Distinct Functions of the Mitogen-activated Protein Kinase-activated Protein (MAPKAP) Kinases MK2 and MK3

Mk2 Mediates Lipopolysaccharide-Induced Signal Transducers and Activators of Transcription 3 (Stat3) Activation by Preventing Negative Regulatory Effects of Mk3

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In LPS-treated macrophages, activation of Stat3 is considered to be crucial for terminating the production of inflammatory cytokines. By analyzing the role of MAPK-activated protein kinase (MK) 2 and MK3 for LPS-induced Stat3 activation in macrophages, the present study provides evidence that MK2 is crucial for Stat3 activation in response to LPS because it prevents MK3 from impeding IFNβ gene expression. Accordingly, LPS-induced IFNβ gene expression is down-regulated in MK2-deficient macrophages and can be reconstituted by additional ablation of the MK3 gene in MK2/3-/- macrophages. This is in contrast to LPS-induced IL-10 expression, which essentially requires the presence of MK2. Further analysis of downstream signaling events involved in the transcriptional regulation of IFNβ gene expression suggests that, in the absence of MK2, MK3 impairs interferon regulatory factor 3 protein expression and activation and inhibits nuclear translocation of p65. This inhibition of p65 nuclear translocation coincides with enhanced expression and delayed degradation of IκBα, whereas expression of IκBα mRNA and protein is impaired in the absence of MK2. The observation that siRNA directed against IκBα is able to reconstitute IκBα expression in MK2/3-/- macrophages suggests that enhanced expression and delayed degradation of IκBα and impaired NFκB-dependent IκBα expression are functionally linked. In summary, evidence is provided that MK2 regulates LPS-induced IFNβ expression and downstream Stat3 activation as it restrains MK3 from mediating negative regulatory effects on NFκB- and interferon regulatory factor 3-dependent LPS signaling.

Recognition of pathogen invasion by macrophages is mediated via activation of receptor complexes specialized to detect pathogen-derived molecular patterns. These receptors represent a front line recognition system required to initiate innate immunity.

In macrophages, LPS induces an inflammatory response via binding to the TLR4/MD2 receptor complex. Subsequently, the activated TLR4 receptor complex elicits its biological actions via activation of an intracellular signaling network that involves activation of the IKK/IκBα/NFκB cascade, of IRF3 or of members of the MAPK family such as p38 MAPK (2). These different pathways are interlinked in the control of the expression of inflammatory gene products at different levels including the level of transcription, mRNA stability, translation, post-translational modification, and protein stability. In macrophages, the activation of Stat3 is crucial for terminating the production of inflammatory cytokines (3, 4), in particular because of its role as the key mediator that transmits the anti-inflammatory effects of IL-10 (5, 6).

Type I interferons (IFNα/β) have been identified as crucial mediators of the LPS-induced production of IL-10 (7), indicating that they are crucial for launching anti-inflammatory effects on macrophages. Consistently, IFNβ-deficient macrophages show an enhanced inflammatory response toward LPS (8). p38 MAPK-mediated activation of MK2 and MK3 is essential for the production of several LPS-induced inflammatory cytokines such as TNFα (9, 10). In addition, upon activation by LPS or TNFα, the p38 MAPK/MK2 cascade also suppresses Stat3 activation by cytokines such as IL-6 (11) via induction of SOCS3 (11–13), warranting that sustained Stat3 activation occurs only in the presence of IL-10, which, in contrast to IL-6, is insensitive toward the inhibitory effects of LPS (14). Congruently, in SOCS3-deficient macrophages IL-6 indeed mediates IL-10-like anti-inflammatory effects (15). This suggests that in macrophages the p38 MAPK/MK2/3 pathway holds a key position in propagating the inflammatory response, because it mediates inflammatory cytokine expression and prevents cytokines such as IL-6 from untimely activating anti-inflammatory signals such as sustained activation of Stat3.

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2 The abbreviations used are: IRF, interferon regulatory factor; BMDM, bone marrow-derived macrophage(s); IFNAR, interferon-α/β receptor; M-CSF, macrophage colony-stimulating factor; IRES, internal ribosome entry site; SDHA, succinate dehydrogenase.
Role of MK2 and MK3 for STAT3 Activation

The present study analyzes the role of MK2 and MK3 for LPS-induced STAT3 activation in macrophages revealing unexpected and novel details on the function of MK3 for the regulation of IFNβ and 1α/β expression. The present manuscript for the first time demonstrates that MK2 and MK3, which so far appeared as closely related, cooperatively acting isoenzymes (10, 16), also have different regulatory roles, with MK2 regulating gene expression by preventing MK3-mediated negative regulatory effects on signaling.

