Kinetic of RelA Activation Controls Magnitude of TLR-Mediated IL-12p40 Induction

Konrad A. Bode,* Frank Schmitz, † Leonardo Vargas,‡ Klaus Heeg,* and Alexander H. Dalpke2*†

IL-12 is a crucial cytokine for dendritic cell-mediated induction of Th 1 cell differentiation. TLR ligands induce IL-12 to differing extents. Stimulation of dendritic cells allowed for the differentiation of three groups of TLRs; potency to induce IL-12 decreased in the order of TLR7/9, TLR3/4, and TLR1/2/6 stimulation. The MAPK, PI3K, and IRF (IFN regulatory factor) signaling pathways could be ruled out to be the cause for the differences in IL-12p40 induction. However, we observed that stimulation of dendritic cells with different TLR ligands resulted in striking differences in the kinetics of NF-κB activation. LPS induced a rapid but short-lived activation of RelA, whereas CpG-DNA stimulation resulted in prolonged RelA activity at the IL-12p40 promoter. Only TLR2 and TLR4 ligands were capable of inducing S536 phosphorylation of RelA, which has been proposed to be responsible for early termination of NF-κB activation. It is suggested that differences in the kinetics of a common TLR signaling module affect the biological response patterns of various TLRs, with IL-12p40 being a gene that needs prolonged NF-κB activation. The Journal of Immunology, 2009, 182: 2176–2184.

Interleukin-12 is a heterodimeric, proinflammatory cytokine composed of two subunits, p35 and p40, of which the latter is exclusively expressed in APCs (1). The main inducers of IL-12p40 expression are components from microorganisms, including cell wall fragments, structure proteins, double-stranded RNA and bacterial DNA, and the foremost ligands of TLRs (1). A main function of IL-12 is induction of the differentiation of naive T cells into effector Th1 cells. Mice lacking IL-12 have a strong defect in cell-mediated immune responses resulting in a high susceptibility to infections with intracellular pathogens including Leishmania major, Listeria monocytogenes, or Toxoplasma gondii (2–4). In contrast, exaggerated expression of Th1-inducing cytokines may contribute to the development and perpetuation of chronic inflammatory and autoimmune diseases.

Pattern recognition receptors are known to be key elements in innate immune responses, detecting conserved molecular structures of microorganisms. Activation of these pattern recognition receptors results in the production of a variety of cytokines and chemokines that differ in dependency of the activating microbes or the components derived thereof (5).

TLRs play a pivotal role in pattern recognition and activation of APCs. All TLRs contain an intracellular Toll/IL-1R (TIR)3 domain that initiates signaling by recruiting one or more of five identified TIR-containing adapter proteins (5). The adapter molecules are responsible for the recruitment of further downstream proteins to the receptor complex, thus initiating intracellular signaling that leads to alterations in gene expression. Four important groups of signaling pathways are key mediators of TLR-induced signal transduction: the NF-κB pathway, the group of MAPKs, the PI3K pathway, and members of the IFN regulatory factor (IRF) family.

NF-κB transcription factors compose central key regulators of inflammation. The NF-κB transcription factor family encompasses five members, the three transactivation domain-containing proteins RelA, RelB, and c-Rel as well as the transcriptionally inactive proteins p50 and p52 (6). The NF-κB/Rel-family molecules are forming different homodimers or heterodimers. In the case of prototypical NF-κB activation, RelA as well as c-Rel-containing dimers are held in the cytoplasm by IκBα, which masks a nuclear localization signal and prevents translocation into the nucleus (6). Upon stimulation, IκBα becomes phosphorylated, ubiquitinylated, and degraded, thereby releasing NF-κB heterodimers that translocate to the nucleus and mediate gene activation (6). NF-κB-induced resynthesis of IκBα serves as a negative feedback mechanism for postinduction repression of NF-κB activation. In addition to the IκBα-dependent regulation of NF-κB activity, covalent modifications such as phosphorylation, acetylation, and ubiquitination of the respective NF-κB molecules are necessary for proper function (7).

The starting point for our interest in the TLR ligand-induced IL-12p40 expression was the observation that despite the activation of a shared set of signal transduction molecules, the biological effects of certain TLR agonists differ considerably. Stimulation of dendritic cells by different TLR ligands resulted in striking differences in the expression of IL-12p40, whereas induction of another proinflammatory cytokine, TNF-α, was comparable. Because IL-12p40 is a key regulator in Th1 polarization,
this phenomenon is of high physiological relevance. The present study shows that kinetics of RelA activation and serine 536 phosphorylation of RelA are crucial control variables that regulate biological activity of different TLRS in terms of IL-12 induction.

Materials and Methods
Phosphotoioate-modified CpG-oligomeric nucleotide 1668 (TCCATGACGTC TCGATGCT) was custom synthesized by TIB Molbiol. LPS from Salmonella enterica serovar Typhimurium was provided by U. Seydel (Research Center Borstel, Borstel, Germany). Pam3CSK4 was obtained from Alexix; R848, poly- \( \text{I}\) icosinopolycyclidic acid, R-FLS, flagellin, and imiquimod were from InvivoGen; NF-κB/RelA, RelB, and c-Rel Abs were obtained from Santa Cruz Biotechnology; Abs against IKKα, IKKβ, phospho-ERK, phospho-JNK, phospho-p38, phospho-IκBα and phospho-S536-RelA were purchased from Cell Signaling Technology. The Ab against β-actin was from Upstate Biotechnology. The inhibitors BAY 11-7082, LFM-A13, Ly 294002, JNKI, SB203580, and PD98059 were from Calbiochem; and the MEK1 inhibitor U0126 was from Cell Signaling Technology.

