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HSV-1/TLR9-Mediated IFNβ and TNFα Induction Is Mal-Dependent in Macrophages

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Abstract
Innate immune response is a universal mechanism against invading pathogens. Toll-like receptors (TLRs), being part of a first line of defense, are responsible for detecting a variety of microorganisms. Among them TLR9, which is localized in endosomes, acts as a sensor for unmethylated CpG motifs present in bacteria, DNA viruses (e.g., HSV-1), or fungi. TLRs differ from one another by the use of accessory proteins. MyD88 adapter-like (Mal) adapter molecule is considered a positive regulator of TLR2- and TLR4-dependent pathways. It has been reported that this adapter may also negatively control signal transduction induced by TLR3 anchored in the endosome membrane. So far, the role of Mal adapter protein in the TLR9 signaling pathways has not been clarified. We show for the first time that Mal is engaged in TLR9-dependent expression of genes encoding IFNβ and TNFα in HSV-1-infected or CpG-C-treated macrophages and requires a noncanonical NF-κB pathway. Moreover, using inhibitor of ERK1/2 we confirmed involvement of these kinases in TLR9-dependent induction of IFNβ and TNFα. Our study points to a new role of Mal in TLR9 signaling through a hitherto unknown mechanism whereby lack of Mal specifically impairs ERK1/2-mediated induction of noncanonical NF-κB pathway and concomitant IFNβ and TNFα production.

Introduction

The proper and rapid recognition of the danger posed by a viral or bacterial infection is the first and extremely important element initiating primary immune response, often conditioning survival of the cell as well as the entire organism. Toll-like receptors (TLRs) are a group of pattern recognition receptors that play a crucial role in "danger" recognition and the induction of immune response.

The family of these receptors consists of 13 members – TLR1–13 – whereas 11 receptors have been characterized in humans so far [1]. TLRs were divided into two groups due to their subcellular localization: receptors present in the cell membrane and in endosomes. Endosomal receptors include TLR3, TLR7–9, TLR11, and

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TLR13 and are mainly responsible for the binding of viral or bacterial nucleic acids.

TLR9 is involved in the recognition of DNA rich in unmethylated CpG structures, present in the genetic material of both higher organisms and microorganisms or viruses [2, 3]. These structures are common in bacteria and viruses, whereas in vertebrates they mainly occur in the promoter regions of genes and provide recruitment sites for proteins necessary for gene transcription. Although TLR9 was discovered as a receptor activated by bacterial genetic material, its important role was reported in the recognition of viruses belonging to the *Herpesviridae* family, such as herpes simplex viruses (HSV-1 and HSV-2), cytomegalovirus, or Epstein-Barr virus [4–7].

Pathogen recognition by the TLR9 receptor is a multistep process initiated by binding of the ligand in the endosomal lumen. Upon ligand binding there is a conformational change in the receptor structure which enables MyD88 adapter docking to the cytoplasmic domain of TLR9, followed by formation of a complex with kinases from the IRAK family, called myddosome [8–10]. This complex triggers a signaling cascade, leading to the translocation of the NF-κB transcription factor into the cell nucleus, which initiates the expression of cytokines and chemokines.

MyD88 is an adapter involved in the signal transduction of most TLRs regardless of their subcellular location. The interaction of MyD88 with TLRs is possible due to the presence of a Toll/interleukin-1 receptor (TIR) region in the adapter protein and the cytoplasmic domain of the receptor. However, in some cases, the interaction of TLRs and MyD88 requires an accessory protein for proper signal transduction [11]. In the case of membrane TLRs (TLR2, TLR4), MyD88 adapter-like (Mal), also termed TIRAP (TIR adapter protein), is involved in this process [12–14].

Mal is a small protein (24–26 kDa) being a "bridging" adapter for MyD88 due to its association with the plasma membrane through a phosphatidylinositol-4,5-bisphosphate-binding motif at the N-terminal region and TIR-TIR interaction in the membrane-proximal compartment [15]. Although Mal is considered a positive regulator of TLR-dependent pathways, it was reported that this adapter molecule may negatively control the signal transduction induced by TLR3 which, unlike TLR2 and TLR4, is anchored in the endosome membrane. Following the activation of TLR3 with a specific ligand, Mal interacts with the IRF7 transcription factor and inhibits its binding to the promoter region of the IFNβ-encoding gene [16].

To date, the exact role of Mal adapter protein in the signaling pathways of other endosomal TLRs, including TLR9, has not been elucidated.

Materials and Methods

Cell Culture and Reagents

Immortalized BMDM cell lines from wild-type (WT) and Mal–/– mice were obtained from Bei Resources. CHME-5 human embryonic microglia were from Pasteur Institute, France. Cells were grown in DMEM with GlutaMAX™ (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and 100 μg/mL Normocin™ (Invivogen). B16-Blue™ IFNα/β cells were from Invivogen and were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Sigma), 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL Normocin, and 100 μg/mL Zeocin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The HSV-1 strain MacIntyre (ATTC VR-539™) was from ATCC. Ultrapure LPS-EB derived from *Escherichia coli* strain O111:B4, CpG-C ODN (M362), and INH-1 ODN were purchased from Invivogen. Inhibitors FR180204 and JSH-23 were purchased from Sigma. Control peptides (CtrlPs) and Mal inhibitory peptides (MalIPs) were from Novus. Buffy coats were kindly provided by the Station of Blood Donation, Fourth Military Hospital, Wroclaw, Poland.

Isolation of Peripheral Blood Mononuclear Cells

Human peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation of buffy coats from healthy blood donors over Lymphoprep™ (Stemcell™ Technologies) according to manufacturer's instructions. Cells were incubated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and 100 μg/mL Normocin and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cell viability was evaluated using Trypan Blue exclusion assay. Viability was always >95%.

Type I IFN Bioassay

Detection of CpG-C- and LPS-induced bioactive murine type I IFNs was assessed using B16-Blue IFNα/β cells, essentially as described by the manufacturer (Invivogen).

ELISA

BMDM cells were stimulated with the indicated ligands. After 16 h, the cell-free supernatants were collected and analyzed for TNFα. Cytokine release was measured as indicated by the manufacturer (R&D Systems®).

Lentiviral Transduction

Control and human *TIRAP* lentiviral shRNA plasmids were from Sigma and the procedure was followed as described [16]. CHME-5 cells (1×10^5 cells/well; 6-well plate) were transduced with control shRNA or *TIRAP* shRNA lentiviral particles and cells were subsequently grown for 1 week under puromycin (10 μg/mL; Invivogen) selection. The efficiency of *TIRAP* knockdown was assessed by qPCR using the following primers: *TIRAP* forward 5′-TTC ACC AAT GCC TGG TCT C-3′, reverse 5′-CTG AAC CAG TCA GCC ATC TT-3′; *HPRT1* forward 5′-TGG AGT CCT ATT GAC ATC GCC AGT-3′, reverse 5′-AAC AAT CCG CCC AAA GGG AAC TGA-3′.