EXPERIMENTAL PROCEDURES

Materials—LPS from *Escherichia coli* 0127:B8 was from Sigma-Aldrich (Munich, Germany); recombinant murine IFNβ was from PBL InterferonSource (Piscataway, NJ); recombinant murine and human IL-6 as well as recombinant murine and human M-CSF were purchased from PeproTech (Hamburg, Germany); oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany); SYBR Green PCR Master Mix was from Applied Biosystems (Darmstadt, Germany); QuantiTect reverse transcription kit was from Qiagen; DMEM supplemented with 1000 g/liter glucose, pyruvate, and GlutaMAX was from Invitrogen (Darmstadt, Germany); penicillin/streptomycin and trypsin/EDTA were from Cytogen (Sinn-Fleisbach, Germany); and FCS was from Perbio Sciences (Bonn, Germany); Dynabeads M-280 streptavidin and Dynal MPC-S were purchased from Invitrogen. The following antibodies were used: monoclonal antibody against GAPDH (Biodesign, Saco, ME) and β-actin (Abcam, Cambridge, UK), polyclonal antibodies specifically recognizing IκBα, lamin A/C, p65 phosphorylated at Ser-536, IRF3 phosphorylated at Ser-396, MK2, MK2 phosphorylated at Thr-222 (also detects human MK3 if phosphorylated at residue Thr-200), p38MAPK phosphorylated at the residues Thr-180 and Tyr-182, STAT3 phosphorylated at Tyr-705 or total murine IRF3 were from Cell Signaling (Frankfurt, Germany); polyclonal antibody against IκBβ (C-20) was from Delta Biolabs (Gilroy, CA) and against SOCS3 was from IBL (Hamburg, Germany); monoclonal antibody against MK3 was from Bethyl Laboratories (Montgomery, TX); polyclonal antibodies against human IRF3 (FL-425), p38MAPK (C-20), p65 (C-20), and STAT3 (C-20) were from Santa Cruz (Heidelberg, Germany); and HRP-conjugated goat anti-rabbit and rabbit anti-mouse were from Dako (Hamburg, Germany).

Immortalized Macrophage Cell Lines—Immortalized murine macrophages were established from bone marrow-derived macrophages (BMDM) that were isolated from mice deficient for MK2 (MK2/−/− MΦ), for both MK2 and MK3 (MK2/3/−/− MΦ), or from wild type animals (WT MΦ) by retroviral infection with a virus encoding both the v-Raf and the v-Myc oncoproteins as described recently (16). The resulting immortalized macrophage populations were 91% (±2.7) positive for the macrophage-specific marker F4/80 and for CD11b as assessed by FACS analysis (data not shown) without significant differences between macrophages derived from wild type animals and those derived from MK2/−/− or MK2/3/−/− animals. The cells were cultivated in DMEM including 1000 mg/liter glucose with glutamax supplemented with 10% heat-inactivated FCS and incubated in a thermoincubator (37 °C and 5% CO2), and medium was changed every day. For the experiments, the cells were seeded on 60- or 100-mm dishes with a final confluence of ~50%.

Preparation and Cultivation of Primary Murine Bone Marrow-derived Macrophages—Mice deficient for MK2, MK3, MK2/MK3, or IFNAR; their genotyping; and maintenance of the colony have been described elsewhere (9, 16–18). To generate WT BMDM, IFNAR-deficient (IFNAR−/− BMDM), MK2-deficient (MK2−/− BMDM), MK3-deficient (MK3−/− BMDM), and BMDM deficient for both MK2 and MK3 (MK2/3−/− BMDM), bone marrow cells (~2 to 3 × 107) were flushed from the femurs of mice. The cells were cultured in 75-cm2 culture flasks in DMEM including 1000 mg/liter glucose supplemented with 10% heat-inactivated fetal calf serum, glu tamax, 100 units/ml penicillin G, 100 mg/ml streptomycin, and 10 ng/ml recombinant murine M-CSF. The cells were incubated in an incubator at humidified atmosphere (37 °C, 5% CO2). Culture medium (10 ml/culture flask) was replaced after 3 days to remove nonadherent cells and cell debris, and at day 6, 30% of the initial volume was added as fresh medium containing 10 ng/ml M-CSF. To achieve a homogenous distribution of the macrophages on the culture dish, the cells were detached after 7 days of culture using trypsin/EDTA solution (incubation for 30 min at 37 °C). Thereafter, the cells were seeded on 60-mm dishes at a density of ~1 × 106 cells/dish, and culture was continued for another 2 days. In the end, this procedure resulted in a yield of ~7–9 × 106 cells at maximum that were 99% (±0.7) F4/80-positive, CD11b-positive, and CD11c-negative macrophages as assessed by FACS analysis (data not shown). The medium was changed to M-CSF-free culture medium 16 h before experiments were performed. The cells were used after a total differentiation period of 9 days.

Preparation and Cultivation of Human Peripheral Blood-derived Macrophages—Human peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected leukocyte-rich buffy coats obtained from healthy blood donors (University of Düsseldorf, Blood Transfusion Service) by a density gradient centrifugation over ficoll-Paque gradient (GE Healthcare) and 12 × 106 cells/dish (100-mm dishes) were seeded. Monocytes were allowed to adhere to the plastic bottom of the dish for 2 h at 37 °C in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. After this incubation period, the nonadherent cells were removed, and the wells were washed with culture medium. The cells were incubated in a thermoincubator (37 °C and 5% CO2). Monocytes were differentiated into macrophages in RPMI 1640 medium supplemented with antibiotics, 10% fetal calf serum, and recombinant human M-CSF (10 ng/ml). Culture medium (6 ml/dish) was replaced after 3 days, and total medium volume was increased by 30% after further 3 days. The cells were used after 9 days of cultivation and differentiation. The medium was changed to M-CSF-free culture medium 16 h before the experiments were performed.

