Induction of In Vitro Reprogramming by Toll-Like Receptor (TLR)2 and TLR4 Agonists in Murine Macrophages: Effects of TLR "Homotolerance" Versus "Heterotolerance" on NF-κB Signaling Pathway Components

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In this study, tolerance induction by preexposure of murine macrophages to Toll-like receptor (TLR)2 and TLR4 agonists was revisited, focusing on the major signaling components associated with NF-κB activation. Pretreatment of macrophages with a pure TLR4 agonist (protein-free *Escherichia coli* (Ec) LPS) or with TLR2 agonists (*Porphyromonas gingivalis* LPS or synthetic lipopeptidem Pam3Cys) led to suppression of TNF-α secretion, IL-1R-associated kinase-1, and IκB kinase (IKK) kinase activities, c-jun N-terminal kinase, and extracellular signal-regulated kinase phosphorylation, and to suppression of NF-κB DNA binding and transactivation upon challenge with the same agonist (TLR4 or TLR2 “homotolerance,” respectively). Despite inhibited NF-κB DNA binding, increased levels of nuclear NF-κB were detected in agonist-pretreated macrophages. For all the intermediate signaling elements, heterotolerance was weaker than TLR4 or TLR2 homotolerance with the exception of IKK kinase activity. IKK kinase activity was unperturbed in heterotolerance. TNF-α secretion was also suppressed in *P. gingivalis* LPS-pretreated, Ec LPS-challenged cells, but not vice versa, while Pam3Cys and Ec LPS did not induce a state of cross-tolerance at the level of TNF-α.

Experiments designed to elucidate novel mechanisms of NF-κB inhibition in tolerized cells revealed the potential contribution of IκBα and IκBε inhibitory proteins and the necessity of TLR4 engagement for induction of tolerance to Toll receptor-IL-1R domain-containing adapter protein/MYD88-adapter-like-dependent gene expression. Collectively, these data demonstrate that induction of homotolerance affects a broader spectrum of signaling components than in heterotolerance, with selective modulation of specific elements within the NF-κB signaling pathway. *The Journal of Immunology*, 2003, 170: 508–519.

Lipopolysaccharide is a predominant, integral structural component of the outer membrane of Gram-negative bacteria and one of the most potent initiators of inflammation. LPS activates monocytes and macrophages to produce cytokines such as TNF-α, IL-1, and IL-6 that, in turn, serve as endogenous inflammatory mediators. An integral component of the LPS receptor complex is Toll-like receptor (TLR)4. Mice that express critical mutations or a targeted mutation in the *tlr4* gene are unresponsive to enterobacterial LPS (1–3). TLR4 is a member of a larger family of signal transducing molecules that share homology with *Drosophila* Toll, a transmembrane protein that mediates dorsal ventral patterning in embryos and antifungal resistance in imagos (4, 5). Ten mammalian TLRs have been identified (6).

Although TLR4 is the principal TLR species involved in LPS signaling, recent studies indicate that TLR2 is the primary signal transducing molecule for LPS from certain nonenterobacterial Gram-negative organisms, including *Porphyromonas gingivalis* (7) and *Leptospira interrogans* (8). TLR1 has also been implicated as a coreceptor for TLR2 in that its coexpression in transfected cells augmented the TLR4-independent response to *Escherichia coli* LPS and *Neisseria meningitidis* lipooligosaccharide (9). However, TLR2 is best recognized as the predominant signal transducing molecule for diverse bacterial lipoproteins and the synthetic lipopeptide, Pam3Cys (6). All TLRs, as well as the IL-1 and IL-18 receptors, share the capacity to bind the intracellular adaptor molecule, MyD88, to a homologous intracytoplasmic domain (6). A second adaptor molecule, Toll receptor-IL-1R domain-containing adapter protein (TIRAP)/MYD88-adapter-like (Mal), mediates TLR4-dependent, but MyD88-independent, signaling. However, TLR3, TLR9, and the IL-1 and IL-18 receptors appear not to engage this pathway to any significant extent (10–12). MyD88 and TIRAP/Mal initiate signaling cascades that lead to recruitment of serine/threonine kinases, IL-1R-associated kinase (IRAK)-1 and/or IRAK-2 (11). Recently, two other serine/threonine kinases, receptor interacting protein 2 and IRAK-4, were also shown to be necessary for cytokine production and NF-κB activation by IL-1R/TLR agonists (13, 14). These kinases, in turn, activate TNFR-associated factor 6-dependent signaling cascade(s) that culminate...
in NF-κB activation (15). However, the precise mechanism(s) by which these downstream signaling cascades function has yet to be elucidated.