First-Strand cDNA Synthesis

BMDM cells (5×10^5 cells/mL; 2 mL), CHME-5 cells (2.5×10^5/mL; 2 mL), or PBMCs (1.5×10^6/mL; 2 mL) were seeded in 6-well plates and grown for 24 h. Cells were then pretreated for 60 min with 10 μM INH-1 ODN, 2.5 μM FR180204, or 10 μM JSH-23 prior to stimulation with 5 μM of CpG-C or 100 ng/mL of LPS-EB for 4 h or infection with HSV-1 at MOI 1. Total RNA was isolated using ReliaPrep™ (Promega) according to the manufacturer's protocol. Isolated RNA (1 μg) was incubated with random hexamer primers (1 μL; 500 μg/mL) at 70 °C for 5 min. Thereafter, the other reaction components were added in the following order: 5 μL of 5× RT buffer, 1.3 μL of 10 mM dNTP, 1 μL of M-MLV reverse transcriptase (Promega), and nuclease-free water to a total volume of 25 μL. Reactions were incubated at 37 °C for 40 min followed by 42 °C for 40 min and heating to 80 °C for 5 min.

qPCR

Total cDNA (10 ng for BMDM cells and PBMCs and 20 ng for CHME-5 cells) was used as starting material for qPCR with CFX Connect™ qPCR system (BioRad) and GoTaq® qPCR Master mix (Promega) with dNTPs, 0.5 μM each. For the amplification of the specific genes the following primers were used: *Ifnb1* forward 5′-GGA GAT GAC GGA GAA GAT GC-3′, reverse 5′-CCC AGT GCT GGA GAA ATT GT-3′; *IFNB1* forward 5′-GCC GCA TTG ACC ATC TAT GA-3′, reverse 5′-GCC AGG TTC TCA ACA ATA G-3′; *Tnf* forward 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′, reverse 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′; *TNF* forward 5′-CAC CAC TTC GAA ACC TGG GA-3′, reverse 5′-CAC TTC ACT GTG CAG GCC AC-3′; *TIRAP* forward 5′-TTC ACC AAT GCC TGG TCT C-3′, reverse 5′-CTG AAC CAG TCA GCC ATC TT-3′; *Myd88* forward 5′-TTC AGC ATT TGG GAG GTA GAG GCA-3′, reverse 5′-GCG AAG CCA AAC AGC TTC TCC TTT-3′; *Tlr9* forward 5′-CAA CCT CCC CAA GAG CCT-3′, reverse 5′-TGC CAT TGG TCA GGG CC-3′. For each mRNA quantification, the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was used as a reference point using the following primers: *Hprt1* forward 5′-GCT TGC TGG TGA AAA GGA CCT CTC TCG AAG-3′, reverse 5′-CCC TGA AGT ACT CAT TAT AGT CAA GGG CAT-3′; *HPRT1* forward 5′-AGC TTG CTG GTG AAA AGG AC-3′, reverse 5′-TTA TAG TCA AGG GCA TAT CC-3′. Real-time PCR data were analyzed using the $2^{-(\Delta\Delta CT)}$ method.

Western Blotting

WT and MalKO BMDM cells were seeded (5×10^5 cells/mL; 2 mL) on a 6-well plate and grown for 24 h. Cells were then treated with 5 μM CpG-C, with or without inhibitors, as indicated and subjected to SDS-PAGE followed by Western blot analysis with anti-phospho-ERK1/2 (Cell Signaling), anti-total-ERK1/2 (Cell Signaling), anti-phospho-p38 (Cell Signaling), anti-total-p38 (Cell Signaling), anti-β-actin (Sigma), anti-IκBα (Santa Cruz Biotechnology), anti-phospho-p105 (Cell Signaling), anti-total-p105 (Cell Signaling), anti-phospho-p100 (Cell Signaling), anti-total-p100, anti-p50 (Cell Signaling), anti-p52 (Cell Signaling), anti-RelA (Cell Signaling), anti-RelB (Cell Signaling), anti-phospho-ATF2 (Cell Signaling), anti-phospho-c-Jun (Cell Signaling), anti-nucleolin (Cell Signaling) antibody, secondary antibodies: IRDye® 800CW goat anti-rabbit IgG (H + L), IRDye® 680RD donkey anti-mouse IgG (H + L). Imaging was performed using the Odyssey® CLx Infrared Imaging System (LI-COR®).

Nuclear Extract and Electrophoretic Mobility Shift Assay

Cells (1×10^6 cells/well; 6-well plate) were washed with ice-cold PBS and disintegrated in ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM sodium orthovanadate, 0.1% NP-40) on ice for 15 min. After centrifugation at 12,000 *g* for 1 min at 4 °C, the supernatants were removed and the nuclear pellets were resuspended in 3× the packed nuclear volume of ice-cold high-salt buffer B (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM PMSF). The samples were gently vortexed at 4 °C for 30 min, centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatants (the nuclear extracts) were saved. Nuclear extracts (10 μg of protein) were incubated with 50 fmol of a double-stranded oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ containing the NF-κB consensus-binding site and end-labeled with IRDye 700 infrared dye according to the manufacturer's protocol (Odyssey EMSA Buffer Kit, LI-COR). Electrophoretic mobility shift assay was performed in a 5% native polyacrylamide gel in TBE buffer (0.5×). The NF-κB-DNA complexes were then analyzed using the Odyssey Infrared Imaging System (LI-COR).

Data Analysis

Statistical analysis was carried out using the unpaired Student *t* test with the SigmaPlot 2001 program. *p* values ≤0.05 were considered to indicate a statistically significant difference.

Results

Mal Adapter Protein Mediates HSV-1-Induced, TLR9-Dependent Antiviral Response

HSV-1 is a neurotropic virus which is considered a major cause of central nervous system infections, such as herpes simplex encephalitis. Since microglial cells are the first cells to respond to viruses within the central nervous system by cytokine and chemokine production before lymphocyte infiltration, we decided to utilize a CHME-5 human microglial cell line in our research [17, 18]. To determine the possible role of Mal adapter protein in the TLR9-dependent signaling pathway, CHME-5 cells with *TIRAP* gene knockdown (MalKD) were generated with the use of the shRNA technique (Fig. 1a). Subsequently, cells were challenged with HSV-1, which is known to be the natural activator of TLR9-mediated immune response [19]. Thereafter, expression of mRNA for cytokines, IFNβ and TNF, was examined by qPCR. It was observed that HSV-1-induced *IFNB1* and *TNF* mRNA expression was markedly impaired in Mal-deficient cells when compared with control cells, suggesting that the Mal adapter is required for signal transduction from TLR9 (Fig. 1b and c, respectively). HSV-1 has the ability to activate other receptors from the Toll-like family, e.g., TLR2 or cytosolic DNA sensors [20–22]. Therefore, INH-1 ODN, a TLR9