Retroviral Gene Transfer—Retroviral gene transfer into MK2/3−/− MΦ was performed as outlined recently (16). Briefly, full-length murine MK2 and MK3 were subcloned into the pMMP-IRES-GFP (kind gift of C. Klein, Hannover Medical School, Hannover, Germany) bicistronic retroviral vector upstream of the IRES. To obtain MK2/3−/− macrophage cell
lines stably reconstituted with either MK2 or MK3, retroviral supernatants were generated by transient transfection of the BD EcoPack 2-293 packaging cell line with bicistronic vectors encoding the gene of interest and GFP as marker and were used for infection. The cells were cultivated as described above.

Transfection of siRNA—For targeted gene knockdown, siRNA specific for murine IκBβ (NFKBIB) or nontargeting control pool 2 were purchased from Dharmacon Inc. (Lafayette, CO). 25 nm siRNA were transfected using DharmaFECT 4 with 2 μl/ml volume according to the manufacturer’s instructions. After an incubation period of 16 h at 37 °C in a thermostimulator, the cells were washed and incubated for 48 h in total.

Preparation of Total Cell Lysates—For the analysis of protein from total protein extracts, the cells were solubilized in Triton lysis buffer (1% Triton, 20 mM Tris/HCl, pH 7.4, 136 mM NaCl, 2 mM EDTA, 50 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM Na3VO4, 4 mM benzamidine, 0.2 mM Pefabloc, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 10% glycerol, and 0.2% SDS) at 4 °C.

Preparation of Cytosolic Lysates and Nuclear Extracts—For cytosolic lysates, the cells were solubilized after removal of medium by the addition of 1% Nonidet P-40 lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, pH 8, 1 mM ZnCl2, pH 4, 1 mM MgCl2, 1 mM Na3VO4, 10% glycerol, 50 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride, 1% Nonidet P-40). After centrifugation, the supernatants were used for immunoblot analysis of cytosolic proteins. For nuclear extracts, the pellets were resuspended in high salt buffer (420 mM NaCl, 20 mM Hepes, pH 7.9, 10 mM KCl, 0.1% Nonidet P-40, 0.1 mM Na3VO4, 20% glycerol, 50 μg/ml 4-(2-aminooethy) benzenesulfonyl fluoride, 1 mM DTT) and sonicated; thereafter 0.2% SDS was added. After centrifugation, the supernatants were used for immunoblot analysis of nuclear proteins.

Immunoprecipitation—Cytosolic fractions of macrophages were prepared as described above and were incubated with a polyclonal antibody specific for IκBβ or with an IgG control antibody as well as with a biotinylated anti-rabbit antibody for 120 min at 4 °C and subsequently incubated with Dynabeads M-280 streptavidin (Invitrogen) for an additional 120 min at 4 °C. The immune complexes were precipitated with the magnetic particle concentrator Dynal MPC-S (Invitrogen) and washed extensively with wash buffer (0.1% Triton, 20 mM Tris/HCl, pH 7.4, 136 mM NaCl, 2 mM EDTA, 50 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM Na3VO4, 4 mM benzamidine, 0.2 mM Pefabloc, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 10% glycerol). Finally, the samples were incubated at 95 °C for 30 min and separated by SDS-PAGE as described above. After blotting, the blots were analyzed using antibodies specific for p65 and IκBβ.

Immunoblotting and Immunodetection—For immunoblot analysis of total protein extracts, the cells were grown in a 60-mm plate and stimulated with LPS or with the respective cytokine at the concentrations indicated. For the analysis of cytosolic lysates and nuclear extracts, the cells were grown in a 100-mm dish using two dishes for one sample. The respective amounts of protein indicated in the figure legends were subjected to SDS gel electrophoresis (8 and 10% polyacrylamide). The electrophoretically separated proteins were transferred onto PVDF membranes by the semidy Western blotting method. Nonspecific binding was blocked with 5% BSA in TBS-T (20 mM Tris/HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween) for 60 min. The blots were incubated overnight at 4 °C with primary antibodies diluted in TBS-T (1:2000). After extensive washing with TBS-T, the blots were incubated with goat anti-rabbit IgG or rabbit anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase and diluted in TBS-T (15000) for 1 h at room temperature. After further rinsing in TBS-T, the immunoblots were developed with the ECL system following the manufacturer’s instructions (PerkinElmer Life Sciences).

RNA Isolation and Real Time PCR—Total cellular RNA was isolated by using the RNeasy miniprep kit (Qiagen) as described in the manufacturer’s instructions. 1 μg of total RNA was reverse transcribed with a QuantiTect reverse transcription kit (Qiagen) using oligo(dT), which included DNase I digestion. cDNA was diluted 1:5, and 1.2 μl of the diluted cDNA was added as template to a final volume of 25 μl including 1× SYBR Green PCR Master Mix according to the manufacturer’s instructions (Applied Biosystems). The following primers were used for real time PCR: mouse IκBα (sense, 5'-ACG AGC AAA TGG TGA AGG AG-3'; antisense, 5'-CCCA GTG GCA GGA AGT CT-3'), mouse IL-10 (sense, 5'-CCA AGC CTT ATC GGA AAT GA-3'; antisense, 5'-TCT CAC CCA GGG AAT TCA AA-3'), mouse IFNβ (sense, 5'-CCCTAT GGA GAT GAC GGA GA-3'; antisense, 5'-ACCCAG TGC TGG AGA AAT TG-3'), and the Fp subunit of the murine succinate dehydrogenase (SDHA) (sense, 5'-TGG GGA GTG CCG TGG TGT CA-3'; antisense, 5'-GTG CCG TCC CCT GTG CTG GT-3'). All of the primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). Specificity of RT-PCR was controlled by no template and no reverse-transcriptase controls. Semiquantitative PCR results were obtained using the ΔCt method. As control gene SDHA was used. Threshold values were normalized to SDHA. Data from at least three independent experiments are presented as means plus standard error of means (S.E.).

