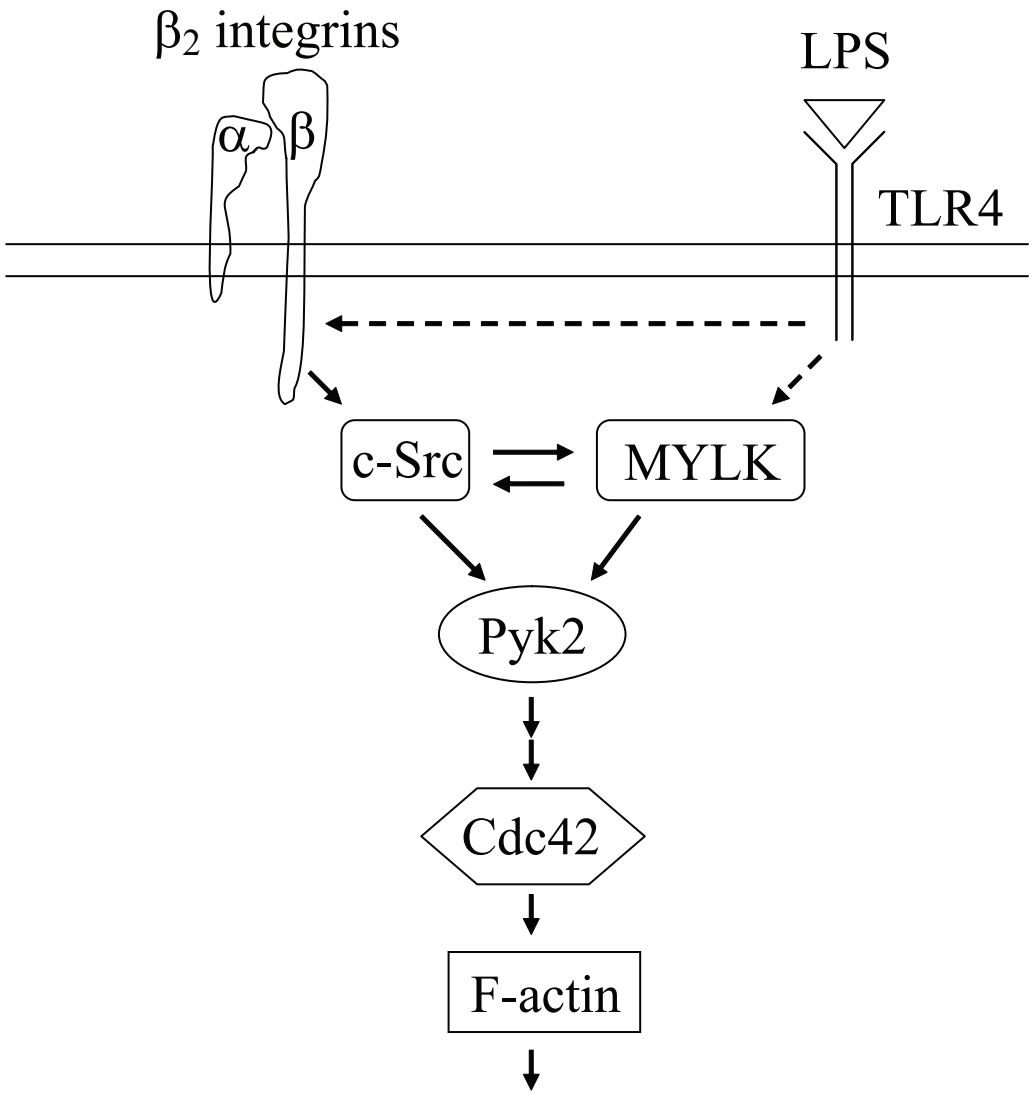
Supplementary Figure 6. Wild type and *Mylk*$^{-/-}$ PMNs were transfected with GFP-MLC and exposed to a uniform concentration (100 nM) of fMLP. Transfection with bone marrow mouse PMNs was performed on an AMAXA Nucleofector Device using manufacturer's suggested protocol. GFP fluorescence and the corresponding Nomarski images are shown. Arrows point to the leading edges and arrowheads point to the trailing edges of polarized PMNs. Bars, 10 μM. Shown are results representative of 3 independent experiments.



Supplementary Figure 7. (**a**) Actin polymerization in WT PMNs pre-treated with or without the inhibitors as indicated. Results are mean of 4 independent experiments. Bars indicate mean ± SEM. Blebbi, blebbistatin. (**b**) PMNs isolated from WT or *Mylk*$^{-/-}$ mice challenged with or without LPS. F-actin content was assayed as described in Methods. Results are mean of 4 independent experiments. Bars indicate mean ± SEM.



Supplementary Figure 8. (**a**) Purified Pyk2 protein was incubated with beads only (B), GST (G), or GST-MYLK (M) and blotted with anti-Pyk2 respectively. $n = 3$, results of a representative experiment are shown. (**b**) Phosphorylation of Pyk2 by MYLK in vitro. Purified Pyk2 or MYLK substrate (LC) was incubated with purified GST-MYLK (Gly1425-Ser1776) kinase in the presence or absence of ML-7. ^{32}P incorporation was measured. Results shown are mean ± SEM of three separate experiments. *, Different from basal; $P < 0.05$. †, inhibition of ML-7 compared to the similar group without inhibitor; $P < 0.05$. (**c**) Phosphorylation of tyrosine kinase Pyk2 in WT PMNs in the presence and absence of ML-7. PMNs were stimulated with LPS (1 μg/ml) at 37 ^{o}C for the times indicated. Equal loading was verified by probing with Pyk2 antibodies. *, increased ($P < 0.05$) p-Pyk2 compared to basal. **, decreased ($P < 0.05$) p-Pyk2 in ML-7 treated PMNs compared to no treated groups post-LPS stimulation. $n = 3$, results of a typical experiment are shown.



Supplementary Figure 9. Inside-out signals from the LPS receptor (TLR4) activate β_2 integrins to an intermediate active state, and induce β_2 integrins to bind to the extracellular matrix. Upon β_2 integrins-matrix binding, outside-in signals then activate the tyrosine kinase, c-Src. In addition to β_2 integrin-matrix binding, MYLK is required for tyrosine kinase c-Src activation. Both MYLK and c-Src further activate Pyk2, which is required for actin polymerization and the full high-affinity and high-avidity activation of β_2 integrins. It remains to be determined in this model how MYLK is activated (dashed lines). One possibility is that LPS binding to TLR4 activates ERK, which can thereby activate MYLK[2] or alternatively, MYLK may be directly phosphorylated and activated by c-Src[3].

Reference:

1. Tang, H. *et al.* Pyk2/CAKbeta tyrosine kinase activity-mediated angiogenesis of pulmonary vascular endothelial cells. *J Biol Chem* **277**, 5441-5447 (2002).
2. Klemke, R.L. *et al.* Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* **137**, 481-492 (1997).
3. Birukov, K.G. *et al.* Differential regulation of alternatively spliced endothelial cell myosin light chain kinase isoforms by p60(Src). *J Biol Chem* **276**, 8567-8573 (2001).