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Abnormal Migration Phenotype of Mitogen-Activated Protein Kinase-Activated Protein Kinase 2\(^{-/-}\) Neutrophils in Zigmond Chambers Containing Formyl-Methionyl-Leucyl-Phenylalanine Gradients\(^1\)

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Time-lapsed video microscopy and confocal imaging were used to study the migration of wild-type (WT) and mitogen-activated protein kinase-activated protein kinase 2 (MK2)\(^{-/-}\) mouse neutrophils in Zigmond chambers containing fMLP gradients. Confocal images of polarized WT neutrophils showed an intracellular gradient of phospho-MK2 from the anterior to the posterior region of the neutrophils. Compared with WT neutrophils, MK2\(^{-/-}\) neutrophils showed a partial loss of directionality but higher migration speed. Immunoblotting experiments showed a lower protein level of p38 mitogen-activated protein kinase and a loss of fMLP-induced extracellular signal-related kinase phosphorylation in MK2\(^{-/-}\) neutrophils. These results suggest that MK2 plays an important role in the regulation of neutrophil migration and may also affect other signaling molecules. The Journal of Immunology, 2001, 167: 3953–3961.

The three major mitogen-activated protein kinase (MAPK)\(^3\) pathways involved in various cellular responses are extracellular signal-related kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (1). The p38 MAPK pathway is activated by LPS (2), the proinflammatory cytokines IL1 and TNF-\(\alpha\) (3), chemotactic factors (4–6), and various stresses (2, 7). It has roles in many cellular processes, including inflammatory responses (8), mRNA stabilization (9), cell division (10), apoptosis (11), and cancer cell invasiveness (12). There are four known isoforms of p38 MAPK termed p38\(\alpha\), p38\(\beta\), p38\(\gamma\), and p38\(\delta\) (13–15). The p38 MAPKs are activated by the upstream kinases through threonine and tyrosine phosphorylation (16–18). The downstream substrates of p38 MAPK include a number of transcription factors such as activating transcription factor 2 (19), CHOP/GADD153 (20), muscle-specific transcription factor 2 (21), and p53 (22), as well as a number of protein kinases such as MAPK-activated protein kinase (MK) 2 (23), MK3 (24), MAPK interacting kinase 2 (25), p38 regulated/activated protein kinase (26), and mitogen- and stress-activated protein kinase (MSK) 1 (27).

Among these protein kinases, MK2 is the first identified and the best-studied substrate for p38 MAPK (28). Fully activated MK2 requires multiple sites of phosphorylation (29, 30). Within cells, MK2 specifically associates with p38 MAPK (31, 32). Two MK2 isoforms of 54 kDa and 46 kDa are known that vary in their C terminus (33). The N terminus of MK2 contains a proline-rich domain that may interact with src homology (SH)3 domain-containing proteins (34). The sequence of the C-terminal region of the p54 isoform of MK2 contains a nuclear localization signal and nuclear export signal (35). MK2 phosphorylates small heat shock protein (HSP) 27 (3), and induces dissociation of HSP27 multimers, thereby regulating the chaperone function of HSP27 and cellular resistance to oxidative stress (36, 37). MK2 also is involved in cytokine-induced mRNA stabilization by an AU-rich region-targeted mechanism (9). In addition to HSP27, other known substrates of MK2 include SRF (38), E47 (39), CREB (40), 5′-lipoxigenase (41), vimentin (42), myosin L chain (43), leukocyte-specific protein (LSP) 1 (44).

Neutrophils play a vital role in defense against infection and in a number of allergic and nonallergic tissue-damaging inflammatory reactions (45, 46). Rapid and transient activation of p38 MAPK and MK2 has been observed in neutrophils treated with chemotactic factors fMLP (4, 5, 6), platelet-activating factor (4), and TNF-\(\alpha\) (6). Treatment of neutrophils with the p38 MAPK \(\alpha\) and \(\beta\) inhibitor SB203580 or its analogs inhibits fMLP-induced MK2 activation (4–6), chemotaxis (4, 5, 6), superoxide production (47, 48), IL-8 expression (6), TNF-\(\alpha\)-induced adhesion (47), cell death (49), and inflammatory responses in vivo (50, 51). Recent work with MK2\(^{-/-}\) mice revealed that MK2 is essential for LPS-induced TNF-\(\alpha\) biosynthesis (52).