Statistical Evaluation—Statistics were calculated using the Prism 5.0 software from GraphPad. Each of the experiments was repeated at least three times. The significance was calculated using a one- or two-way analysis of variance test together with a Bonferroni test. p < 0.05 was considered to be significant.

RESULTS

MK2 Is Crucial for LPS-mediated STAT3 Activation—In macrophages, LPS induces an activation of STAT3 (Fig. 1, A and B), which was delayed compared with the immediate STAT3 activation observed in response to IL-6 (Fig. 1C). As demonstrated in Fig. 1, in human and mouse macrophages, STAT3 activation becomes detectable 80–120 min after the addition of LPS to the culture medium at the earliest and lasts for at least 6–7 h.

Previous data indicated that MK2, which is rapidly activated in response to LPS, is important for induction of SOCS3 by TNFα (19) and LPS and thus mediates negative regulatory sig-

3 C. Ehlting, N. Ronkina, M. Gaestel, D. Häussinger, and J. G. Bode, unpublished observations.
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FIGURE 1. LPS induces delayed activation of STAT3 but early activation of IRF3 and the p38MAPK/MK2/MK3 pathway. PBMC-derived human macrophages (A) and a murine macrophage cell line (RAW 264.7) (B) were stimulated with 100 ng/ml LPS as outlined in the figure, whereas PBMC-derived human macrophages (C) were treated with IL-6 (200 units/ml) as depicted. After the time periods indicated, total protein extracts were prepared, and 80 μg were subjected to immunoblot analysis. Protein phosphorylation and/or expression was assessed as indicated using antibodies specifically recognizing STAT3-Tyr705, IRF3-Ser396, p38-Thr180/Tyr182, MK2-Thr222, and antibodies recognizing total proteins of STAT3, IRF3, MK2, MK3, p38MAPK, SOCS3, and GAPDH. Of note, the antibody recognizing MK2-Thr222 also recognizes the homologous phosphorylation of human MK3 (MK3-Thr200).

FIGURE 2. LPS-induced but not IL-6- or IFNβ-induced STAT3 activation is impaired in the absence of MK2 but restored to wild type levels upon additional deletion of the MK3 gene. A, immortalized macrophages derived from wild type (Mφ WT) and MK2−/− (Mφ MK2−/−) bone marrow-derived murine macrophages were treated with 100 ng/ml LPS as indicated. B–D, BMDM were prepared from wild type, MK2−/−, MK3−/−, or MK2/3−/− animals and treated with 100 ng/ml LPS (for B and C) or with 200 units/ml IL-6 (for C) for the time periods indicated. E, Mφ WT, Mφ MK2−/−, and Mφ MK2/3−/− were treated with 200 units/ml IFNβ for the time period indicated. After the respective time periods, the total protein extracts were prepared, and 80 μg of protein was subjected to immunoblot analysis. Protein phosphorylation and/or expression was assessed as indicated using antibodies specifically recognizing STAT3-Tyr705, p38-Thr180/Tyr182, and antibodies that recognize total protein of STAT3, MK2, MK3, p38MAPK, SOCS3, and GAPDH.

nals on STAT3-dependent cytokine signaling. We used MK2-deficient macrophages to address, whether MK2 is also involved in the control of delayed STAT3 activation after LPS treatment. As demonstrated in Fig. 2 (A and B), LPS-mediated activation of STAT3 was strongly affected in MK2-deficient macrophages but not in macrophages that lack MK3, a member of the MK family that is closely related to MK2 (Fig. 2C). This role of MK2 in mediating STAT3 activation is specific for LPS, because activation of STAT3 in response to cytokines such as IL-6 or IFNβ (Fig. 2, D and E) is not significantly affected upon ablation of the MK2 gene. At a first glance, these data suggest that MK2 but not MK3 is of key importance for mediating
delayed and sustained STAT3 activation in response to LPS. Because MK3 has been reported to cooperate with MK2 in regulation of TNFα expression (16), LPS-induced STAT3 activation was further assessed in macrophages depleted of both MK2 and MK3 (MK2/3−/−). Unexpectedly, and in contrast to the single ablation of the MK2 gene, combined ablation of MK2 and MK3 (Fig. 2B) resulted in the recovery of LPS-induced STAT3 activation almost back to wild type levels. This observation suggests a yet unknown interrelationship between MK2 and MK3, in which knock-out of MK2 results in an imbalance of the system permitting otherwise unapparent negative regulatory effects of MK3.