Prior exposure to LPS both in vitro and in vivo can lead to desensitization of immune cells to subsequent challenge with LPS, a phenomenon that has been referred to as “endotoxin tolerance.” One of the main characteristics of LPS tolerance in vitro is a change in the pattern of inflammatory gene expression in cells of myeloid lineage, when responses to a single or two sequential LPS exposures are compared (16). For example, restimulation of monocytes and macrophages previously exposed to LPS fails to elicit TNF-α, IL-1β, IL-6, IL-12, and Jun B gene expression, although other genes, including IL-10, IL-1R antagonist, and TNFRII, are expressed at normal or elevated levels (17). Induction of an endotoxin tolerant phenotype is not specific to the initiating action of LPS because engagement of TLR/IL-1R family members, other than TLR4, has also been found to result in macrophage refractoriness to subsequent LPS challenge. The capacity of a stimulus that does not share structural homology with LPS, yet elicits the tolerant phenotype, has been referred to as “cross-tolerance.” For example, pretreatment of murine peritoneal macrophages with IL-1β results in inhibition of NF-κB and AP-1 transactivation in response to LPS (18). LPS-induced NF-κB DNA binding, c-jun N-terminal kinase (JNK) kinase activity, and TNF-α secretion were significantly inhibited in murine macrophages pre-exposed to a mycoplasma lipopeptide, macrophage-activating lipopeptide (MALP)-2, which signals through TLR2 (19). Pretreatment of macrophages with another bacterial product, lipoteichoic acid (LTA), results in inhibition of TNF-α secretion induced by LPS (20). Desensitization of macrophages to LPS challenge can also be achieved by other non-TLR stimuli. For example, pre-exposure of human macrophages to 25-hydroxycholesterol, the product of oxidativemodification of low density lipoproteins involved in atherogenesis, inhibits NF-κB binding to the TNF-α promoter and TNF-α mRNA expression in response to LPS restimulation (21). Human macrophages derived from patients after hemorrhage, surgery, trauma, and blood transfusion manifest suppressed responses to LPS ex vivo (22–24). These stresses also inhibit NF-κB DNA binding and/or suppress TNF-α gene expression in response to LPS challenge. Thus, various exogenous and endogenous stimuli can induce a hyporesponsive state to a challenge with enterobacterial LPS.

Many studies have sought to determine the molecular mechanisms that underlie endotoxin tolerance induced by LPS or other stimuli. Clearly, disruption of the major signaling components leading to NF-κB translocation have been identified in LPS-pre-treated macrophages, including IRAK-1, IκBα, and IκBβ (18, 19, 25). Using an Ab that detects TLR4 in complex with MD-2, a molecule that facilitates signaling through TLR4 (26), Nomura et al. (27) correlated a reduction in cell surface TLR4/MD-2 complex expression with development of the tolerant phenotype; however, diminished TLR4/MD-2 expression was very transient, and was not observed at low doses of LPS that are fully capable of inducing a tolerant phenotype in vitro (16). Furthermore, cells engineered to overexpress TLR4 and MD-2 are readily tolerized by LPS (28). Lastly, if down-regulation of TLR4/MD-2 was the fundamental mechanism by which desensitization to LPS occurred, then one would predict a global shutdown of LPS-induced gene expression, rather than the complex pattern of suppressed and overexpressed genes that is observed. In cells pretreated with the TLR2 agonist, MALP-2, tolerance to LPS was observed, but was not associated with down-regulation of TLR4/MD-2 expression (19).