In this paper, we studied the migration of wild-type (WT) and MK2\(^{-/-}\) neutrophils in Zigmond chambers containing fMLP gradients. Confocal images of polarized WT neutrophils showed an intracellular gradient of phospho-MK2 from the anterior to the posterior regions of the neutrophils. Compared with WT neutrophils, the MK2\(^{-/-}\) neutrophils showed impaired directionality but...
Materials and Methods

Materials

MK2<sup>−/−</sup> mice were generated at Martin Luther University (Halle, Germany; Ref. 52). Breeding pairs of MK2<sup>−/−</sup> mice were transferred to the University of Connecticut Health Center and maintained in sterile microisolator cages on standard mouse chow in a 12-h light-dark cycle. C57BL/6J WT control mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in similar conditions. In each experiment, age- and sex-matched experimental and control mice were used. All experiments involving the mice were approved by the University of Connecticut Health Center Animal Care Committee.

The following materials were purchased: NIM2 reagents from Cardinal Associates (San Francisco, CA); crystalline BSA, lyssolecithin, normal goat serum, PMA, FMLP, fibrinogen, vitronectin, and thiglycollate from Sigma (St. Louis, MO); rhodamine-phalloidin, FITC-phalloidin, and Slowfade reagent from Molecular Probes (Eugene, OR); peroxidase and FITC-conjugated goat anti-rabbit immunoglobulin from Jackson Immunoresearch (West Grove, PA); rat monoclonal anti-GR1 from Accurate Chemical and Scientific (Westbury, NY); Percoll and [γ-<sup>32</sup>P]ATP from Amersham Pharmacia Biotech (Piscataway NJ); parafomaldehyde from Polysciences (Warrington, PA); Abs to p38, phospho-p38, ERK 1/2, and phospho-ERK 1/2 from New England Biolabs, (Beverly, MA); and HSP25 from Stessgen, (Victoria, British Columbia, Canada).

Isolation of neutrophils

Murine bone marrow neutrophils were prepared with Percoll gradients as described previously (53). In some experiments, mouse peripheral blood neutrophils were purified by using NIM2 reagents from anticoagulant (0.05 M EDTA)-treated blood obtained by cardiac exsanguination. In both cases, after isolation, neutrophils were suspended in Hanks buffer solution (0.14 M NaCl, 5.4 mM KCl, 1 mM Tris, 1.1 mM CaCl<sub>2</sub>, 0.4 mM MgSO<sub>4</sub>, and 1 mM HEPES, pH 7.2) containing 1 mg/ml BSA. Granulocyte differential counts were made by staining as analyzed by flow cytometry and detection of mRNA by RT-PCR for myeloperoxidase, elastase, cathepsin, lysozyme, lactoferrin, and gelatinase showed similar levels in WT and MK2<sup>−/−</sup> neutrophils (data not shown). There was no significant difference between the WT and MK2<sup>−/−</sup> mice in their neutrophil counts with either peripheral blood or bone marrow (data not shown).

Videomicroscopy of migrating neutrophils

Time-lapsed videomicroscopy was used to examine neutrophil movements in Zigmound chambers. In these chambers, neutrophils purified from peripheral blood were recorded crawling in the absence or presence of FMLP gradients. The microscope was equipped with differential interference contrast optics and a ×20 objective. Images were captured at 5-s intervals with a PXL-EEV37 CCD camera (Photometrics, Waterloo, Ontario, Canada) and Metamorph Imaging software (West Chester, PA). Individual cell tracings were made from the captured images. From these tracings, the final position of a neutrophil relative to its starting position was graphed. On these graphs, a positive X distance reflects travel up the gradient. Absolute Y values represents lateral deviation along the gradient.