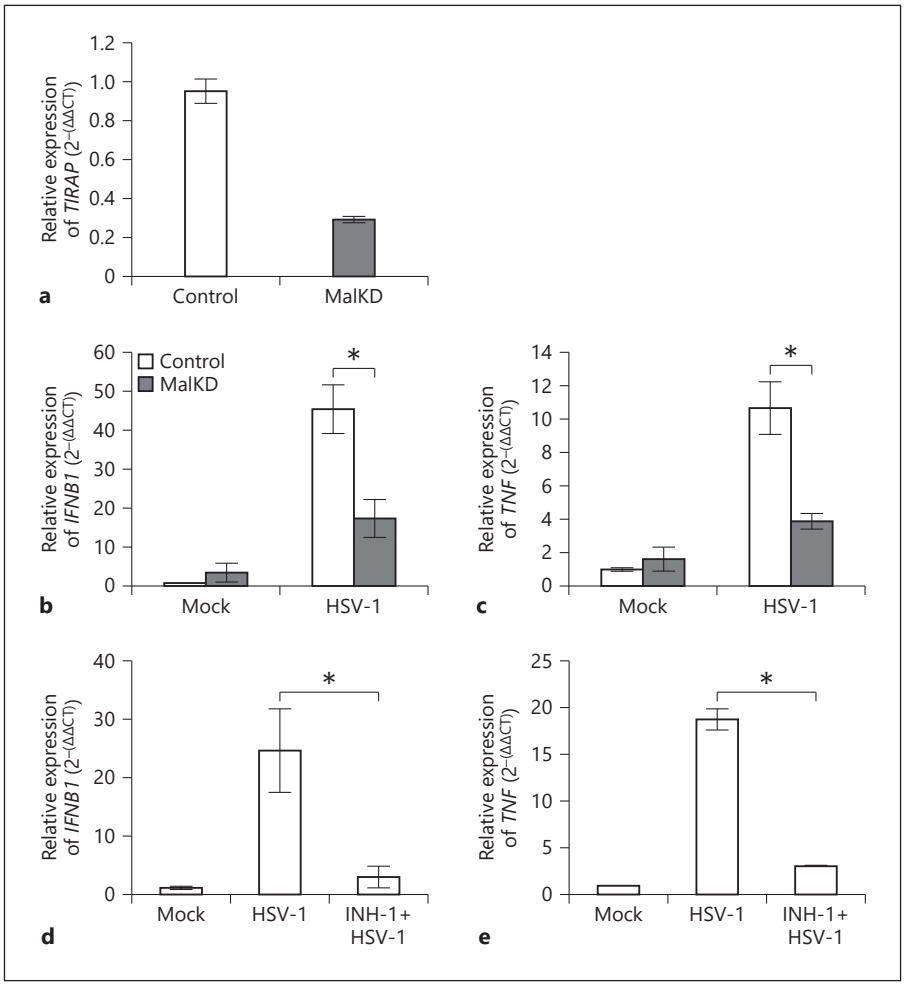

Fig. 1. Mal adapter protein mediates HSV-1-induced, TLR9-dependent antiviral response. **a** Knockdown of Mal expression in the CHME-5 cell line. CHME-5 cells were transduced with shRNA specific for *TIRAP* gene (MalKD) or scrambled shRNA (Control). Cells were cultivated with puromycin. Total RNA was isolated and relative expression of *TIRAP* was assayed with qPCR ($2^{-(\Delta\Delta CT)}$). **b**, **c** Activation of *IFNB1* and *TNF* expression by infection of CHME-5 microglial cells with HSV-1. Cells with *TIRAP* knockdown and control cells were infected with HSV-1 at MOI 1 for 8 h. Thereafter, total RNA was isolated and relative expression of *IFNB1* (**b**) and *TNF* (**c**) was assayed with the use of qPCR. *HPRT1* was used as a reference gene. Data are compiled from at least three independent experiments, each experiment being performed in duplicate (mean ± SD). **d**, **e** Specificity control of TLR9 activation by HSV-1. CHME-5 cells were infected with HSV-1 at MOI 1 for 8 h with or without INH-1 ODN (10 μM), added 1 h prior to infection. Subsequently, total RNA was isolated and the relative expression of *IFNB1* (**d**) and *TNF* (**e**) was assayed with the use of qPCR ($2^{-(\Delta\Delta CT)}$). Relative expression values were normalized to the *HPRT1* reference gene. Data are compiled from at least three independent experiments, each experiment being performed in duplicate (mean ± SD). * $p < 0.001$ (unpaired Student *t* test).

synthetic inhibitory oligonucleotide, was employed to confirm that cytokine expression in CHME-5 cells infected with HSV-1 is induced in a TLR9-dependent fashion. As shown in Figure 1d and e, the level of both *IFNB1* and *TNF* expression was significantly reduced in cells stimulated with INH-1 ODN prior to HSV-1 infection, suggesting that the immune response elicited by HSV-1 is dependent on TLR9.

A similar effect of Mal on *IFNB1* gene expression was observed in primary cells – human PBMCs. PBMCs were stimulated with a synthetic TLR9 ligand, C-type oligonucleotide containing unmethylated CpG sequences (CpG-C ODN) or HSV-1 along with the use of the MalIP. In cells prestimulated with the blocking peptide, expression of the *IFNB1* determined by qPCR was reduced in comparison to the cells treated with the CtrlP (Fig. 2a and b).

Next, to confirm that HSV-1-induced cytokine expression is mediated by TLR9, INH-1 ODN was used. It was observed that the induction of *IFNB1* gene expression was suppressed in cells pretreated with INH-1 ODN in comparison with cells infected with HSV-1 alone, which indicates that HSV-1 activated TLR9 in PBMCs (Fig. 2c).

Together, the data suggest that Mal adapter protein is engaged in TLR9-induced signal transduction in human microglia and PBMCs when stimulated with viral particles or synthetic ligand.

Knockout of Mal Abrogates IFNβ and TNF Production in BMDM Cells

To determine the exact mechanism of regulation of the TLR9 signaling pathway by Mal, immortalized macrophages derived from bone marrow of Mal-deficient mice (BMDM MalKO) and WT mice (BMDM WT) were utilized. Cells were stimulated with CpG-C ODN and analyzed by qPCR. We found that in CpG-C ODN-stimulated BMDM MalKO cells the expression of *Ifnb1* and *Tnf* genes was reduced in comparison with WT cells (Fig. 3a and b, respectively). Subsequently, it was checked wheth-