BALB/c mice and C57BL/6 mice were purchased from Charles River Laboratories; IRF-1-deficient mice (strain Ivl/P, C57BL/6, and C3H/Hej mice, and C3H/Bl6 mice) were obtained from The Jackson Laboratory; and IRF-3 and IRF-7, gifts from T. Taniguchi (University of Tokyo, Tokyo, Japan), were crossed with IRF-1-deficient animals to generate IRF-1/-3/-7 triple-deficient mice. IRF-7, gifts from T. Taniguchi (University of Tokyo, Tokyo, Japan), C3H/Hej mice (8) were from The Jackson Laboratory; and IRF-3 and Bruton’s tyrosine kinase (Btk)-deficient mice (13, 14) were obtained from F. Greten (Technical University Munich, Germany), and Bruton’s tyrosine kinase (Btk)-deficient mice (15, 16) were obtained from L. Vargas (Karolinska Institute, Stockholm, Sweden).

Generation of primary bone marrow-derived dendritic cells (BMDCs)
Dendritic cells were prepared from female 4- to 10-week-old mice as described by Inaba et al. (17) with minor modifications. Briefly, bone marrow was collected from femurs and tibiae. Cells were placed in 70-cm² tissue culture flasks in differentiation medium (RPMI 1640 medium supplemented with 10% FCS, 50 mM 2-ME, the antibiotics penicillin G (100 IU/ml), and streptomycin sulfate (100 IU/ml) and 200 U/ml GS-CMF). After 24 h, nonadherent cells were collected, washed, and counted. The cells (10³) were then seeded into 175-cm² tissue culture flasks in differentiation medium. On day 5, fresh differentiation medium was added and on day 9 nonadherent, immature dendritic cells (CD11c⁺, B220⁻) were harvested. Culture supernatant of a GM-CSF-transfected cell line was used as a source of GM-CSF.

Western blotting
After stimulation, cells were lysed for 30 min on ice in 100 μl of lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Igepal, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μM each aprotinin, leupeptin, and pepstatin, 1 mM Na₃VO₄, and 1 mM NaF). Lysates were cleared by centrifugation at 4°C for 10 min at 11,000 x g. Equal amounts of the lysates were fractionated by 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked with TBS (pH 7.8), 5% nonfat dry milk, and 0.05% Tween 20 and were blotted with the indicated Abs. Detection was by ECL (Amersham). Abs were used according to the manufacturer’s protocol.

Determination of cytokine secretion
Cell-free supernatant was harvested and analyzed for cytokines by commercially available ELISA kits (OptEIA; Becton Dickinson).

Quantitative RT-PCR
Total RNA was isolated using Qiagen RNeasy kits (Qiagen) or HighPure RNA kits (Roche), which included DNase I digestion. A total of 1 μg of RNA was reverse transcribed with a cDNA synthesis kit (MBI Fermentas) using oligo(dt) primer. cDNA was diluted 1:4 and 2.5 μl were used as a template in 25 μl of TaqMan-PCR mix according to the manufacturer’s protocol (reagents were from Applied Biosystems). Quantifications were made using fluorogenic probes (FAM/TAMRA; Eurogentec). The specificity of RT-PCR was controlled by nontemplate and no-control RTs. Quantitative PCR results were expressed relative to the housekeeping gene β-actin (1/2^{ΔΔCt}) (where Ct is threshold cycle).

Chromatin immunoprecipitation assay (ChIP)
ChIP was conducted as described by Saccani et al. (19). Briefly, cells were fixed by adding formaldehyde to a final concentration of 1% (37% HCHO in 10% methanol; Calbiochem). After 7 min, crosslinking was stopped by adding glycine to a final concentration of 125 mM. The cells were transferred to a 1.5-ml tube, centrifuged, and washed three times with ice-cold PBS. Nuclei were isolated by incubating cells in L1 buffer (50 mM Tris (pH 8.0), 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol supplemented with protease inhibitors) for 5 min on ice and by centrifuging for 5 min at 3,000 rpm and 4°C. Nuclei were resuspended in L2 buffer (50 mM Tris (pH 8.0), 5 mM EDTA, and 1% SDS). For chromatin fragmentation, samples were treated with ultrasonic power (DNA fragments between 0.5 and 1.2 kbp). Samples were centrifuged for 5 min at 13,000 rpm at 4°C and the supernatants were transferred into new tubes and diluted 10 times with D buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 200 mM NaCl, and 0.5% Nonidet P-40). Extracts were precleared for 2 h with 60 μl of protein A-agarose. Immunoprecipitation was conducted overnight at 4°C using 2 μg of Ab. Immunocomplexes were collected with 20 μl of salmon sperm- saturated protein A-agarose for 30 min. Immunocomplexes were washed four times (5 min) with high-salt buffer (20 mM Tris (pH 8.0), 2 mM EDTA, 0.1% SDS, 1% Nonidet P-40, and 0.5 M NaCl) and three times with no-salt buffer (1X TE containing 10 mM Tris and 1 mM EDTA). Immune complexes were extracted in 120 μl 1X TE containing 2% SDS and protein/DNA crosslinks were reverted by heating at 65°C. DNA was purified using a QIAquick kit (Qiagen). One-twentieth of the immunoprecipitated DNA was used in quantitative PCR. For normalization, DNA was isolated from the precleared primary solution and one-twentieth of the DNA was used in PCR. Results were normalized by subtracting the obtained input DNA Ct value from that of the specific target gene. The ΔCt value inversely correlated with the transcription factor association at the region being amplified.