LPS-inducible Expression of IL-10 and IFNβ Is Differentially Regulated by MK2 and MK3—Recently it has been demonstrated that activation of the IFNAR is crucial for LPS-induced IL-10 expression and subsequent STAT3 activation (7). In line with this, IFNAR-deficient macrophages do not exhibit LPS-induced STAT3 activation (Fig. 3). We next addressed the question whether type I interferon expression is regulated by MK2-MK3 interaction. As demonstrated in Fig. 4A, LPS-
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FIGURE 5. Combined inactivation of MK2 and MK3 by blocking p38MAPK activity results in inhibition of LPS-induced IL-10 expression, whereas expression of IFNβ, as well as STAT3 activation, is not affected. Because of the lack of phosphospecific antibodies specifically recognizing MK3-Thr200 phosphorylated at threonine, activation of MK3 was analyzed in human macrophages because the antibody used to detect MK2-Thr222 also recognizes the human but not mouse MK3 in its phosphorylated state. A and B, human PBMC (A) and murine bone marrow-derived macrophages (B) were generated and pretreated for 16 h with 1 μM SB203580 and subsequently stimulated with 100 ng/ml LPS for the times indicated. Total protein lysates were prepared and separated on SDS page. The blots were analyzed using antibodies specifically recognizing STAT3-Tyr705, MK2-Thr222, or antibodies recognizing total protein of STAT3, MK2, MK3, and GAPDH. C and D, bone marrow-derived macrophages generated from wild type mice were pretreated with 1 μM SB203580 for 16 h. Thereafter, the cells were stimulated with 100 ng/ml LPS for the times indicated. Total RNA extracts were prepared and subjected to RT-PCR for IFNβ, IL-10, and IL-12 mRNA expression (Fig. 5, C and D). Comparable results were obtained from the analysis of LPS-induced IFNβ and IL-10 mRNA expression in SB203580-pretreated human PBMC-derived macrophages (data not shown). Semiquantitative PCR results were obtained using the ΔCt method. Cytokine mRNA was normalized to SDHA, and relative mRNA levels were expressed as fractions of the normalized value of the control, which was set to 100%. The data are presented as the means ± S.E. (n = 3). p values smaller than 0.05 were considered significant.

duced IFNβ expression was substantially reduced in MK2−/− macrophages in comparison with wild type macrophages. This down-regulation of LPS-mediated IFNβ expression again was dependent on the presence of MK3 (Fig. 4A).

Because the turnover of the IFNβ transcript is strictly regulated (20, 21) and MK2 is known to be important for regulation of transcript stability, the influence of MK2 on IFNβ transcript stability was assessed. The data depicted in Fig. 4B suggest that in macrophages neither MK2 nor MK3 significantly influences IFNβ transcript stability induced by LPS treatment.

In contrast to IFNβ, LPS-mediated IL-10 mRNA expression was strongly impaired in MK2−/− as well as in MK2/3−/− macrophages (Fig. 4C) and reflects the effects of MK2-dependent stabilization of IL-10 mRNA (Fig. 4D). Thus, MK2 regulates IL-10 expression apparently independent of MK3 by controlling IL-10 transcript stability. These observations further suggest that LPS-induced STAT3 activation in MK2/3−/− macrophages is independent of IL-10 and is most likely based on IFNβ-mediated activation of IFNAR, which is able to directly mediate STAT3 activation (Fig. 2E).

Corroborating the above conclusions, combined inactivation of MK2 and MK3 by pretreatment of human or mouse macrophages (Fig. 5, A and B) with the p38MAPK inhibitor SB203580 strongly inhibits LPS-induced IL-10 expression, whereas the induction of IFNβ expression is not affected (Fig. 5, C and D). Moreover, in line with the findings in MK2/3−/− macrophages (Fig. 2B), LPS-inducible STAT3 activation is not significantly affected in SB203580-pretreated macrophages (Fig. 5, A and B), although IL-10 expression is inhibited.

IRF3 Protein Expression and IRF3 Activation in Response to LPS Is Impaired in MK2-deficient but Not in MK2/3-deficient Macrophages—The observations described above suggest that MK2/3-mediated regulation of LPS-induced IFNβ gene expression occurs on the level of transcription. To further understand the underlying molecular mechanisms, we asked whether negative regulation of IFNβ expression in MK2-deficient macrophages is due to impairment of signaling events, which are essential for the transcriptional regulation of IFNβ gene expression. We focused on the two components IRF3 and NFκB, which are essential parts of the transcriptional complex that regulates IFNβ gene transcription (22–24). Fig. 6 (A and B) demonstrates that the activating phosphorylation of IRF3 at serine residue 396 is strongly impaired in MK2−/− macrophages. To some extent, this down-regulation of IRF3 phos-
phorylation in MK2−/− macrophages is due to reduced IRF3 protein expression (Fig. 6, A and B). In contrast, ablation of MK3 had almost no effect on IRF3 expression and/or its activation (Fig. 6C). In turn, ablation of both MK2 and MK3 was able to override the effect of the single MK2 deficiency and resulted in IRF3 protein levels and IRF3 phosphorylation that were almost comparable with wild type macrophages, as illustrated in Fig. 6 (A and B). These data suggest that in the absence of MK2, the sole presence of MK3 negatively influences IRF3 protein expression and its activation and that under normal conditions MK2 precludes MK3 from mediating these negative regulatory effects. To prove that MK3 indeed mediates negative regulatory effects on IRF3 function in the absence of MK2, MK3 was stably reconstituted in MK2/3−/− macrophages by retroviral gene transfer. In support of the proposed inhibitory activ-