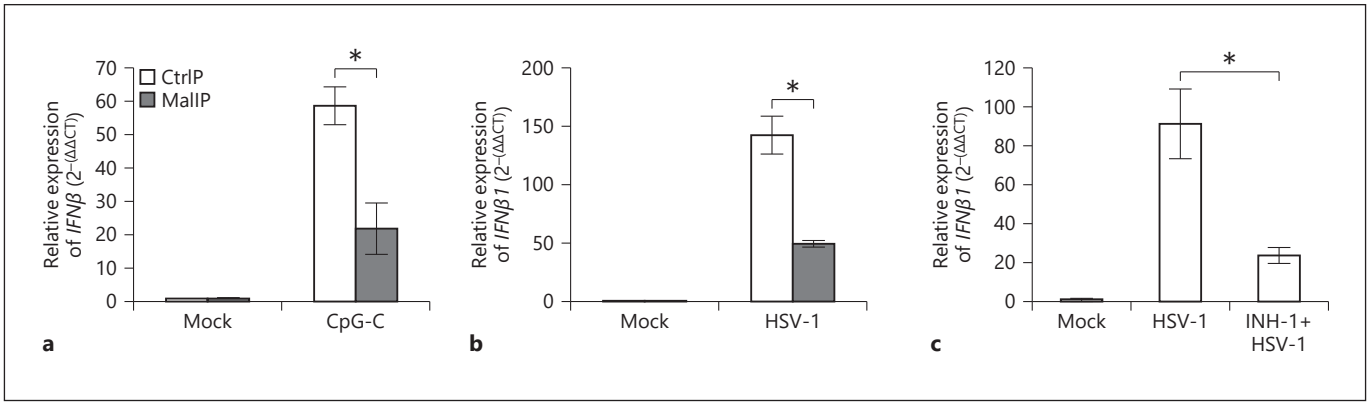

Fig. 2. a, **b** Suppression of Mal inhibits TLR9-dependent expression of *IFNB1* in human PBMCs. Human PBMCs, isolated from buffy coats, were pretreated with MalIP or CtrlP for 24 h. Thereafter, cells were stimulated with CpG-C (5 μM) for 4 h (**a**) or infected with HSV-1 MOI 1 for 8 h (**b**). Total RNA was isolated and relative expression of *IFNB1* was assayed with qPCR ($2^{-(\Delta\Delta CT)}$). *HPRT1* was used as a reference gene. **c** Specificity control of TLR9 activation by HSV-1. PBMCs were infected with HSV-1 MOI 1 for 8 h with or without INH-1 ODN (10 μM), added 1 h prior to infection. Subsequently, total RNA was isolated and relative expression of *IFNB1* was assayed with the use of qPCR ($2^{-(\Delta\Delta CT)}$). Relative expression values were normalized to the *HPRT1* reference gene. The results are representative of at least three independent experiments. * $p < 0.001$ (unpaired Student *t* test).

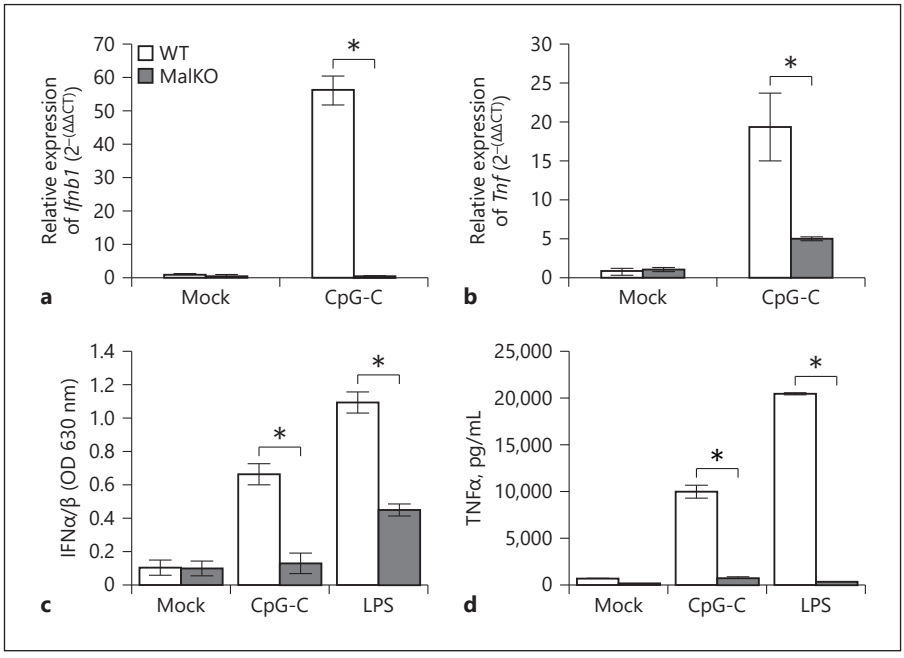

Fig. 3. a, **b** Knockout of *Mal* abrogates the production of IFNβ and TNFα in murine macrophages. WT and Mal-deficient (MalKO) BMDM cells were stimulated with the TLR9 ligand CpG-C (5 μM) for 4 h. Thereafter, total RNA was isolated and reverse transcribed. The relative expression of *Ifnb1* and *Tnf* was assayed with the use of qPCR ($2^{-(\Delta\Delta CT)}$). Relative expression values were normalized to the *Hprt1* reference gene. **c**, **d** Level of biologically active type I IFNs and TNFα in BMDM cells stimulated with the TLR9 ligand. WT and MalKO BMDM cells were stimulated with CpG-C (5 μM) for 24 h. Subsequently, the level of IFNα/β or TNFα in culture medium was measured with the use of bioassay and ELISA, respectively. The results are representative of at least three independent experiments. * $p < 0.001$ (unpaired Student *t* test).

er the data obtained for RNA correlated with the amount and activity of the corresponding proteins. The bioassay based on the B16-Blue cell line was used to measure bioactive murine type I IFNs, whereas TNFα release was assessed using ELISA test. Confirming the qPCR data, synthesis of biologically active interferons and production of TNFα in response to TLR9 ligand were significantly inhibited in MalKO cells (Fig. 3c and d, respectively).

Furthermore, to check whether the Mal knockout does not affect mRNA level of TLR9 and MyD88, which is a TLR9 adapter protein, we analyzed gene expression level. As shown in Table 1, neither *Tlr9* nor *Myd88* expression was significantly altered in MalKO cells in comparison with WT cells. These results suggest that observed impaired cytokine production by MalKO resulted from the lack of Mal expression only.