EMSA
Nuclear extracts were prepared as described by Andrews and Faller (20). EMSAs were performed using the LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer’s protocol with minor modifications; instead of the provided binding buffer, a 5X binding buffer containing 100 mM HEPES (pH 8.0), 250 mM KCl, 2.5 mM DTT, 25 μM EDTA, 5 mM MgCl₂, and 25% glycerin was used. Sequence of the biotinylated NF-κB probe was 5’-AGTTGAGGGGACTTTCCCAGGC-3’. For semiquantitative analysis of the changes of NF-κB DNA-binding activity, sequential dilutions of nuclear extracts of 20 min LPS-stimulated BMDCs was generated. The quantification of the bands was performed using the BIO-VISION video documentation system and the BIO-1D advanced software from PEQLAB Biotechnologie. The NF-κB-binding activity observed after 40 min of LPS stimulation was set to 100% maximal DNA-binding activity. For the supershift experiment, nuclear extracts were preincubated in the binding buffer for 15 min with 1 μg of the respective Ab before adding the DNA probe.

TransAM transcription factor ELISA
For the NF-κB ELISA, nuclear extracts were prepared and analyzed according to the manufacturer’s protocol (Active Motif).

Statistical analysis
All experiments were repeated at least three times. If not stated otherwise mean ± SD is shown. Significant differences were evaluated by the unpaired Student’s t test with two-tailed distributions. Values of p < 0.05 were considered significant and are indicated by one asterisk (*).

Results
Differential potency of various TLR ligands to induce IL-12p40 in dendritic cells
To examine differences in the cytokine response patterns of varying TLRS, the capacity of different TLR ligands to induce IL-12p40 in BMDCs was tested. Fig. 1A shows that CpG-DNA (TLR9) and R848 (TLR7) are strong inducers of IL-12p40 expression, whereas LPS (TLR4) and polyinosinic-polycytidylic acid (TLR3) were significantly less active. Moreover, TLR2/TLR6 and TLR2/TLR1 stimulation by lipoteichoic acid, R-FLS,
or Pam3CSK4 was largely ineffective in the induction of IL-12p40. To stress the physiological relevance of the findings, we further tested the bioactive IL12p70 with similar results (Fig. 1B). In contrast, all of the TLR ligands investigated induced TNF-α and IL-6 secretion in a comparable range (Fig. 1, C and D). TLR ligand-mediated secretion of IL-12p40 was not detectable until 4 h after stimulation and increased nearly linearly during the next 10 h (Fig. 1E), whereas TNF-α secretion started very rapidly but ceased already 8 h after initial stimulation (Fig. 1F).

To get a first insight into the underlying mechanisms for this differing induction of IL-12p40, quantitative RT-PCR experiments were performed (Fig. 1G). Stimulation of BMDCs with CpG-DNA, LPS, and Pam3CSK4 showed striking differences in the capacity to induce IL-12p40 mRNA, resulting in the order TLR9 > TLR4 > TLR1/2 and confirming the data obtained for secreted protein. Again, the tested TLR ligands were equally active when examining the induction of TNF-α mRNA (Fig. 1H). To confirm that differences of IL-12p40 mRNA upon stimulation with CpG-DNA and LPS were due to different transcription rates rather than changes in mRNA stability, we measured the IL-12p40 pre-mRNA concentration (unspliced or partially spliced RNAs). CpG-DNA induced 30- and 34-fold higher concentrations of IL-12p40 pre-mRNA than LPS at 0.5 and 2 h of stimulation, respectively (data not shown).

MAPKs are not responsible for the differential induction of IL-12p40 secretion

At least four important groups of signaling pathways are involved in TLR ligand cell activation, including MAPKs, the PI3K/Akt pathway, members of the IRF family, and the NF-κB pathway. Previous reports suggested ERK activation to be an important regulator of IL-12p40 (21). We therefore tested the activation patterns of the three groups of MAPKs (Fig. 2A). BMDCs were stimulated for 10–120 min with CpG-DNA, LPS, or Pam3CSK4. Stimulation of BMDCs with LPS or Pam3CSK4 resulted in a time-dependent phosphorylation of JNK, ERK, and p38 that was already detectable after 5–10 min, increased up to 20 min, and vanished after 40 min. In contrast, CpG-DNA induced a delayed and much weaker phosphorylation of p38, ERK, and JNK. Preincubation of BMDCs with the pharmacological p38 inhibitor SB203580 and the JNK inhibitor JNKI resulted in a strong inhibition of the IL-12p40 expression; however, the relative differences of IL-12p40 upon stimulation with the different TLR stimuli were preserved (Fig. 2, B and C). Preincubation of BMDCs with both MEK1 inhibitors, UO126 and PD98059, resulted in a slightly increased IL-12p40 expression, again without affecting the proportion of IL-12p40 secretion after stimulation with CpG-DNA, LPS, and Pam3CSK4 (Fig. 2D).

Inhibition of the PI3K/Akt pathway does not impair the differential induction of IL12p40 expression

Stimulation of BMDCs with CpG-DNA, LPS, and Pam3CSK4 resulted in a weak phosphorylation of Akt with no difference in the activation kinetics between the three ligands (Fig. 2A). The inhibition of the PI3K/Akt pathway with the pharmacological Akt inhibitor SB203580 and the JNK inhibitor JNKI resulted in a strong inhibition of the IL-12p40 expression; however, the relative differences of IL-12p40 upon stimulation with the different TLR ligands were preserved (Fig. 2G). Preincubation of BMDCs with both MEK1 inhibitors, UO126 and PD98059, resulted in a slightly increased IL-12p40 expression, again without affecting the proportion of IL-12p40 secretion after stimulation with CpG-DNA, LPS, and Pam3CSK4 (Fig. 2D).