FIGURE 6. MK3 negatively regulates IRF3-dependent signaling in the absence of MK2. A and C, WT BMDM, MK2−/− BMDM, and MK2/3−/− BMDM (A) and MK3−/− BMDM (C) were treated with LPS (100 ng/ml) as indicated. B, WT Mφ, MK2−/− Mφ, and MK2/3−/− Mφ were treated with LPS (100 ng/ml) for the time period indicated. D and E, MK2/3−/− Mφ were stably transfected with a vector co-expressing MK3 (D and E) or MK2 (E) and GFP for control via an IRES or with the same vector expressing GFP for control using retroviral gene transfer. The cells were treated with LPS (100 ng/ml) for the time periods indicated, total protein lysates were prepared, and 80 μg of protein were analyzed by immunoblot using antibodies specifically recognizing IRF3-Ser396, STAT3-Tyr705, MK2-Thr222, and total IRF3, STAT3, MK2, or MK3. Expression of GAPDH (B, D, and E) and β-actin (A and C) was analyzed for loading control.

MK2 Prevents MK3 from Impeding Nuclear Translocation of NFKB p65—Apart from the involvement of IRF3, transcriptional regulation of the IFNβ gene also requires activation and nuclear translocation of NFKB. To assess whether MK2 and MK3 regulate NFKB activation, we analyzed p65 nuclear translocation in wild type, MK2−/−, and MK2/3−/− macrophages. Notably, in MK2-deficient macrophages, p65 nuclear translocation is reduced and occurs with substantial delay when compared with wild type controls (Fig. 7, A and B). This alteration leads to a desynchronization of p65-mediated signals in comparison with the different other signaling events elicited by LPS, such as the activation of IRF3, and therefore might be responsible for disturbed transcriptional regulation of gene expression. In this context, it is interesting to note that not only the strength but also the kinetics of NFκB expression (25, 26). Consistently, p65 is almost undetectable in macrophages when analyzed after 15 (Fig. 7, A and B) or 20 min (Fig. 7C). As shown in Fig. 7 (A and C), the delay in nuclear translocation of p65 was almost completely reversed upon additional ablation of the MK3 gene in MK2/3−/− macrophages. These data suggest that in macrophages, MK3 counteracts nuclear translocation of p65 in response to LPS in the absence of MK2. Consistent with this,
reintroduced MK3, but not MK2, in MK2/3−/− macrophages restores the phenotype of MK2−/− macrophages with impaired nuclear translocation of p65 (Fig. 7D).

In MK2-deficient Macrophages, Delayed Degradation of IκBβ Is Responsible for Impaired Expression of IκBa—Nuclear NFκB regulates the expression of a multitude of different genes, either as the leading regulatory factor or as a part of a transcriptional complex that essentially requires additional factors to gain optimal functionality. One of the target genes that is primarily regulated by NFκB is its own inhibitor IκBa, which is induced as part of an autoregulatory feedback loop (27, 28). Consequently, as a result of impaired NFκB activation, expression of IκBa is strongly decreased in nonstimulated MK2−/− macrophages when compared with wild type (Figs. 7A and 8, A and C) and also in MK3-reconstituted MK2/3−/− macrophages when compared with GFP control cells (Fig. 8B). The observation that reintroduction of MK3 into MK2/3−/− macrophages also results in a down-regulation of IκBa expression again emphasizes that MK3 exerts negative regulatory effects on NFκB-dependent regulation of gene expression in the absence of MK2.

In contrast to IκBa, IκBβ expression is enhanced in MK2−/− (Figs. 7A and 8, C and D) and MK2/3−/− macrophages (Figs. 7A and 8D) when compared with wild type macrophages. Moreover, LPS-inducible degradation of IκBβ is substantially delayed in MK2−/− macrophages when compared with WT and MK2/3−/− macrophages (Figs. 7A and 8D). Thereby, in MK2-deficient macrophages, the time course of IκBβ degradation bridges the time period when IκBa is absent in the cytoplasm and matches to the delayed appearance of p65 within the nucleus in MK2−/− macrophages (Fig. 7A). According to this, co-immunoprecipitation experiments using cytoplasmic lysates indicate that in contrast to wild type and MK2/3−/−
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In macrophages, the binding of p65 to IκBβ and the LPS-induced degradation of IκBβ is prolonged in MK2−/− macrophages (Fig. 8D). Hence, the data suggest that impaired early nuclear translocation of p65 in MK2-deficient macrophages is due to delayed degradation of IκBβ, which interacts with the same spectrum of Rel proteins as IκBα (29). This assumption is further substantiated by the observation that in MK2−/− macrophages, expression of the NFκB target gene IκBα is almost completely restored back to those observed in wild type macrophages if enhanced IκBβ expression (Fig. 8C, third and fourth lanes) is knocked down back to wild type protein levels using IκBβ-specific siRNA (Fig. 8C, compare first and second lanes with fifth and sixth lanes).

**DISCUSSION**

In macrophages, STAT3 is considered to be the major mediator of anti-inflammatory effects (3–5). Its sustained activation in response to LPS is the result of a complex and yet only partially understood feedback loop (3–6) involving activation of IFNAR and IFNAR-dependent release of IL-10 (7, 30). By analyzing the role of MK2 and MK3 for LPS-inducible STAT3 activation in macrophages, the present study reveals unexpected and novel details on the function of MK3, which so far appeared as closely related, cooperatively acting isoenzyme of MK2 with an identical recognition sequence (10, 16). The present manuscript indicates for the first time that MK2 and MK3 also have different regulatory roles, with MK2 regulating gene expression by preventing MK3-mediated negative regulatory effects on LPS-induced signaling (for a schematic summary see Fig. 9).