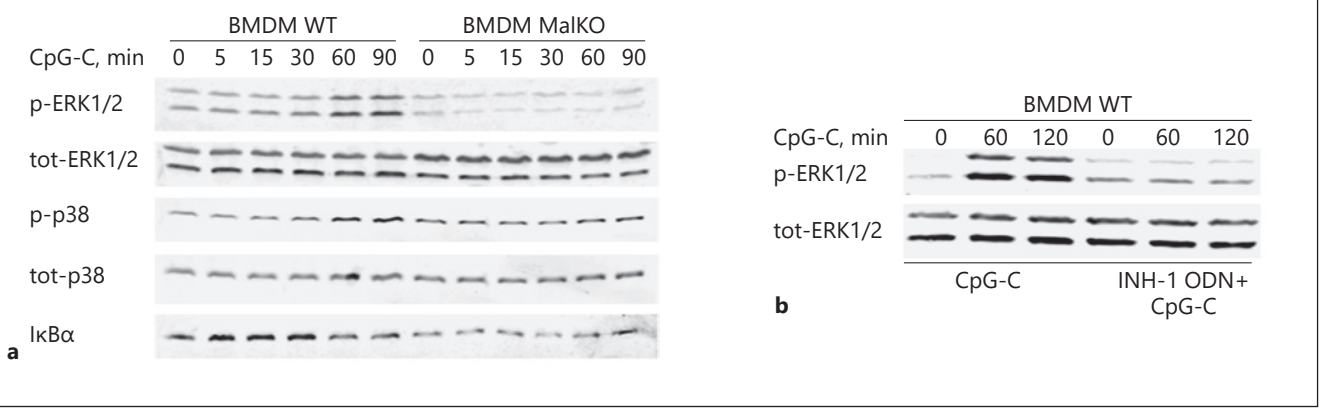

Fig. 4. a TLR9-dependent ERK1/2 activation is regulated by Mal in BMDM cells. WT and MalKO BMDM cells were stimulated with CpG-C (5 μM) for the indicated time. Cell lysates were subjected to SDS-PAGE. Detection of proteins was conducted with the use of specific antibodies and secondary antibodies conjugated with IR dye. **b** Specificity control of the ERK1/2 phosphorylation in WT and MalKO BMDM cells treated with TLR9 ligand. WT and MalKO BMDM cells were stimulated with CpG-C (5 μM) for the indicated time in the presence or absence of TLR9 inhibitor added 1 h prior to stimulation (INH-1 ODN, 10 μM). Cell lysates were subjected to SDS-PAGE. Protein detection was performed using specific antibodies and appropriate secondary antibodies conjugated with IR dye. Visualization was performed using the Odyssey CLx Imaging System LI-COR. The results are representative of at least three independent experiments.

Table 1. *Myd88* and *Tlr9* expression levels in WT and MalKO BMDM cells

	$\Delta C(T)$ [$C(T)_{GEN} - C(T)_{HPRT}$]	
	BMDM WT	BMDM MalKO
Myd88	4.94±0.03	5.09±0.14
Tlr9	4.95±1.19	5.17±0.52

TLR9-Dependent ERK1/2 Activation Is Regulated by Mal in BMDM Cells

In the next step, the ability of TLR9 to promote activation of classical signaling pathways in BMDM cells was assessed using Western blotting, as judged by phosphorylation or degradation of key proteins. Only the difference in late phosphorylation of ERK1/2 in WT versus MalKO BMDM cells was evident after TLR9 stimulation (Fig. 4a).

Next, to verify whether the activation of ERK1/2 upon CpG-C stimulation was dependent on TLR9, INH-1-ODN was used to block the receptor. Pretreatment of BMDM cells with INH-1, followed by TLR9 agonist stimulation, caused strong suppression of ERK1/2 phosphorylation, indicating that CpG-C specifically activated TLR9 without cellular stress induction (Fig. 4b).

It is well known that stimulation of TLRs leads to activation of NF-κB transcription factor. In resting cells NF-κB remains in the cytoplasm due to sequestration by IκBα. Therefore, we decided to examine whether IκBα is degraded in BMDM cells after TLR9 activation. Unexpectedly, IκBα degradation was observed neither in WT nor in MalKO cells stimulated with CpG-C, suggesting that induction of IFNβ and TNFα by TLR9 did not require the canonical mechanism of NF-κB activation. Furthermore, no differences in phosphorylation of p38 kinase were observed (Fig. 4a).

To explore the potential of ERK1/2 to mediate TLR9-dependent gene activation, we examined the expression of *Ifnb1* and *Tnf* in WT cells stimulated with CpG-C in the presence or absence of a specific inhibitor of these kinases – FR180204 [23]. As shown in Figure 5a, the production of mRNA for *Ifnb1* was abolished in cells treated with FR180204 prior to CpG-C, which was reflected in the production of the biologically active interferons (Fig. 5b). Also, a significant reduction in mRNA expression for *Tnf* was observed in cells pretreated with the ERK1/2 kinase inhibitor (Fig. 5c).

Taken together, these findings suggest that absence of Mal adapter protein prevents TLR9-dependent ERK1/2 activation and subsequent IFNβ and TNFα production.

ERK1/2 Is Phosphorylated in a Mal-Dependent Fashion in CHME-5 Cells

Considering the fact that previous experiments utilized a synthetic oligonucleotide in murine BMDM cells,

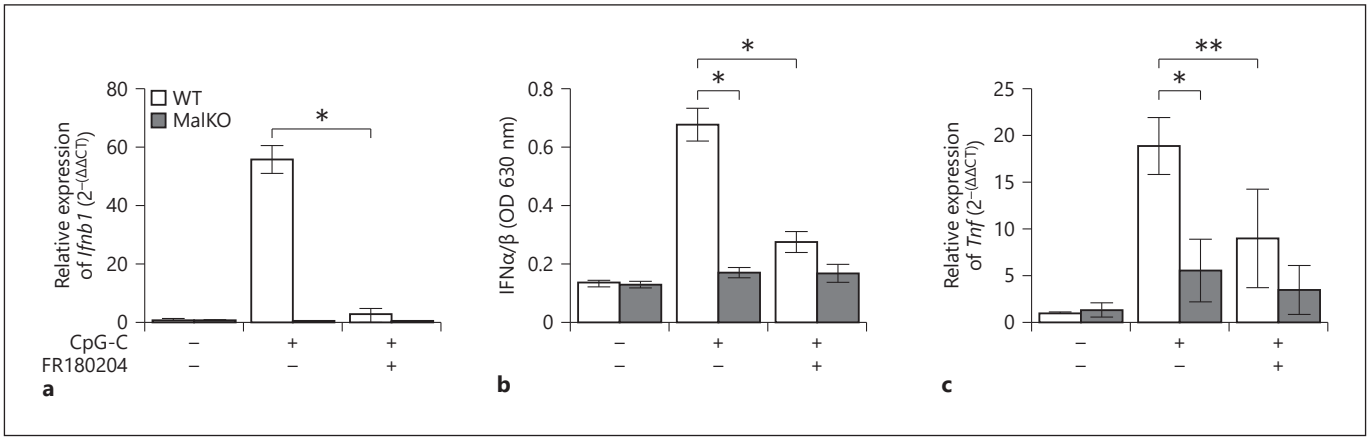

Fig. 5. Effect of ERK1/2 inhibition on *Ifnb1*, *Tnf* expression, and biologically active type I IFN release in BMDM cells. Cells were stimulated with CpG-C at 5 μM for 4 h in the presence or absence of an ERK1/2 inhibitor FR180204 (2.5 μM) added 1 h prior to stimulation. Next, total RNA was isolated and relative expression of *Ifnb1* (**a**) or *Tnf* (**c**) was assayed with qPCR ($2^{-(\Delta\Delta CT)}$) using *Hprt1* for normalization. Cells were stimulated with CpG-C at 5 μM for 16 h in the presence or absence of an ERK1/2 inhibitor FR180204 (2.5 μM) added 1 h prior to stimulation. Subsequently, the level of type I interferons in supernatants was determined by a biological method (**b**). Data are compiled from at least three independent experiments, each experiment being performed in duplicate (mean ± SD). * $p < 0.001$, ** $p < 0.05$ (unpaired Student *t* test).