Confocal microscopy of migrating neutrophils

For indirect immunofluorescence, neutrophils were fixed in Zigmound chambers by the careful removal of chemotaxis buffers and replacement with 2.4% parafomaldehyde in PBS for 15 min at 37°C (54). This was followed by incubation with a solution of 0.1% lyssolecithin in PBS containing 1% normal goat serum and Abs to the protein of interest (1/100 dilution). After 30 min at room temperature, the cells were washed and incubated in lyssolecithin/PBS containing FITC-conjugated goat anti-rabbit immunoglobulin and rhodamine-phalloidin. After this incubation, cells were washed and coverslips mounted with a drop of Slowade fade reagent. These samples were then examined using a Zeiss (Oberkochen, Germany) confocal imaging system with excitation wavelengths of 488 and 568 nm and emission filters to detect FITC (515–520 nm) and rhodamine (590 nm). The pinhole of the confocal microscope was set at 25 μm to minimize staining differences attributable to cell thickness (55). Measurements of staining intensity were obtained from original images using NIH Image software (http://rsb.info.nih.gov/nih-image).

F-actin polymerization assay

WT or MK2<sup>−/−</sup> neutrophils were stimulated with FMLP (10<sup>−5</sup> M) for the indicated times and stained with FITC-phalloidin for flow cytometric analysis to detect F-actin content as described (56). Analysis on a FACSScan cytometer (BD Biosciences, Mountain View, CA) was performed with a linear scale fluorescence channel (FL1). The level of F-actin polymerization stimulated by FMLP was determined by the increase of mean fluorescence over time zero.

Adhesion assay

Determination of neutrophil adhesiveness with or without FMLP (10<sup>−5</sup> M) or PMA (2.5 ng/ml) treatments was performed essentially as described (57). Briefly, bone marrow neutrophils (3 x 10<sup>4</sup> in 100 μl) were incubated (37°C in 5% CO<sub>2</sub> atmosphere) in wells of a microtiter plate previously coated with fibrinogen (100 μg/ml overnight at 4°C). At the indicated times, samples were removed and the plates inverted and centrifuged on Whatman (Clifton, NJ) #3 filter paper. The remaining cells were quantified by measuring membrane acid phosphatase. The 100% standard was determined by measuring the value of a sample (100 μl) of the original cell suspension.

In vitro MK2 kinase activity assay

Bone marrow-derived neutrophils from WT and MK2<sup>−/−</sup> mice were suspended in Hanks buffer. Cells were preincubated (5 min at 37°C) and then treated with buffer, 5 x 10<sup>−5</sup> M FMLP, or 0.1 μg/ml PMA for the indicated times. Stimulation was stopped by the addition of an equal volume of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% NP40, 150 mM NaCl, and 2 mM diisopropyl fluorophosphate) followed by incubation on ice for 20 min. Cell lysates were centrifuged (13,000 × g, 10 min) and 20 μl of the supernatants added to a kinase assay mixture (20 μl, 20 mM HEPES, pH 7.3, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 5 μM sodium orthovanadate, 5 μM okadaic acid, 2 mM DTT, 0.2 mM [γ-<sup>32</sup>P]ATP (10<sup>5</sup> cpm/pmol), and 1 μg HSP25). The phosphorylation reaction was allowed to proceed for 30 min at 37°C. Samples were analyzed by SDS-PAGE (10%), and phosphorylation of HSP25 was detected by autoradiography.

Peritoneal neutrophil influxes assay

Mice were injected i.p. (1 ml) with sterile solutions of thioglycollate (2.4%), FMLP (10<sup>−5</sup> M), or PBS. Four hours after the injections, the mice were sacrificed. The peritonea of WT and MK2<sup>−/−</sup> mice were rinsed (3 times), and total neutrophils recovered were counted.

Immunoblots

Neutrophils (2 x 10<sup>6</sup>) were lysed with sample buffer, boiled, and resolved in 10% PAGE. Samples then were analyzed as immunoblots with Abs to the proteins of interest (1:1000) followed by peroxidase-conjugated goat anti-rabbit immunoglobulin (1:5000). Bound Abs were visualized with the ECL detection system. In some experiments, immunoreactivity of the protein bands was determined by densitometric scanning with NIH Image software.