The data suggest that in macrophages MK2 is important for delayed STAT3 activation in response to LPS (Figs. 2, A and B, and A) because it controls IFNβ expression (Fig. 4A), which in turn is required for IFNAR-dependent activation of STAT3 (Fig. 3). Thereby, MK2 does not directly control IFNβ gene expression (Fig. 4, A and B) but rather prevents MK3 from inhibiting signaling events (Figs. 6 and 7) essential for induction of IFNβ gene expression (22–24). Thus, in the absence of MK2, MK3 is demonstrated to be responsible for impaired IRF3 function (Fig. 6) and inhibition of nuclear translocation of the p65 subunit of NFκB (Fig. 7), both of which are reported to be crucial for transcriptional control of IFNβ gene expression (22–24).

In line with the data reported herein, impaired p65 nuclear translocation and reduced p65 NF-κB DNA binding capacity as a result of MK2 depletion has recently also been demonstrated in human umbilical endothelial cells (31). However, the underlying molecular mechanisms identified to be responsible for impaired nuclear translocation of NFκB differ substantially between human umbilical endothelial cells and macrophages; thus, in human umbilical endothelial cells, impaired nuclear translocation of p65 has been reported to be due to an accelerated rescue of IκBα protein expression that results in an enhanced and premature removal of p65 from the nucleus (31).
FIGURE 9. Schematic summary of the regulation of LPS-induced STAT3 activation by MK2 and MK3. The present work suggests that in macrophages, MK2 regulates LPS-induced expression of IFNβ by preventing MK3 from exerting negative regulatory effects on NFκB- and IRF3-dependent signaling. Both IRF3 and the p65 subunit of NFκB are essential components of the transcriptional complex that mediates enhanced transcription of the IFNβ gene in response to LPS (22–24). As indicated by the data, MK2 maintains IRF3-dependent LPS signaling because it deters MK3 from impeding IRF3 protein expression and its activation in response to LPS (step 1 in the scheme). Furthermore, MK2 warrants timely and unimpaired nuclear translocation of p65 in the absence of MK2. Thereby MK2 assures accurate NFκB nuclear translocation by controlling basal protein levels of IκBα and by preventing MK3 from impeding its degradation (step 2). Hence, MK2 deficiency results in a MK3-dependent desynchronization of the LPS-induced activation of important components of the transcriptional complex that enhances transcription of the IFNβ gene (step 3) and therefore inhibits IFNβ expression. Apart from IFNβ, p65 also controls the expression of other NFκB target genes such as IκBα (step 4), which is part of an autoregulatory feedback loop. As a consequence of the hampered NFκB activation in MK2−/− macrophages, IκBα gene expression is likewise impaired. Previous reports (7) and the data provided herein indicate that LPS-induced IL-10 expression and IL-10-induced activation of STAT3 requires activation of the IFNAR (step 5), which is also capable of directly mediating IFNβ-induced STAT3 activation (step 6). Hence, MK2 also controls LPS-mediated activation of STAT3 in an indirect way as it ensures IFNβ expression. In addition, the data indicate that MK2 (independent from MK3) is required for the transcriptional regulation of IFNβ (step 7). This may further explain the observation that additional deletion of the MK3 gene in MK2/3−/− macrophages reconstitutes LPS-induced expression of IFNβ but not of IL-10. Interestingly, in MK2/3−/− macrophages rescued IFNβ expression, and subsequent IFNAR activation is sufficient to mediate LPS-induced activation of STAT3 independent from IL-10, which is not restored, indicating that under defined conditions IFNAR activation alone is sufficient to mediate STAT3 activation in response to LPS. Corresponding to this, combined inhibition of MK2 and MK3 activation by blocking their common upstream activator p38MAPK using the compound SB203580, supposed to specifically inhibit catalytic activity of p38MAPK, does not inhibit LPS-induced IFNβ expression and subsequent STAT3 activation, although it inhibits IL-10 expression.

Contrariwise, in macrophages ablation of the MK2 gene unmasks negative regulatory properties of MK3 (Fig. 7), resulting in delayed and impaired nuclear translocation of p65 and a significant reduction of IκBα transcript and protein (Figs. 7, A and B, and 8, A and B) levels. Thereby, it is likely that downregulation of IκBα expression reflects impaired NFκB activation, because it is known to be NFκB-dependently induced as a part of an autoregulatory feedback loop (27).