Given these discrepancies, coupled with the fact that many previous studies were conducted using commercial LPS preparations that were contaminated with non-LPS microbial components that activate cells via TLR2 (7), and perhaps, through other TLRs, we sought to carry out a systematic comparison of a highly purified, pure TLR4 agonist, Ec K235 LPS, and two previously well-characterized TLR2 agonists, synthetic Pam3Cys and highly purified, natural P. gingivalis (Pg) LPS, for the capacity to induce a tolerant phenotype in mouse macrophages. The focus of the present study was to define the effects of TLR homotolerance (i.e., the tolerizing stimulus and the challenge stimulus use the same TLR) and TLR heterotolerance (i.e., the tolerizing and secondary stimuli use different TLRs) on the major components of the NF-κB activating pathway. Our results indicate that TLR homo- and heterotolerance differ significantly in strength of induction and their relative capacities to modulate various components along the NF-κB signaling pathway, and result in differential TNF-α secretion. Moreover, during this analysis, two proteins were identified that may contribute to tolerance by inhibiting the interaction of NF-κB with DNA.

Materials and Methods

Mice

C3H/OUj and C57BL/6j females, 4- to 6-wk-old, were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments were conducted in compliance with guidelines set forth by the National Institute of Health Guide for Care and Use of Laboratory Animals, with Uniformed Services University of Health Sciences, and University of Maryland-Baltimore institutional (Institutional Animal Care and Use Committee) approval.

TLR4 and TLR2 agonists

Protein-free Ec K235 LPS (18) was used as the prototypic TLR4 agonist. Repurified, protein-free LPS from Pg (7) and synthetic lipoprotein S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH, trihydrochloride (Pam3Cys; EMC Microcollections, Tubingen, Germany) were used as TLR2 agonists.

Antiserum

Rabbit antisera raised against synthetic peptides were used and most have been described previously (29–31). anti-mouse p50 (no. 1263), p65 (no. 1207), p52 (no. 1495), c-Rel (no. 1266), RelB (no. 1319), IκBα (no. 751), IκBβ (no. 3279), and IκB kinase (IKK) β (no. 4165). Anti-IRAK-1 Ab was from Cell Sciences (Norswood, MA). Anti-β-actin antiserum was from Chemicon International (Temecula, CA). Ab specific for the activated forms of JNK 1,2 and extracellular signal-regulated kinase (ERK) 1,2 were from Promega (Madison, WI).

Cell culture and transient transfection

Thiglycollate-elicited murine peritoneal macrophages were obtained and cultured as described before (18). Human embryonic kidney cells (HEK 293) were purchased from the American Type Culture Collection (Manassas, VA). The RAW 264.7 macrophage cell line was kindly provided by Dr. G. Feldmann (Food and Drug Administration, Bethesda, MD). Cells were cultured in DMEM (BioWhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO2 in air. The endotoxin content in the medium was <0.01 EU/ml, according to the manufacturers. Cells passaged >20 times were not used. Transient transfection of cells with the DNA constructs listed below were performed using FuGene6 (Roche, Indianapolis, IN) or SuperFect (Qiagen, Valencia, CA) according to manufacturers’ instructions.

DNA constructs

The NF-κB-responsive reporter plasmid, pELAM-Luc, was a kind gift of Dr. D. T. Golenbock (University of Massachusetts, Amherst, MA). The pCMV-1/β-galactosidase (β-gal) plasmid was described previously (32–34) and used for monitoring transfection efficiency and normalization. The expression vectors pCDNA3.1+ and pCDNA3.1+ IκBα were described previously (31).

Cell fractionation and EMSA

Conditions for cell fractionation and EMSA have been described previously (29, 30).
**Reporter assay**

NF-κB driven luciferase activity was measured as described previously (18). The ratio of luciferase relative light units divided by β-gal relative light units was calculated for each sample.

**Western blot analysis**

Thirty micrograms of total protein were incubated in NuPAGE sample buffer for 2 min at 85°C, resolved on 10% tricine SDS-PAGE (Invitrogen, Carlsbad, CA), and transferred to “Westran” polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). Immunoreactive proteins were detected with an ECL system (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, U.K.).