Members of the IRF family are not responsible for the differential induction of IL-12 secretion in dendritic cells observed after stimulation with various TLR ligands

It has been shown that IRF family members such as IRF-1 and IRF-2 are involved in the regulation of IL-12p40 (22). However, Schmitz et al. recently showed that in BMDCs of IRF-1 knockout mice, the differences in CpG-DNA- and LPS-induced IL12p40 expression still could be observed (23). To rule out the possibility that IRF-3 and IRF-7 might contribute, we also analyzed IRF-1/IRF-3/IRF-7 triple-knockout BMDCs (Fig. 2F). Again, the differences in IL-12p40 expression upon stimulation with the different TLR ligands, CpG-DNA and LPS, were not altered, indicating that IRF-1, IRF-3, and IRF-7 are not responsible for the different potency in IL-12p40 induction.

FIGURE 1. Differential induction of IL-12p40 by TLR-dependent stimuli. A–D, BMDCs were stimulated with CpG-DNA (1 μM), LPS (100 ng/ml), polyinosinic-polycytidylic acid (PIC) (50 μg/ml), Pam3CSK4 (P3C) (10 μg/ml), lipoteichoic acid (LTA) (10 μg/ml), and R-FSL (10 μg/ml) for 14 h. IL-12p40 (A), IL-12p70 (B), TNF-α (C), and IL-6 (D) were determined in supernatants by ELISA. E and F, BMDCs were stimulated as above for the indicated times. IL-12p40 (E) and TNF-α (F) were measured in the supernatant (mean ± SD; n = 3). G and H, BMDCs were stimulated with LPS (100 ng/ml) or CpG-DNA (1 μM) and Pam3CSK4 (10 μg/ml). After the indicated time, mRNA was isolated and the expression of IL-12p40 (G) and TNF-α (H) was quantified by quantitative RT-PCR. The relative expression of IL-12p40 mRNA and TNF-α mRNA in comparison with the mRNA encoding the housekeeping gene β-actin is shown (mean ± SD; n = 4).
NF-κB signaling is decisive for IL-12p40 regulation upon TLR stimulation

To get insight into the function of the NF-κB signal pathway for IL-12p40 expression, NF-κB activation was inhibited at the level of IkBα phosphorylation and degradation. Using the IKKα/β inhibitor BAY 11-7082, it could be shown that inhibition of IKKα/β reduced the TLR induced IL-12p40 expression down to 15% of the control values (Fig. 2G). Again, it was observed that the disruption of whole NF-κB signaling decreased TLR-induced IL-12p40 expression but did not affect the differential induction of TLR-mediated IL-12p40 expression.

NF-κB activation dramatically differs in its kinetics upon stimulation of TLR2/4/9

Next, differences in TLR ligand-induced NF-κB activation were investigated. After stimulation of dendritic cells with CpG-DNA, LPS, or Pam3CSK4, the latter two stimuli induced a pronounced and early IkBα phosphorylation resulting in a strong IkBα degradation that started 5–10 min after initial stimulation and lasted until 40–60 min (Fig. 3A). CpG-DNA stimulation, in contrast, induced a hardly detectable IkBα phosphorylation and a late and weak IkBα degradation.

Apart from the degradation of IkBα, the degradation of IkBβ could as well be responsible for NF-κB activation. To check this possibility, IkBβ degradation was measured additionally. There was no major CpG-DNA-, LPS-, or Pam3CSK4-induced IkBβ degradation detectable within the first 2 h after stimulation (Fig. 3A).

Oscillating time course of TLR-ligand induced nuclear NF-κB-binding activity

Having shown that the proximal kinetics of NF-κB activation (IkBα phosphorylation and degradation) differed considerably between various TLR ligands, we next investigated downstream NF-κB signaling in detail. As already shown for LPS stimulation by others (24), TLR ligand-induced NF-κB DNA-binding activity followed an oscillating time course (Fig. 3, B and C). Corresponding to the observed rapid IkBα degradation upon LPS stimulation, an early increase of nuclear NF-κB DNA-binding activity was observed. In contrast, CpG-DNA-induced NF-κB DNA-binding activity increased much later. As shown by supershift, both RelA and c-Rel were responsible for the measured NF-κB DNA-binding activity (Fig. 3D). When we analyzed multiple EMSA experiments quantitatively, we consistently observed that the kinetics of NF-κB-binding activity differed upon CpG-DNA or LPS stimulation. The latter induced early and strong activity, whereas CpG-DNA resulted in delayed but prolonged activity with the nearly exclusive ability to produce late binding activity (at 4–5 h poststimulation) (Fig. 3C). To measure the specific contribution of the respective NF-κB subunits to the overall NF-κB DNA-binding activity, an ELISA-based assay was used. As shown in Fig. 3E, LPS stimulation resulted in an early increase of nuclear RelA DNA-binding activity whereas the CpG-DNA-induced RelA DNA-binding activity increased much later. The RelB DNA-binding activity increased after 5 h, but there was no difference between stimulation by CpG-DNA and stimulation by LPS (data not shown). Unfortunately, the c-Rel assay did not work with murine extracts.