Results

Intracellular staining pattern of phospho-MK2

It recently has been found that signaling molecules involved in chemotaxis can be asymmetrically distributed in migrating cells (58, 59). Because p38 MAPK pathway may be involved in neutrophil chemotaxis (5, 6), we wondered whether an intracellular gradient of phospho-MK2 may also exist in migrating neutrophils. Thr<sup>317</sup> of the p54 isoform of MK2 (Thr<sup>314</sup> of the p46; Ref. 60) has been shown as the major phosphorylation site of p38 MAPK, and its phosphorylation is required for activation and nuclear export of MK2 (29, 34). We have previously prepared anti-phosphopeptide Abs that recognize the Thr<sup>317</sup>-phosphorylated form of MK2 in neutrophils stimulated by FMLP (56, 60). We used these antiphospho-MK2 Abs to examine the intracellular staining patterns of phospho-MK2 in polarized WT mouse neutrophils. Phospho-MK2 was found to be colocalized with F-actin in the leading front of the
polarized neutrophils (Fig. 1A, middle). When measured from anterior to posterior (Fig. 1B, top, a and b), the intensity of phospho-MK2 staining decreased rapidly toward the posterior region, showing a pattern of intracellular gradient. When examined laterally (Fig. 1B, top, c and d), no internal gradient of phospho-MK2 staining was observed. The results indicate that phospho-MK2 is primarily found in the lamelipodia regions of the neutrophil. Intracellular F-actin staining of polarized WT neutrophils showed a decrease of staining from the anterior to posterior regions, although not as dramatic as the phospho-MK2 (Fig. 1B, bottom). No phospho-MK2 staining was observed in the confocal images of MK2−/− neutrophils (Fig. 1A, bottom).

**fMLP-induced phosphorylation and activation of MK2 in murine neutrophils**

The antiphospho-Thr317 MK2 Abs and an in vitro kinase assay were used to demonstrate that stimulation with fMLP activates MK2 in murine neutrophils. The Abs to phospho-Thr317 MK2 were used in immunoblots and showed that stimulation of murine...
bone marrow neutrophils with fMLP caused a time-dependent increase in the phosphorylation of the p46 and p54 protein bands (Table I and Fig. 1C). These two protein bands correspond to the two isoforms (M, 46,000 and M, 54,000) of MK2 in mouse (34, 52). Stimulation with PMA (10 min) also induced increased phosphorylation of p46 and p54 (Table I and Fig. 1C). No phospho-MK2 immunoreactivity was observed in MK2−/− neutrophils (Fig. 1C). The in vitro MK2 kinase assay with mouse HSP25 as a substrate demonstrated a large increase in MK2 activity in the cell lysates of fMLP- or PMA-stimulated WT but not MK2−/− neutrophils (p < 0.005, n = 3; Fig. 1D). These results demonstrate that fMLP induces the phosphorylation and activation of MK2 and suggest that MK2 is the major kinase for HSP25 in mouse neutrophils.

**ERK and p38 MAPK pathways in MK2−/− neutrophils**

It has been known that fMLP and PMA stimulate both p38 MAPK (4, 5, 6) and ERK (57) pathways in human neutrophils. We examined both kinases in WT and MK2−/− mouse neutrophils on fMLP and PMA stimulation. Phosphorylation of ERK (p42/44) was observed in WT neutrophils stimulated with fMLP or PMA (Fig. 2A, left). Compared with WT neutrophils, the phosphorylation of ERK was greatly reduced when stimulated by fMLP and partially reduced when stimulated by PMA (Fig. 2A). This is not attributable to a low protein level of ERK in the MK2−/− neutrophils, as the protein levels of ERK present in both WT and MK2−/− neutrophils were similar (Fig. 2A, right). This suggests that MK2 is required for activation of upstream signaling molecules involved in ERK phosphorylation stimulated by fMLP.

MK2 and p38 MAPK are intracellular binding partners (31, 32, 33), so we compared the phosphorylation and protein levels of p38 MAPK in WT and MK2−/− neutrophils. A time-dependent increase in p38 MAPK phosphorylation was observed after fMLP or PMA stimulation of WT neutrophils (Fig. 2B, left). Compared with WT neutrophils, a lower level of p38 MAPK phosphorylation increase was observed in fMLP- or PMA-stimulated MK2−/− neutrophils (Fig. 2B left). Immunoblots with Abs to the nonphospho-p38 MAPK showed that the lower level of phospho-p38 MAPK may be attributable to the lower protein level of p38 MAPK in the MK2−/− neutrophils (69 ± 5.6% of WT; Fig. 2B, right). These results suggest that MK2 may regulate the intracellular protein level of p38 MAPK.