**In vitro kinase assay**

IKKβ was immunoprecipitated with 1 μl of antiserum no. 4165. After three washes with TNT buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM EDTA, pH 8.0, 1% Triton X-100) and three washes in kinase buffer (20 mM HEPES, pH7.4, 10 mM MgCl2, 2 mM MnCl2), precipitated kinases were incubated with either wild-type or S32/36A mutant human recombinant IκBα (1–71 aa) in the presence of 0.5 μM [γ-32P]ATP for 30 min at 30°C. Products of the kinase reactions were separated on a 10–20% tricine gradient gel (NOVEX, San Diego, CA), dried, and exposed to XOMAT film (Kodak, Rochester, NY). IRAK-1 was immunoprecipitated with 2 μg/ml affinity purified Ab that does not cross-react with other members of the IRAK family. The kinase assay was performed as described previously (35).

**ELISA**

Murine TNF-α in macrophage culture supernatants was detected with a murine TNF-α ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions.

**Analysis of mRNA**

Total RNA was isolated using the RNeasy purification kit (Qiagen, Valencia, CA). For Northern blot analysis, 10 μg of total RNA was separated on 0.7% agarose gel, transferred onto a Hybond N+ nylon membrane, UV cross-linked, and hybridized with a 32P-labeled DNA fragment corresponding to 314–750 bp of murine IκBα (AB 047549). This DNA fragment was generated by RT-PCR from RNA isolated from RAW 264.7 cells stimulated with 30 ng/ml of LPS for 1 h. Filters were exposed to XOMAT film for 7 days.

**Statistical analysis**

Statistical analysis of data was performed using SPSS 10.1.3 software (Chicago, IL). Data were subjected to one-way ANOVA with post hoc comparison test. A value of p ≤ 0.05 was accepted as the level of significance.

**Results**

**TNF-α secretion by tolerized macrophages**

TNF-α secretion by macrophages is inhibited in TLR2 and TLR4 homotolerance and Pg LPS and Ec LPS heterotolerance, but is not affected in Ec LPS and Pam3Cys heterotolerance. Both TLR2 and TLR4 agonists are potent inducers of TNF-α (37). Many previous studies have shown that TLR4 homotolerance results in markedly decreased levels of secreted TNF-α (16, 19, 20, 38, 39). However, only a few studies have been conducted using TLR2 agonists as “tolerizing” agents in macrophages, e.g., Pg LPS (40), MALP2 (19), or LTA (20), and only two studies have examined the effects of “cross-tolerance” between TLR2 and TLR4 agonists (19, 20). However, in some of these studies, commercial preparations of enterobacterial LPS and LTA were used that likely contained contaminants that initially engaged TLRs other than TLR4 or TLR2, respectively. In addition, MALP2, used in the study by Sato et al. (19), was later demonstrated to require both TLR2 and TLR6 for signaling (41). Given the possibility that the observed results were confounded by the engagement of multiple TLRs during the initial “tolerizing” exposure, we sought to revisit the issue of TLR2 and TLR4 homo- and heterotolerance by using highly purified agonists that had been well-characterized with respect to TLR2 or TLR4 specificity. Therefore, we first analyzed how exposure of murine peritoneal macrophages to TLR2 (Pg LPS or Pam3Cys) or the TLR4 agonist, protein-free Ec K235 LPS, influences subsequent production of TNF-α upon restimulation of cells with these agonists. The concentration of each agonist was chosen based on the ability to elicit comparable NF-κB DNA binding (data not shown).

**IFN-β mRNA is suppressed in TLR4 homotolerance, but is not affected in TLR2/TLR4 heterotolerance**

We recently demonstrated that in contrast to TNF-α mRNA, IFN-β mRNA is strongly up-regulated by TLR4 agonists, but not TLR2 agonists, and that its expression is MyD88-independent, but TRAP/Mal-dependent (36). IFN-β gene expression was measured following TLR homo- and heterotolerance induction as described for Fig. 1A. As reported previously, Ec LPS induced strong steady-state IFN-β mRNA, while neither Pg LPS nor Pam3Cys were active (Fig. 1D). More importantly, IFN-β gene expression was completely inhibited by TLR4 homotolerance, but not by prior exposure to either TLR2 agonist.