LPS-induced RelA recruitment to the IL-12p40 promoter is rapidly terminated

To gain more insight into the way TLR-induced NF-κB activation influences the IL-12p40 expression, LPS and CpG-DNA induced
RelA and c-Rel recruitment to the IL-12p40 promoter were assessed using a ChIP assay. In the early stage (20 min after stimulation), RelA and c-Rel recruitment to the IL-12p40 promoter could only be observed after LPS stimulation (Fig. 4), which is in accordance with the kinetics of CpG-DNA- and LPS-induced NF-κB-binding activity. At later time points, 100 to 300 min, CpG-DNA and LPS stimulation induced c-Rel recruitment to the IL-12p40 promoter, whereas prolonged RelA recruitment to the IL-12p40 promoter could only be observed after CpG-DNA stimulation. LPS-induced RelA recruitment to the IL-12p40 promoter was markedly decreased already 100 min after stimulation, although at this time point the LPS-induced nuclear NF-κB-binding activity was comparable to that observed after CpG-DNA stimulation. CpG-DNA- and LPS-induced C/EBPβ recruitment to the IL-12p40 promoter showed as expected a faster C/EBPβ promoter binding after LPS stimulation, whereas no difference in C/EBPβ promoter binding was observed after CpG-DNA and LPS stimulation at later time points (Fig. 4).

**TLR2 and TLR4 but not TLR9 ligands induce phosphorylation of serine 536 in RelA.**

The obvious question now was as follows: which mechanism is responsible for the early termination of RelA binding after LPS stimulation? NF-κB activation is classically terminated by the re-synthesis of new IκBα molecules entering the nucleus and transporting the NF-κB molecules into the cytoplasm. Indeed, we observed a much stronger induction of IκBα mRNA upon LPS stimulation than for CpG-DNA stimulation (Fig. 5A). Recently, Lawrence and coworkers showed that LPS induced phosphorylation of RelA at serine 536, which in turn led to destabilization and degradation of RelA (25). LPS induced serine 536 phosphorylation in RelA immediately (10 min) after stimulation, and this phosphorylation could be observed for ~30–60 min (Fig. 5B). Strikingly, phospho-S536-RelA could not be observed upon stimulation with CpG-DNA. The total amount of RelA was not affected. To further analyze this observation, we stimulated cells with various TLR ligands. Phospho-S536-RelA could only be observed after stimulation with the TLR2 ligand Pam3CSK4 and the TLR4 ligand LPS, but not following TLR9 (CpG-DNA) or TLR7 (R848; imiquimod) stimulation (Fig. 5C). This pattern correlated perfectly with the observed capacity to induce IL-12 (Fig. 1A).

For a better understanding of the function of phospho-S536-RelA, the signal pathways involved in the phosphorylation should be investigated. Using BMDCs from TLR2-, TLR4-, and MyD88 knockout mice and TRIF domain-containing adaptor-inducing IFN-β (TRIF)-defective LPS2 mice, it could be shown that the
LPS/Pam3CSK4-induced RelA phosphorylation was a TLR-dependent effect (data not shown). However, neither single deficiency in the adaptor molecule MyD88 nor TRIF abolished pS536-RelA generation (Fig. 5, A and B).

Concerning TLR-dependent but TRIF- and MyD88-independent signal transduction, MAL (TIR domain-containing adaptor protein or TIRAP) might be the possible adaptor molecule because it is used by TLR2 as well as TLR4. Mansell and coworkers (26) showed a MAL-dependent, MyD88-independent phosphorylation of RelA that was mediated by Btk. Using Btk knockout mice it was shown that stimulation of BMDCs with LPS or Pam3CSK4 still resulted in S536 phosphorylation of RelA, albeit at lower efficacy (Fig. 6A). Confirming the hypothesis that phospho-S536-RelA regulates NF-κB kinetics and IL-12p40 secretion, we observed that BMDMs from Btk−/− mice showed increased IL-12p40 secretion upon LPS and Pam3CSK4 stimulation, which is in accordance with reduced phospho-S536-RelA. Still, both stimuli were not as effective as CpG-DNA (Fig. 6B).

In bone marrow-derived macrophages of IKKα-defective IKKα/AA mice (in which serine residues 176 and 180 of the activating loop of IKKα have been replaced by alanine residues) LPS lost its ability to induce serine 536 phosphorylation of RelA (13, 25). In this study we analyzed BMDCs from such IKKα AA mutant mice with respect to LPS- and Pam3CSK4-induced pS536-RelA. Surprisingly, in BMDCs pS536-RelA generation was not affected in these mice and was comparable to that in wild-type

![FIGURE 5](image_url)

**FIGURE 5.** Only TLR2 and TLR4 ligands, but not ligands of other TLRs, induce serine 536 phosphorylation of RelA. A. BMDCs were stimulated with CpG-DNA (1 μM) and LPS (100 ng/ml). After the indicated times, mRNA was isolated and the expression of IL-12p40 (continuous line) and β-actin (dotted line) was quantified by quantitative RT-PCR. The relative expression of IL-12p40 mRNA and β-actin in comparison to the mRNA encoding the housekeeping gene β-actin is shown. Mean + SD; n = 3. B, BMDCs were stimulated with CpG-DNA (CpG; 1 μM) and LPS (100 ng/ml) for the indicated times. Similar amounts of cellular extracts were blotted with Abs for IL-12p40 and β-actin. C, BMDCs were stimulated with CpG-DNA (CpG; 1 μM), LPS (100 ng/ml), Pam3CSK4 (P3C; 10 μg/ml), R848 (1 μg/ml), polyinosinic-polycytidylic acid (PIC; 50 μg/ml), or imiquimod (IQ; 20 μg/ml) for 10 min. Similar amounts of cellular extracts were blotted with Abs for RelA phosphorylated at serine 536 (pS536RelA) and β-actin. D and E, BMDCs of MyD88 knockout (MyD) and wild-type (WT) mice (D) and LPS2 (LP2) and wild-type (WT) mice (E) were stimulated as indicated with CpG-DNA (1 μM) and/or LPS (100 ng/ml) for 10 min. Similar amounts of cellular extracts (10 μg/ml) were blotted with Abs for phospho-S536-RelA and β-actin. Results are representative of three independent experiments.
nuclear NF-κB-binding activity and RelA recruitment to the IL-12p40 promoter, and finally resulted in high IL-12p40 secretion (Fig. 7).