**Migration of MK2−/− neutrophils**

The intracellular staining pattern of MK2 in neutrophils in fMLP gradients (Fig. 1A) suggests that MK2 may be involved in regulating neutrophil chemotaxis. To examine chemotaxis, in vitro, time-lapsed video microscopy was used to analyze the migration of WT and MK2−/− neutrophils on BSA-coated coverslips in fMLP gradients as described by Zigmond (61). MK2−/− neutrophils migrated faster (49 μm/min ± 12.7, n = 86 vs WT 29 μm/min ± 5.4, n = 55) but much less directionally toward fMLP than WT neutrophils (54 vs 100% WT, Fig. 3A). Similar results were obtained when the mouse chemokine KC was used as the chemoattractant (not shown). Without a fMLP gradient, the random migration speed of MK2−/− neutrophils is also higher than WT (8.0 ± 3.7 vs 3.6 ± 1.4 μm/min).

The speed of cell migration is influenced by the substrate on which the cell crawls. Therefore, we examined the migration speeds of WT and MK2−/− neutrophils on various substrate molecules. Both WT and MK2−/− neutrophils showed reduced migration speeds in fMLP gradients when coverslips coated with fibrinogen or vitronectin instead of BSA were used for the chemotaxis assays (Fig. 3A). However, when using fibrinogen or vitronectin coated coverslips, the MK2−/− neutrophils still showed higher migration speed (Fig. 3B) and less directionality toward fMLP than did WT neutrophils (data not shown), similar to what was observed with BSA-coated coverslips (Fig. 3A). These results suggest the abnormal migration phenotype of MK2−/− neutrophils is not dependent on the substrates used in the assays.
Morphological examination of migrating WT and MK2−/− neutrophils

We examined the morphology of WT and MK2−/− neutrophils to better understand the nature of the chemotactic defects observed in vitro. Tracings of the boundaries of individual migrating neutrophils indicated that MK2−/− neutrophils, in fMLP gradients, extended their leading fronts further than WT at each time interval (50 s; Fig. 4A). In addition, the MK2−/− neutrophils had twice as many membrane protrusions as WT and showed lateral and rearward protrusions that were not detected in WT (Table II).

The morphologies of WT and MK2−/− neutrophils fixed during migration in fMLP gradients were examined by F-actin staining with confocal microscopy. The WT neutrophils showed polarized morphologies with heads (lamelipodia) and tails (Fig. 4B, left). The MK2−/− neutrophils showed polarized cell bodies but had multiple protrusions, especially side protrusions and dramatic misalignments of their heads and tails (Fig. 4B, right). These results suggest that MK2 regulates cell morphology during chemotaxis.

Adhesion of WT and MK2−/− neutrophils to fibrinogen

Adhesion strength can affect migration speed, so WT and MK2−/− neutrophil adhesiveness was quantitated with fibrinogen as a substrate. Compared with WT, MK2−/− neutrophils showed slightly reduced fMLP-stimulated adherence (25.6 ± 6.4% MK2−/− vs 33.1 ± 7.7% WT at 60 min; Fig. 5A). Adherence induced by PMA was not reduced in MK2−/− neutrophils (66.0 ± 6.4% MK2−/− vs 63.1 ± 10.0% WT at 60 min; Fig. 5B). These results suggest that the abnormal migration phenotype of MK2−/− neutrophils described above is not attributable to a large change of adhesiveness induced by fMLP.

FACs analysis of fMLP-induced F-actin polymerization of WT and MK2−/− neutrophils

To ensure that the fMLP receptor was functional on MK2−/− neutrophils, the F-actin polymerization response to fMLP was examined. Both the rate and extent of F-actin polymerization in fMLP-stimulated WT and MK2−/− neutrophils are similar when assayed by FACs (Fig. 6).