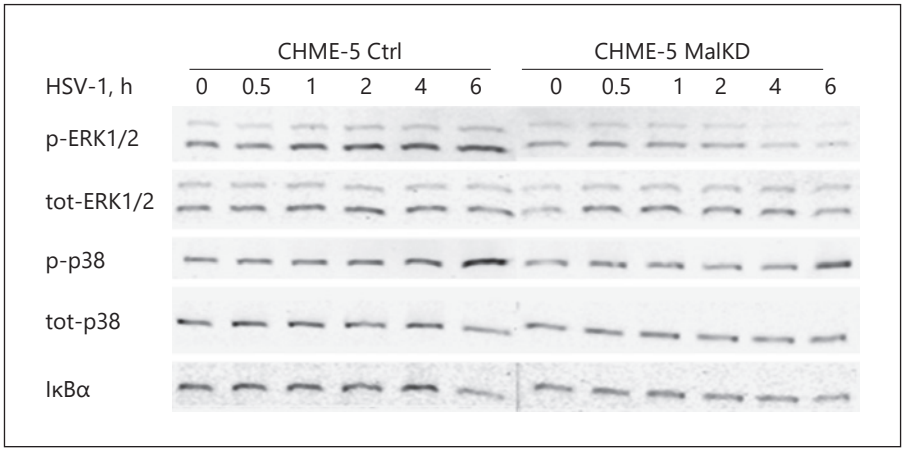

Fig. 6. ERK1/2 is phosphorylated in a Mal-dependent fashion in CHME-5 cells. Control and MalKD CHME-5 cells were infected with HSV-1 at MOI 1 for the indicated time. Cell lysates were subjected to SDS-PAGE. Detection of proteins was conducted with the use of specific antibodies and secondary antibodies conjugated with IR dye. Immunoreactivity was determined using the Odyssey Infrared Imaging System (LI-COR).

we decided to check whether similar effects would be observed in a human cell line using viral-dependent activation of TLR9. Therefore, CHME-5 cells were infected with HSV-1 and the activation of ERK1/2 and p38 kinase as well as the degradation of IκBα were determined as previously.

Infection with HSV-1 caused late activation of ERK1/2 kinases (1 h after infection) in the control cells unlike in the Mal-deficient cells (MalKD) in which phosphorylation of these kinases was not found. No differences were observed in the activation of p38 kinase, which was phosphorylated 6 h after infection with HSV-1, both in control and MalKD cells.

Furthermore, no degradation of the IκBα inhibitory protein was detected, suggesting that in CHME-5 cells, stimulation of TLR9 does not trigger the classical activation of NF-κB (Fig. 6), similarly to BMDM cells.

TLR9-Induced Activation of NF-κB in Macrophages Is Mal-Dependent

Given that degradation of IκBα in BMDM cells stimulated with CpG-C was not observed and based on published reports indicating that after TLR9 activation NF-κB is translocated to the cell nucleus, we decided to check whether the noncanonical NF-κB pathway is activated by CpG-C in BMDM cells and whether Mal adapter protein

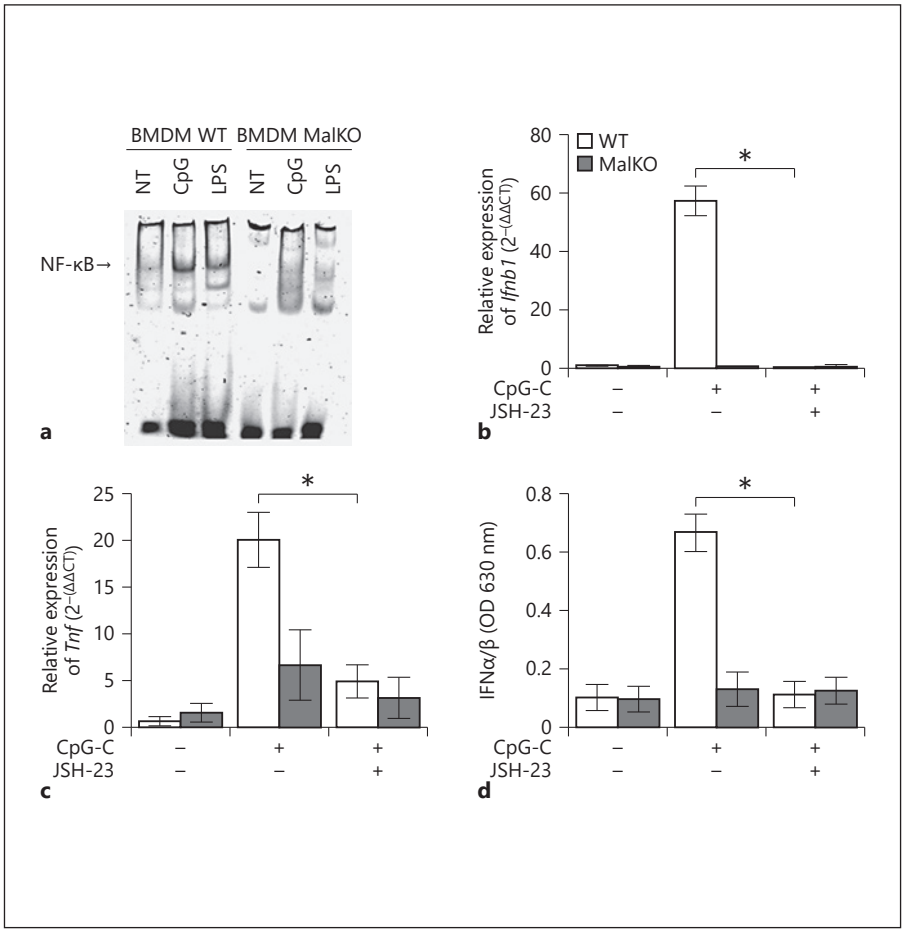

Fig. 7. TLR9-induced activation of NF-κB in macrophages is Mal-dependent. **a** Translocation of the NF-κB transcription factor in BMDM cells treated with TLR9 ligand. WT and MalKO BMDM cells were stimulated with CpG-C (5 μM) for 2 h. The nuclear fractions were then isolated and incubated with specific, fluorescently labeled oligonucleotide probes to bind the NF-κB factor. The samples were electrophoresed on a polyacrylamide gel. Visualization was perform ed using the Odyssey CLx Imaging System (LI-COR). The results presented are representative of at least three independent experiments. **b–d** Effect of inhibition of NF-κB translocation on *Ifnb1*, *Tnf* expression, and biologically active type I IFN release in BMDM cells. Cells were stimulated with CpG-C (5 μM) for 4 h, in the presence or absence of JSH-23 (10 μM), the NF-κB nuclear translocation inhibitor, added 1 h prior to stimulation. Next, total RNA was isolated and relative expression of *Ifnb1* (**b**) or *Tnf* (**c**) was assayed with qPCR ($2^{-(\Delta\Delta CT)}$) using *Hprt1* for normalization. Cells were stimulated with CpG-C at 5 μM for 16 h in the presence or absence of JSH-23 (10 μM) added 1 h prior to stimulation. Subsequently, the level of type I interferons in supernatants was determined by a biological method (**d**). Data are compiled from at least three independent experiments, each experiment being performed in duplicate (mean ± SD). $^*p < 0.001$.

is engaged in this process [24]. Using the electrophoretic mobility shift assay method, we found that in MalKO BMDM cells stimulated with CpG-C, the DNA-binding activity of NF-κB was markedly reduced in comparison with control cells.