The stimulus-dependent early termination of LPS-induced RelA recruitment to the IL-12p40 promoter occurred independently of the NF-κB-induced IkBα resynthesis. IkBα-independent, gene-specific termination of the RelA promoter binding has already been shown in previous studies. In particular, a promoter-specific, proteasome-dependent degradation of DNA-bound RelA has been described in the fibroblast cell line 3T3 (33). Although the responsible ubiquitin ligase has not been identified to date, it is likely that enzymes such as PD-LIM2 or SOCS1 are involved (34, 35). As demonstrated in the present work, inhibition of proteasomal degradation by MG132 resulted in a substantial increase in the phosphorylation of RelA at serine 536, suggesting that a proteasomal pathway participates in the control of RelA phosphorylated at this residue. This may also include the possibility that RelA, if phosphorylated at S536, is subjected to enhanced degradation, which in turn would result in decreased RelA-dependent transcriptional activation of respective target genes as has been shown for embryonic fibroblasts (25). However, the only regulatory mechanism described to be involved in the regulation of proteasomal ubiquitination-mediated degradation of RelA is the protective interaction of Pim1 with RelA (36). The direct link between RelA S536 phosphorylation-mediated destabilization of RelA and ubiquitin-dependen
dent RelA degradation has not been shown so far. It is conceivable that other effects of the RelA S536 phosphorylation, such as altered interaction with other transcription factors, are also responsible for the observed early termination of RelA IL12p40 promoter binding. To further confirm the causal relationship of the TLR2- and TLR4-induced specific serine 536 phosphorylation of RelA for the early termination of the RelA promoter binding in BMDCs, different knockout mice have been tested. Among those, only in BMDCs from Btk−/− mice could a reduction of LPS- or Pam3CSK4-induced phospho-S536-RelA be observed. Interestingly, IKKα was not operative in dendritic cells, which contrasts with findings reported for macrophages (25). However, it is well known that macrophages are in general less effective in IL-12 secretion, and increased pS536-RelA might contribute to this observation. In addition to IKKα, IKKβ, and Btk, an IKKε- and TBK1-dependent phosphorylation of RelA at serine 536 has been described (37). Because IKKε and TBK 1 are involved in TLR-induced type I IFN

Discussion
Marked differences in the magnitude of IL-12p40 secretion in dendritic cells by different TLRs have been described repeatedly (28–30). The aim of the present work was to clarify the mechanisms that might be responsible for this differential capacity to induce IL-12. Using pharmacological inhibitors and BMDCs from knockout mice, the MAPK, the PI3K, and the IRF signal pathway could be ruled out to be causative for the observed differences. In the present work it could be shown that the stimulation of BMDCs with different TLR ligands resulted in striking differences in kinetics of NF-κB activation. Stimulation of BMDCs with LPS and Pam3CSK4 induced an early IkBα phosphorylation resulting in a pronounced IkBα degradation and a fast and strong NF-κB activation, whereas CpG-DNA induced a weak and delayed IkBα degradation resulting in a decelerated NF-κB activation. We could further show that LPS induced a rapid but short-lived activation of RelA, whereas CpG-DNA stimulation resulted in prolonged RelA activity at the IL-12p40 promoter.

Saccani et al. discriminated in a recent paper between two kinds of genes with respect to the kinetics of stimulus-induced NF-κB promoter recruitment (31). The first subset of target genes showed fast NF-κB recruitment to constitutively and immediately accessible promoters (pulse-type genes), whereas the second group of target genes showed late NF-κB recruitment to promoters that required stimulus-dependent modifications in chromatin structure. TNF-α belongs to the first group of genes, whereas IL-12p40 is a representative of the second group of genes (28, 32). In accordance with this classification, stimulation with TLR ligands resulted in an early TNF-α production whereas the IL-12p40 induction showed delayed kinetics. We therefore propose a model that discriminates between pulse type and delayed type genes to explain the phenomenon of differential TLR-induced IL-12p40 expression (Fig. 7). In contrast to the induction of TNF-α where only a short binding of NF-κB to the promoter is sufficient, activation of IL-12p40 expression needs a prolonged binding of NF-κB to its promoter. TLR2 and TLR4 induce rapid serine 536 phosphorylation of RelA, which in turn results in early termination of RelA promoter binding and thereby an insufficient IL-12p40 induction. In accordance with this hypothesis, we could show that in BMDCs from Btk knockout mice with a strongly reduced RelA S536 phosphorylation after LPS or Pam3CSK4 stimulation, the differences in TLR induced IL-12p40 expression markedly diminished. In contrast, stimulation of TLRs7/9 lacked phospho-S536-RelA, showed prolonged nu-

FIGURE 7. Model of differential TLR-induced RelA promoter recruitment. “Pulse-type genes” TNF-α showed fast NF-κB recruitment to constitutively and immediately accessible promoters, whereas “delayed-type” genes (IL-12p40) showed late NF-κB recruitment to promoters that required stimulus-dependent modifications in chromatin structure. In contrast to the induction of TNF-α, where only a short binding of NF-κB to the promoter is sufficient, activation of IL-12p40 expression needs a prolonged binding of NF-κB to its promoter. TLR2 and TLR4 induce rapid serine 536 phosphorylation of RelA, which in turn results in early termination of RelA promoter binding and thereby an insufficient IL-12p40 induction.
expression and TLR2 ligands are not or only extremely weak inducers of type I IFN, it is unlikely that IKKe and TBK1 are responsible for the observed induction.