Neutrophil influxes

To evaluate the migration phenotype in vivo, mice were injected with chemotactic stimuli. Injections of fMLP but not thioglycollate into the peritoneal cavities of MK2−/− mice resulted in the influxes of higher numbers of peritoneal neutrophils (4 h after injection) when compared with WT mice (5.94 ± 0.86 × 106 MK2−/− (n = 4) vs 1.86 ± 0.25 × 106 WT (n = 4) with fMLP injection; Fig. 7).

Discussion

During cell migration, intracellular signals are activated via surface receptors at the leading edge (62, 63). Recent studies that fluorescently tagged the G-protein Gβ (59) and the plekstrin homology domain of kinase AKT/protein kinase B (58) shows that...
these signaling molecules are localized to regions of the cell receiving the strongest chemotactic signals. These regions also contain pseudopods and are the sites of actin polymerization. Therefore, it has been suggested that these molecules are part of the compass that directs neutrophil movement (64). In this study, we observed that in WT neutrophils, phospho-MK2 colocalized with F-actin and formed an intracellular gradient during neutrophil chemotaxis in fMLP gradients. Additionally, the loss of MK2 resulted in the loss of directionality during migration. Therefore, phospho-MK2 may be part of the compass directing the neutrophil during chemotaxis.

In addition to a loss of directionality, MK2/−/− neutrophils showed a higher migration speed than WT neutrophils. Similar to what we observed, microinjection of a dominant-negative Cdc42 mutant into Bacl.2F5 macrophages enhances migration speed but eliminates polarization during chemotaxis toward colony stimulating factor-1 (65). Additionally, mice lacking the nonreceptor tyrosine kinases Abl and Arg showed that these kinases negatively control cell migration through the regulation of Crk and CAS adapter protein complexes (66).

The control of directionality and migration speed may be related. MK2 may regulate speed and directionality through its unique primary structure and the phosphorylation of its cytoskeletal substrates. The N terminus of MK2 contains a proline-rich domain that may interact with SH3 domain-containing proteins localized to the actin cytoskeleton. Previously, we identified LSP1, an F-actin cross-linking protein, as a major substrate for MK2 in neutrophils (44). A recent study showed that the expression levels of LSP1 could modulate the migration speed of monocyte-differentiated U937 cells (67). We speculate that MK2 may regulate both directionality and migration speed by controlling the phosphorylation of an F-actin cross-linking protein such as LSP1. A weaker cross-linked F-actin cytoskeleton may have a less stiff membrane, which will produce more membrane protrusions during migration.

Table II. Protrusive activity of fMLP stimulated WT and MK2/−/− neutrophils

| Neutrophil Genotype | Average No. of Protrusions per Neutrophil | Forward Protrusions (%) a | Lateral Protrusions (%) a | Rearward Protrusions (%) a |
|---------------------|------------------------------------------|--------------------------|-------------------------|--------------------------|
| WT                  | 0.9 ± 0.3                                | 100                      | 0                       | 0                        |
| MK2/−/−             | 2.1 ± 0.9                                | 22.0 ± 0.9               | 52.1 ± 0.8              | 25.0 ± 0.9               |

a The direction of protrusions was determined by the angle of the protrusion in relation to the source of fMLP. Forward, 315–45°; lateral, 46–135° or 226–314°; rearward, 136–225° (75).
However, the multiple membrane protrusions in chemokine KC gradients (68). Normal fMLP-induced actin polymerization in WT and MK2 protein level in the cell, perhaps through their binding in-neutrophils. This suggests that MK2 may help maintain p38 fMLP- and PMA-induced adhesion of WT and MK2−/− neutrophils. The development of MK2−/− neutrophils (Table I). This could also explain the appearance of MK2−/− neutrophils in confocal images, characterized by multiple protrusions and the dramatic misalignment of fronts and tails (Fig. 4B). Recently we reported that LSP1−/− neutrophils have reduced directionality and multiple membrane protrusions in chemokine KC gradients (68). However, the LSP1−/− neutrophils have a reduced migration speed. This suggests that other potential cytoskeletal substrates for MK2 such as myosin L chain (43), vimentin (42), and HSP25 (37, 38) also may be important.