It is well known that NF-κB is strongly activated by LPS in a Mal-dependent fashion. Thus, cells were stimulated with LPS as a control. As previously, NF-κB binding activity was induced only in WT cells (Fig. 7a). Next, we wanted to check whether inhibition of NF-κB translocation would affect the expression of CpG-C-induced cytokines. For this purpose, the pharmacological inhibitor of NF-κB – JSH-23 – was used. As shown in Figure 7b and d, treatment of cells with JSH-23 prior to stimulation with CpG-C ODN abolished *Ifnb1* gene expression as well as biologically active interferon release. Moreover, *Tnf* gene expression was also significantly decreased when cells were pretreated with JSH-23 (Fig. 7c).

Based on these observations, we decided to further explore the activation of the NF-κB transcription factor. The active NF-κB is a dimer composed of two proteins from two families: NF-κB (p50, p52), which occurs in the form of inactive precursors p105 and p100, respectively, and Rel (c-Rel, RelA [p65], RelB) [25]. Therefore, to check the Mal-dependent activation of NF-κB, we investigated the phosphorylation of the p105 and p100 proteins. In the CpG-C-stimulated WT cells, presence of the phosphorylated form of p105 was observed, in contrast to MalKO cells, whereas the p100 protein was phosphorylated neither in WT nor in MalKO cells (Fig. 8a). The formation of the active form of NF-κB leads to its translocation to the cell nucleus; thus, we examined the presence of NF-κB subunits in nuclear fractions obtained from CpG-C-stimulated cells. Interestingly, in WT cells translocation of the RelA and c-Rel subunits was observed, as was p105 phosphorylation and a corresponding increase in the p50 protein level. In contrast, in the nuclear fractions obtained from MalKO cells only RelA was identified after TLR9 activation, which indicated that the formation of the c-Rel/p50 complex was dependent on the Mal protein (Fig. 8b).

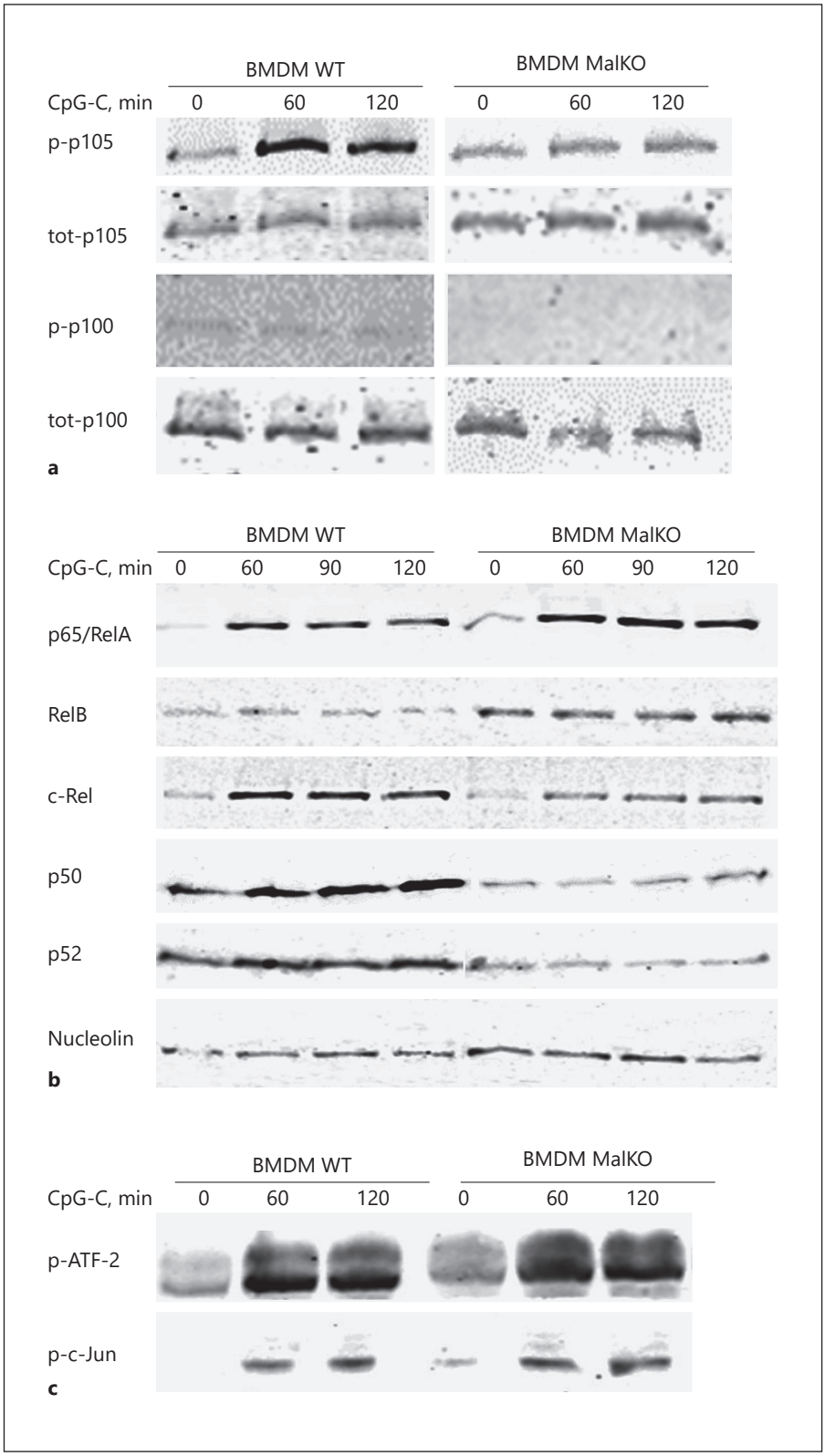

Fig. 8. Activation of NF-κB and AP-1 transcription factor subunits in BMDM cells treated with TLR9 ligand. WT and MalKO BMDM cells were stimulated with CpG-C (5 μM) for the indicated time. Subsequently, cell lysates (**a**, **c**) or nuclear fractions (**b**) were subjected to SDS-PAGE. Detection of individual proteins was carried out with the use of specific antibodies and appropriate secondary antibodies conjugated with IR dye. Visualization was performed using the Odyssey CLx Imaging System (LI-COR). The results are representative of at least three independent experiments.