TLR ligand-induced NF-κB activation follows an oscillating kinetic. It was the group of Hoffmann and coworkers that already showed in 2002 (38) that stimulation of Jurkat T cells with TNF-α induced an oscillating activation of NF-κB. TNF-α-induced IKK activation leads to phosphorylation, ubiquitination, and degradation of IκBα, resulting in the release of NF-κB dimers. NF-κB-induced resynthesis of IκBα is responsible for negative feedback and postinduction repression of NF-κB activation. During ongoing stimulation, the alternation of NF-κB activation and termination results in an oscillating time course of NF-κB activation. In cells containing IκBα, IκBβ, and IκBe, this oscillation is dumped very soon by the counteraction of IκBβ and IκBe, whereas in IκBβ- and IκBe-deficient cells the oscillation has a prolonged progression. Similar oscillation has already been described for LPS-induced NF-κB activation (24). The LPS-induced NF-κB oscillation observed in the present work fits into the picture of a dumped oscillation, whereas the CpG-DNA-induced NF-κB activation showed an increasing NF-κB DNA-binding activity in the second peak of the oscillation, a phenomenon that could not be explained by an alternation of IκBα degradation and resynthesis.

TLR ligand-induced activation of BMDCs resulted in an increase of NF-κB DNA-binding activity. Using supershift experiments we could show that in accordance with earlier publications (39), the NF-κB heterodimers RelA/p50 and c-Rel/p50 were responsible for the NF-κB activation within the first 5 h after stimulation. Later, however, RelA/p50 and c-Rel/p50 homodimers contribute only little to the NF-κB DNA-binding activity observed after 5 h or longer poststimulation (data not shown). Preliminary data using mass spectroscopy indicate that additional binding partners to the NF-κB enhancer element might exist that could be responsible for this late induced NF-κB DNA-binding activity. A change in NF-κB dimer composition has already been described previously by Saccani and colleagues and was shown to affect the specificity of induction of NF-κB dependent genes (40).

In the present work the RelA recruitment to the IL-12p40 promoter was more pronounced compared with the IL-12p40 promoter recruitment of c-Rel. This finding is in contrast to an early publication of Sanjabi and co-workers who showed that NF-κB activation of the IL-12p40 promoter was mostly dependent on the activation of c-Rel homodimers and that the knockout of c-Rel genes resulted in an almost complete loss of IL-12p40 expression (41). An important difference with regard to the work of Sanjabi et al. is that in the cited study macrophages were analyzed whereas in the present work dendritic cells were used as a model system. Indeed, CpG-DNA- and LPS-induced IL-12p40 expression was reported to be similar in splenic DCs of c-Rel knockout and control mice (42). This implies that the usage of NF-κB family members differs between the various cell types.

TLR2 and TLR4 receptors are located on the cell surface, whereas TLR9 is located in the endosome (5). For TLR2- and TLR4-induced receptor activation direct ligand binding is sufficient, whereas for the endosomal receptors, including TLR7–9, ligand uptake and endosomal maturation are prerequisites (43, 44). The delayed activation of NF-κB but also the retarded activation of the MAPKs JNK and p38 could be explained by the complex activation kinetics of the endosomal TLR9 ligand. Another intriguing explanation was recently provided by Yao et al. (45) that described two parallel IL-1–mediated signaling pathways for NF-κB activation, a TAK1-dependent and TAK1-independent MEKK3-dependent pathway. Depending on the activation of TAK1, NF-κB activation could be brought about either with or without IκBα degradation. Preliminary experiments by us confirm an exclusive activation of TAK1 by TLR2/4 but not by TLR9.

Taken together, we show that TLR9 ligands induce prolonged activity of NF-κB due to a lack of destabilizing S536 phosphorylation in RelA. This plays an important biological role, as it explains the propensity of these but not other TLR ligands to induce high amounts of IL-12, which in turn leads to in vivo Th1 polarization. Moreover, this study shows that alteration of the activation kinetics of one and the same signaling module results in strong differences in the overall biological effects.
21. Hacker, H., H. Mischak, G. Hacker, S. Eser, N. Prenzel, A. Ullrich, and H. Wagner. 1999. Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. *EMBO J.* 18: 6973–6982.

22. Salkowski, C. A., K. Kopydlowski, J. Blanco, M. J. Cody, R. McNally, and S. N. Vogel. 1999. IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice. *J. Immunol.* 163: 1529–1536.

23. Schmitz, F. A. Heit, S. Guggemoos, A. Krug, J. Mages, M. Schiennmann, H. Adler, I. Drexler, T. Haas, R. Lang, and H. Wagner. 2007. Interferon-regulatory factor 1 controls Toll-like receptor 9-mediated IFN-β production in myeloid dendritic cells. *Eur. J. Immunol.* 37: 315–327.