**IRAK-1 activation is inhibited by TLR homotolerance and heterotolerance**

It was previously shown that activation of IRAK-1, a key kinase in Toll/IL-1R NF-κB signal transduction, is inhibited in macrophages rendered endotoxin-tolerant in vitro (25, 27). Therefore, we next analyzed how pretreatment of macrophages with Ec LPS or Pg LPS would influence the activation of IRAK-1 in response to subsequent challenge with these agonists. Fig. 2, A (autoradiogram) and B (densitometry measurements), illustrates that both the TLR4 (Ec LPS) and TLR2 (Pg LPS) agonists induce a comparable IRAK-1-associated kinase activity in medium-pretreated macrophages, as evidenced by phosphorylation of immunoprecipitated IRAK-1. Pre-exposure of cells to Ec LPS results in significant inhibition of IRAK-1 activation followed by the second challenge with the same agonist (i.e., TLR4 homotolerance), but slightly less inhibition when Pg LPS was used as the challenge stimulus (p = 0.014 at 30 min). Pretreatment of cells with TLR2 agonist, Pg LPS, also significantly inhibited IRAK-1 activation upon either TLR4 or...
was 6 pg/ml. D

isolated and analyzed by RT-PCR with IFN-(Ec), 100 ng/ml Pg LPS (Pg), or 100 ng/ml Pam3Cys (P3C). Total RNA was

to

cipitates by Western blot did not reveal differences in the IRAK

peripheral macrophages from C3H/OuJ mice were treated as indicated and levels of secreted TNF-

were assessed by ELISA as described in Materials and Methods. B

restimulated with medium ( ), 10 ng/ml Ec LPS ( ), or 100 ng/ml Pam3Cys ( ) for 7 h. Shown are results (mean ± SEM) of seven independent experiments. C, Cells were pretreated with medium only, 1000 ng/ml Pg LPS, or 100 ng/ml Pam3Cys for 24 h, washed, and restimulated with medium ( ), 10 ng/ml Ec LPS ( ), or 100 ng/ml Pam3Cys ( ) for 7 h. Shown are results (mean ± SEM) of four independent experiments. * A significant difference from the corresponding challenge in medium-pretreated cells (*, p ≤ 0.05). The minimal detectable TNF-α level was 6 pg/ml. D, Cells were treated with 10 ng/ml Ec LPS, 1000 ng/ml Pg LPS, or 100 ng/ml Pam3Cys for 24 h, after that cells were washed with warm medium and restimulated for 1 h with either medium (M), 10 ng/ml Ec LPS (Ec), 100 ng/ml Pg LPS (Pg), or 100 ng/ml Pam3Cys (P3C). Total RNA was isolated and analyzed by RT-PCR with IFN-β- or GAPDH-specific primers.

FIGURE 1. TNF-α secretion is inhibited in TLR homotolerance, but differentially modulated in TLR heterotolerance. Peritoneal macrophages derived from C3H/OuJ mice were treated as indicated and levels of secreted TNF-α were assessed by ELISA as described in Materials and Methods. A, Cells were pretreated with medium only (M), 10 ng/ml Ec LPS, or 100 ng/ml Pam3Cys for 24 h. After that, cells were washed three times with warm medium and restimulated with medium ( ), 10 ng/ml Ec LPS ( ), or 100 ng/ml Pam3Cys ( ) for 7 h. Shown are results (mean ± SEM) of seven independent experiments. B, Macrophages were pretreated with medium only, 10 ng/ml Ec LPS, or 100 ng/ml Pg LPS for 24 h, washed, and restimulated with medium ( ), 10 ng/ml Ec LPS ( ), or 100 ng/ml Pg LPS ( ) for 7 h. Shown are results (mean ± SEM) of six independent experiments. C, Cells were pretreated with medium only, 1000 ng/ml Pg LPS, or 100 ng/ml Pam3Cys for 24 h, washed, and restimulated with medium ( ), 10 ng/ml Ec LPS ( ), or 100 ng/ml Pam3Cys ( ) for 7 h. Shown are results (mean ± SEM) of four independent experiments. * A significant difference from the corresponding challenge in medium-pretreated cells (*, p ≤ 0.05). The minimal detectable TNF-α level was 6 pg/ml. D, Cells were treated with 10 ng/ml Ec LPS, 1000 ng/ml Pg LPS, or 100 ng/ml Pam3Cys for 24 h, after that cells were washed with warm medium and restimulated for 1 h with either medium (M), 10 ng/ml Ec LPS (Ec), 100 ng/ml Pg LPS (Pg), or 100 ng/ml Pam3Cys (P3C). Total RNA was isolated and analyzed by RT-PCR with IFN-β- or GAPDH-specific primers.