Of note, in MK2−/− macrophages, delayed nuclear translocation of p65 and impaired expression of p65 target genes such as IκBα is accompanied by an enhanced expression and a decelerated LPS-induced degradation of IκBβ (Fig. 7A). IκBβ is reported to interact with the same spectrum of Rel proteins as IκBα (28, 29) and is able to retain p65 in the cytoplasm of unstimulated cells (32) as also demonstrated herein (Fig. 8D). Depletion of IκBβ results in increased basal NFκB DNA binding activity, and its expression inhibits NFκB activation (32). Moreover, studies on cell lines deficient for IκBα and IκBε with IκBβ as the most prominent member of the IκB family, suggest that in contrast to IκBα, IκBβ mediates delayed but sustained activation of NFκB (33). Therefore, one may conclude that in MK2-deficient macrophages, IκBβ is responsible for impaired...
and delayed activation of p65 and down-regulation of IκBα expression. This assumption is supported by the observation that knockdown of IκBβ expression in MK2−/− macrophages by specific siRNA (Fig. 8C) restores expression of IκBα back to protein levels observed in wild type macrophages. However, in contrast to IκBα, this restoration of NFκB-dependent gene expression by suppressing IκBβ expression was not sufficient to rescue IFNβ expression in MK2−/− macrophages (data not shown). This indicates that re-establishment of NFκB-mediated signaling alone would not be sufficient to reconstitute LPS-inducible IFNβ expression in MK2−/− macrophages because this requires the activation of additional transcription factors, such as IRF3 (24).

Studies using IκBβ knock-out animals indicated that IκBβ has a dual function in regulation of inflammatory gene expression. Thus, apart from cytoplasmic sequestration of NFκB in unstimulated cells and NFκB release during the early stages of stimulation, IκBβ also plays a crucial role as a transcriptional regulator of inflammatory cytokine expression in vivo (32, 34). In this context, the previously described hypo- or unphosphorylated form of IκBβ seems to be particularly important. Upon stimulation, this form is newly synthesized (35) and forms a transcriptional active DNA-binding complex with p65 and c-Rel (32). This complex has been suggested to be required for expression of inflammatory cytokines, such as TNFα, IL-1β, IL-6, and IL-12 (32, 34). Whether IκBβ also plays a role for the transcriptional regulation of type I interferon expression is unknown. However, in MK2-deficient macrophages basal IκBβ expression is up-regulated (Figs. 7A and 8C), but not diminished, and therefore rather represents a situation of basal IκBβ overexpression accompanied by impaired NFκB activation (32) and not an IκBβ knock-out situation with basal NFκB activation being up-regulated.

Apart from p65 activation, the activation of IRF3 is also impaired in MK2−/− macrophages (Fig. 6). In analogy to p65, impaired function of IRF3 in the absence of MK2 is demonstrated to be also due to negative regulatory effects of MK3. Accordingly, impaired IRF3 function can be almost completely rescued by additional ablation of the MK3 gene in MK2/3 knock-out situation with basal NFκB activation being strongly affected. Thereby, it is likely that reduced activation of IRF3 in MK2−/− macrophages is to a major degree due to down-regulation of IRF3 protein levels. However, despite substantial efforts, we were not able to clarify the molecular mechanisms responsible for down-regulation of IRF3 protein levels in MK2−/− macrophages. Our own data demonstrate that reduction of IRF3 protein expression observed in MK2−/− macrophages is due neither to impaired transcription nor to reduced IRF3 transcript stability and does not involve enhanced MG132-sensitive proteasomal degradation, caspase-mediated protein degradation or bafilomycin-sensitive proteolysis (data not shown). Furthermore, pull-down assays and co-immunoprecipitation studies indicate that, in contrast to p38MAPK, which has been demonstrated to be stabilized by its interaction with MK2 (36), IRF3 interacts with neither MK2 nor MK3 (data not shown). Hence, it is also unlikely that regulation of IRF3 protein stability based on direct protein/protein interaction with either MK2 or MK3 plays a role. Protein kinase assays, using recombinant IRF3 as a substrate, further suggest that LPS-inducible phosphorylation of IRF3 is TBK1-dependent but occurs independently of MK2 (data not shown).

As schematically summarized in Fig. 9, the data provide strong evidence that MK2 controls LPS-inducible IFNβ gene expression and subsequent IFNAR-mediated activation of STAT3 by neutralizing negative regulatory effects of MK3 on LPS-induced p65 and IRF3-mediated signaling. The data further indicate that in MK2/3−/− macrophages, IFNAR-dependent STAT3 activation occurs independently from IL-10, because in contrast to IFNβ, impaired IL-10 expression is not restored upon additional deletion of MK3 in MK2/3−/− macrophages. These differences between IFNβ and IL-10 are suggested to be due to the fact that the IL-10 transcript requires the presence of MK2 for stabilization (Fig. 4D), which is in line with the regulation previously reported for other transcripts such as IL-6 (37). In contrast to IL-10, MK2 deficiency has no effect on the stability of the IFNβ transcript (Fig. 4B), suggesting that regulation of IFNβ by MK2 and MK3 does not occur on the level of transcript stability. Because in MK2/3−/− macrophages IFNβ expression, but not IL-10 expression, is restored, the data further indicate that in MK2/3−/− macrophages, LPS-inducible activation of STAT3 occurs independently from IL-10. The fact that LPS indeed is able to activate STAT3 IL-10-independent in the absence of MK2 and MK3 activity is further substantiated by the finding that combined inactivation of MK2 and MK3 by blocking p38MAPK activity does not affect STAT3 activation (Fig. 5, A and B), although LPS-induced expression of IL-10 (Fig. 5D) but not of IFNβ (Fig. 5C) is strongly affected. Thereby, it is likely that in MK2/3−/− macrophages, activation of IFNAR by IFNβ is sufficient to mediate LPS-induced STAT3 activation IL-10-independent, because IFNβ-induced STAT3 activation is not impaired in MK2−/− or MK2/3−/− macrophages (Fig. 2E).

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