24. Covert, M. W., T. H. Leung, J. E. Gaston, and D. Baltimore. 2005. Achieving stability of lipopolysaccharide-induced NF-κB activation. *Science* 309: 1854–1857.

25. Lawrence, T., M. Bebien, G. Y. Liu, V. Nizet, and M. Karin. 2005. IKKε limits macrophage NF-κB activation and contributes to the resolution of inflammation. *Nature* 434: 1138–1143.

26. Mansell, A., R. Smith, S. L. Doyle, P. Gray, J. E. Fenner, P. J. Crack, S. E. Nicholson, D. J. Hilton, L. A. O’Neill, and P. J. Hertzog. 2006. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat. Immunol.* 7: 148–155.

27. Yang, F., E. Tang, K. Guan, and C. Y. Wang. 2003. IKKβ plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. *J. Immunol.* 170: 5630–5635.

28. Albrecht, I., T. Tapmeier, S. Zimmermann, M. Frey, K. Heeg, and A. Dalpke. 2004. Toll-like receptors differentially induce nucleosome remodelling at the IL-12p40 promoter. *EMBO Rep.* 5: 172–177.

29. Cowdery, J. S., T. H. Leung, L. A. O’Neill, P. S. Myung, and G. A. Koretzky. 1999. Differential regulation of the IL-12 p40 promoter and of p40 secretion by CpG DNA and lipopolysaccharide. *J. Immunol.* 162: 6770–6775.

30. Hermann, C., I. Spreitzer, N. W. Schroder, S. Morath, M. D. Lehnert, W. Fischer, C. Schutt, R. R. Schumann, and T. Hartung. 2002. Cytokine induction by purified lipoteichoic acids from various bacterial species—role of LBP, sCD14, CD14 and limits of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat. Immunol.* 7: 148–155.

31. Saccani, S., S. Pantano, and G. Natoli. 2001. Two waves of nuclear factor κB recruitment to target promoter. *J. Exp. Med.* 193: 1351–1359.

32. Tanaka, T., M. J. Grusby, and T. Kaisho. 2007. PDLIM2-mediated termination of transcription factor NF-κB activation by intranuclear sequestration and degradation of the p65 subunit. *Nat. Immunol.* 8: 584–591.

33. Saccani, S., I. Marazzi, A. A. Beg, and G. Natoli. 2004. Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor κB response. *J. Exp. Med.* 200: 107–113.

34. Natoli, G., and S. Chiocca. 2008. Nuclear ubiquitin ligases, NF-κB degradation, and the control of inflammation. *Sci. Signal.* 1: e1.

35. Saccani, S., G. F. Scaglione, A. A. Beg, and G. Natoli. 2004. Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor κB response. *J. Exp. Med.* 200: 107–113.

36. Tanaka, T., M. J. Grusby, and T. Kaisho. 2007. PDLIM2-mediated termination of transcription factor NF-κB activation by intranuclear sequestration and degradation of the p65 subunit. *Nat. Immunol.* 8: 584–591.

37. Ryo, A., F. Suizu, Y. Yoshida, K. Perrem, Y. C. Liou, G. Wulf, R. Rottapel, S. Yamaoka, and K. P. Lu. 2003. Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol. Cell.* 12: 1413–1426.

38. Huss, H. A., Dorrie, M. L., Schmitz, E. Hoffmann, K. Resch, and M. Kracht. 2004. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-κB at serine 536 is mediated by multiple protein kinases including IkB kinase (IKK)α, IKKβ, and an unknown kinase and couples p65 to TATA-binding protein-associated factor II13-mediated interleukin-8 transcription. *J. Biol. Chem.* 279: 55633–55643.

39. Hoffmann, A., A. Levchenko, M. L. Scott, and D. Baltimore. 2002. The IκB-NF-κB signaling module: temporal control and selective gene activation. *Science* 298: 1241–1245.

40. Murphy, T. L., M. G. Cleveland, P. Kulesza, J. Magram, and K. M. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF-κB half-site. *Mol. Cell Biol.* 15: 5258–5267.

41. Saccani, S., S. Pantano, and G. Natoli. 2003. Modulation of NF-κB activity by exchange of dimers. *Mol. Cell* 11: 1563–1574.

42. Sanjabi, S., K. J. Williams, S. Saccani, L. Zhou, A. Hoffmann, G. Ghosh, S. Gerondakis, G. Natoli, and S. T. Smale. 2005. A c-Rel subdomain responsible for enhanced DNA-binding affinity and selective gene activation. *Genes Dev.* 19: 2138–2151.

43. Grumont, R., H. Hochrein, M. O’Keeffe, R. Gugasyan, C. White, I. Caminschi, W. Cook, and S. Gerondakis. 2001. c-Rel regulates interleukin 12 p70 expression in CD8+ dendritic cells by specifically inducing p35 gene transcription. *J. Exp. Med.* 194: 1021–1032.

44. Dalpke, A. H., S. Zimmermann, I. Albrecht, and K. Heeg. 2002. Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo. *Immunology* 106: 102–112.

45. Hacker, H., H. Mischak, T. Mielke, S. Liptay, R. Schmid, T. Sparwasser, K. Heeg, G. B. Lipford, and H. Wagner. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 17: 6230–6240.

46. Yoo, J., T. W. Kim, J. Qin, Z. Jiang, Y. Qian, H. Xiao, Y. Lu, W. Qian, M. F. Gulin, N. Szemere, et al. 2007. Interleukin-1 (IL-1)-induced TAK1-dependent NF-κB activation pathways bifurcate at IL-1 receptor-associated kinase modification. *J. Biol. Chem.* 282: 6075–6089.