Additionally, to ensure that the TLR9- and Mal-dependent cytokine expression is induced by NF-κB, we examined the activation of AP-1 transcription factor, which is another activator of IFNβ and TNFα gene transcription, in addition to NF-κB. The key components of the AP-1 factor are ATF-2 and c-Jun; therefore, we investigated the phosphorylation of these proteins in WT and MalKO cells stimulated with TLR9 ligand. As shown in Figure 8c, upon TLR9 stimulation both AP-1 subunits were phosphorylated in WT and MalKO cells, and the level of activated proteins was equal despite the presence or absence of Mal protein. Taken together, these findings clearly indicate that the noncanonical mechanism of NF-κB activation is triggered by TLR9 and regulated by Mal adapter protein in macrophages.

Discussion

Since its discovery, the Mal adapter protein has been considered an accessory protein utilized solely by TLRs anchored in the cell membrane. This assumption was based not only on pioneering experiments on cells with the knockout of the Mal-encoding gene, but also on characteristic structural features of Mal, precisely the presence of the N-terminal motif that binds 4,5-bisphosphate, which predominantly occurs in lipid rafts of the cell membrane [12, 13, 15].

In 2010, Mal was shown to participate in the TLR3-dependent signaling pathway; however, in this particular case, it acted as a negative regulator since cells lacking this protein showed increased expression of the interferon-encoding gene [16].

Here, we show for the first time that Mal is involved in TLR9-dependent expression of genes encoding IFNβ and TNFα through the activation of ERK1/2 kinases. Furthermore, we demonstrate that the noncanonical NF-κB pathway is required for TLR9-induced, Mal-regulated inflammatory response.

Our findings are consistent with results shown by Bonham et al. [26], which indicate that Mal can anchor not only to cell membrane lipid rafts, but also to endosomal vesicles. This fact supports the hypothesis that Mal associates with the TIR domain of all TLRs that engage the MyD88 adapter in their signaling pathway.

The initial research was carried out employing HSV-1, the natural ligand of TLR9, which propagates in nerve cells. The protection of the central nervous system from pathogens crossing the blood-brain barrier is driven mainly by microglia. Therefore, CHME-5 cells, a human microglial cell line, served as a model for the research.

Various elements are involved in the recognition of this virus, starting from receptors specific to the capsid decorating proteins to cytosolic sensors of viral nucleic acid. Among TLRs, TLR9 and TLR2 are involved in the recognition of the HSV-1 virus elements. TLR2 is the receptor for capsid proteins, while TLR9 recognizes viral DNA [17, 22].

To date, only Bonham et al. [26] have postulated that Mal is involved in the regulation of the TLR9-dependent immune response to HSV-1. In agreement with this report, we showed that HSV-1-induced inflammatory response, manifested by IFNB1 and TNF expression, depends on TLR9 and is regulated by Mal protein. Our data demonstrated that TLR9 inhibitory ODN and Mal knockdown abolished cytokine production in response to TLR9 stimulation. Moreover, the same effect was observed in PBMCs treated with MalIP. MalIP directly interacts with the Mal TIR domain, which prevents its recruitment to the myddosome and subsequent cytokine induction. Thus, the distinct mechanisms of Mal "switch-off" result in the convergent experimental observations.

There are three subsets of ODNs: multimeric CpG-A, monomeric CpG-B, and CpG-C – combining the features of forms A and B [27]. Structural differences between these oligonucleotides determine different migration times through endosomal vesicles, resulting in the expression of different cytokine repertoires. CpG-A ODNs, having polyG tails, form aggregates and, due to their complex structure, persist longer in early endosome. Activation of the TLR9 receptor by CpG-A leads to recruitment of the MyD88 adapter protein followed by IRF7-dependent production of type I IFNs. The monomeric structure of CpG-B facilitates its fast transition to late endosome, where it binds TLR9 and activates the MyD88/TRAF6/IRAK4/NF-κB signal pathway followed by expression of cytokines, such as IL-1 and TNFα [28–30]. CpG-C does not form aggregates due to lack of polyG tails, but it has a palindromic sequence that determines the activation of TLR9 in early and late endosomes. In the study, we used CpG-C because it shares the functions of CpG-A and CpG-B. Cytokine expression was significantly reduced in Mal-deficient cells as compared to WT cells, which is in line with the results obtained by Bonham et al. [26].

To explain the mechanism of this phenomenon, we studied the activation of the TLR/Mal/MyD88-dependent pathway and found that ERK1/2 phosphorylation in Mal-deficient cells was abrogated. In the light of available data, it remains controversial whether activation of

ERK1/2 kinases leads to TLR9-dependent cytokine induction. Earlier studies showed that CpG failed to induce substantial phosphorylation of ERK within 1 h in J774 cells [31]; however, others indicated that TLR9 activates ERK1/2 in murine BMDM cells with delayed kinetics, e.g., by a mechanism involving autocrine ROS signaling [32]. Moreover, the negative role of ERK1/2, regulated by Tpl2 kinase was postulated in the induction of IFNβ production [33]. On the other hand, our experiments clearly demonstrated that CpG-C-stimulated ERK1/2 phosphorylation was evident only in WT cells and that the inhibitor of ERK1/2 caused suppression of cytokine production. Moreover, we observed altered NF-κB activation that depended on Mal protein.

NF-κB transcription factor is activated by all known TLR ligands and plays a pivotal role in the immune response by regulation of cytokine and chemokine expression. The canonical route of NF-κB signaling involves degradation of IκBα inhibitory subunit and formation of RelA/p50 heterodimer [25]. Based on our experiments showing differences in the translocation of NF-κB subunits to the cell nucleus in WT and MalKO cells, it can be assumed that TLR9/MyD88/Mal activation does not lead to stimulation of the canonical NF-κB signaling pathway, but rather to formation of the c-Rel/p50 dimer. Interestingly, studies using mice with p50, RelA, and c-Rel knockout indicate that NF-κB plays a minor role in the activation of IFNβ gene expression [34]. In other reports, indirect involvement of NF-κB in the regulation of IFNβ production was observed, particularly in the expression of the gene encoding YY1 transcription factor. The YY1 factor may act either as an activator or repressor of transcription depending on the site of attachment to the IFNβ gene promoter. In the case of TLR3, which is the only TLR functioning without MyD88 engagement, it was shown that the RelB and c-Rel subunits are activated, which leads to induction and translocation of YY1 to the nucleus, where it acts as a transcriptional repressor, preventing the binding of IRF transcription factors to the IFNβ gene enhancer [35]. Our data showed that JSH-23 (the inhibitor of NF-κB) nearly abolished the cytokine expression induced by CpG-C. Thus, we postulate that c-Rel/p50 plays a positive role in the Mal-regulated TLR9-dependent pathway.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J. Zyzak developed the concept, designed and performed the experiments, analyzed the data, and prepared the figures and the initial version of the manuscript. M. Mitkiewicz, E. Leszczyńska, and P. Reniewicz designed and performed the experiments and analyzed the data. P.N. Moynagh participated in results interpretation and manuscript preparation. J. Siednienko conceived the study, supervised the project, analyzed the data, and corrected the manuscript.

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