The protein level of p38 MAPK was reduced in the MK2−/− neutrophils. This suggests that MK2 may help maintain p38 MAPK protein level in the cell, perhaps through their binding interactions (33). MK2 also may regulate the stability or expression of p38 MAPK mRNA. In contrast to the finding of a reduced level of p38 MAPK, a reduction of ERK protein level was not observed in MK2−/− neutrophils. However, the phosphorylation of ERK induced by fMLP was greatly reduced in the MK2−/− neutrophils. Recent studies indicate that ERK can be activated by RAS/RAF/mitogen-activated protein/extracellular signal-related kinase kinase (MEK) pathway as well as AKT/PAK/MEK pathway (69, 70). mitogen-activated protein/extracellular signal-related kinase kinase In human neutrophils, it has been shown that MEK2 is the major upstream kinase of ERK (71). Further work is required to study the effects of MK2 knockout on the low protein level of p38 MAPK and the loss of fMLP-induced ERK phosphorylation.

Treatment of neutrophils with SB203580 or its analogs has been shown to inhibit neutrophil fMLP-induced MK2 activation and chemotaxis as assayed by Boyden chambers (4, 5, 6). The downstream targets of p38 MAPK include MK2 (23), MK3 (24), MNK2 (25), PRAK (26), and MSK1 (27). If MK2 is the major downstream target of p38 MAPK for regulating neutrophil migration, one would expect a similar phenotype between MK2−/− and SB203580-treated WT neutrophils. In this study, we observed that the phenotype of MK2−/− neutrophils undergoing chemotaxis in Zigmond chambers containing fMLP gradients showed a partial loss of directionality but enhanced migration speeds. Several possibilities need to be considered. First, in MK2−/− neutrophils a loss of fMLP-induced ERK phosphorylation was observed. In contrast, SB203580 does not inhibit fMLP-induced ERK phosphorylation (72). The MEK inhibitor PD98059 does not inhibit neutrophil chemotaxis (73), suggesting that ERK activation is not required for chemotaxis. Second, in addition to MK2, other kinase substrates (MK3, MNK2, PRAK and MSK1) of p38 MAPK may also be involved in chemotaxis. One of them, MK3, is known to also phosphorylate HSP25 (74). However, the near-complete loss of HSP25 kinase activity in MK2−/− neutrophils (Fig. 1C) suggests that MK3 is not a major HSP25 kinase in mouse neutrophils. Third, the structure of MK2 contains a proline-rich domain that may bind to SH3 domain-containing molecules. A complete loss of MK2 may have results different from simply inhibiting the activation of MK2. Finally, a partial loss of p38 MAPK protein was observed in MK2−/− neutrophils. We cannot rule out that this may alter the phenotypes of MK2−/− neutrophils. The development of MK2−/− neutrophils appears to be normal as shown by adhesion assay (Fig. 5), fMLP-induced F-actin polymerization (Fig. 7), and FACs analysis of GR1 expression (data not shown).

We saw no difference in the rate or extent of actin polymerization in neutrophils stimulated with fMLP and analyzed by FACs. However, we did observe an abnormal migration phenotype of MK2−/− neutrophils in Zigmond chambers containing fMLP gradients. This suggests that migration speed and direction are not
determined solely by actin polymerization. In fact, fibroblasts from gelsolin−/− mice have reduced motility despite increased levels of F-actin (76). It is likely that cross-linking of F-actin filaments and intracellular calcium levels (77) also play roles in controlling neutrophil speed. Compared with WT mice, MK2−/− mice showed higher influxes of neutrophils into their peritoneal cavities after fMLP injection. In Zigmond chambers containing fMLP gradients, MK2−/− neutrophils migrated with higher speed but less direction. The discrepancy between these in vitro and in vivo results could be attributable to 1) in vivo the peritoneal cavity is a large target area and the increase in migration speed compensates for the lack of directionality; and 2) neutrophil chemotaxis in two-dimensional Zigmond chambers in vitro may be different from the in vivo transmigration across endothelial cells followed by migration through the three-dimensional intercellular matrixes.

Originally, MK2 was found to be involved in the cellular responses to stress (7, 2), bacterial LPS (2), and the proinflammatory cytokines IL-1 and TNF-α (3). Our results indicate that MK2 plays a regulatory role in IMLP-induced neutrophil migration and may also affect other signaling molecules.

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