TLR2 restimulation ( p ≤ 0.05). Analysis of IRAK1 immunoprecipitates by Western blot did not reveal differences in the IRAK protein content between control and tolerant cells (data not shown). Thus, IRAK-1-associated kinase activity is significantly suppressed in both homo- and heterotolerance.

IKKβ kinase activity is inhibited in TLR homotolerance, but not in TLR heterotolerance

The next downstream event in the cascade leading to NF-κB activation is the phosphorylation of the NF-κB inhibitor, IκB, by a specific kinase complex that is composed of two serine/threonine kinases, IKKα and IKKβ, and a scaffolding protein, IKKγ (NF-κB essential modulator or NEMO). Of these, IKKβ is considered to be the primary IκB kinase because mice deficient in this protein demonstrate no IκB phosphorylation in response to various inflammatory stimuli (15). Using an in vitro kinase assay, we measured the capacity of IKKβ to phosphorylate its substrate, IκBα, in macrophages subjected to TLR homotolerance or heterotolerance. Fig. 3 illustrates that the kinase activity of this protein is rapidly stimulated by both TLR2 (Pg LPS and Pam3Cys) and TLR4 (Ec LPS) agonists within 1 h in medium-pretreated macrophages, with maximum IKKβ kinase activity observed between 30 and 60 min of treatment with all three agonists. In vitro phosphorylation of the substrate, IκBα, in response to Ec LPS was completely abolished in macrophages pre-exposed to Ec LPS (i.e., TLR4 homotolerance); however, this same pretreatment did not inhibit kinase activity induced by either TLR2 agonist. Similarly, if macrophages were pre-exposed to either Pg LPS or Pam3Cys, no significant elevation in IKKβ kinase activity was observed in response to subsequent challenge with either of these TLR2 agonists, while challenge with Ec LPS led to kinase activation comparable to that observed in medium-pretreated, Ec LPS-stimulated cells (Fig. 3). Expression of IKKβ and the scaffold protein IKKγ was studied by Western blot and was comparable in medium- and agonist-pretreated cells (data not shown). To quantify this effect of TLR2 or TLR4 pretreatment on IKK kinase activity, we analyzed the densitometric results of two additional experiments conducted at the 45-min time point. Fig. 3B shows that only TLR homotolerance is induced significantly ( p ≤ 0.05) at the level of IKK kinase activity. Thus, IKKβ kinase activity is inhibited in TLR2 or TLR4 homotolerance, but is not affected in TLR heterotolerance.

Activation of JNK and ERK kinases is perturbed in TLR2 and TLR4 tolerance

Positive cooperation between activated IKK and a member of the mitogen-activated protein kinase (MAPK) superfamily, JNK, in the regulation of NF-κB transcriptional activation was demonstrated recently (42) and occurs at the step of IκB modification that precedes its degradation, i.e., IKK phosphorylates IκB on two conserved serine residues, while JNK regulates the abundance and activity of β-TrCP, a protein that mediates IκB ubiquitination through recruitment of a ubiquitin ligase (42, 43). Activation of JNK and other members of the MAPK family of proteins were demonstrated to be perturbed in Ec LPS homotolerance, as well as in Ec LPS and MALP2 heterotolerance (18, 19). Therefore, we analyzed the activity of JNK 1,2 and another MAPK, ERK 1,2, that share a common upstream kinase, MAPK kinase kinase (MEKK)1. Fig. 4 illustrates that both the TLR4 agonist (Ec LPS) and TLR2 agonist (Pg LPS) induce rapid and comparable phosphorylation of JNK 1,2 and ERK 1,2. Pretreatment of macrophages with Ec LPS prevents JNK 1,2 and ERK 1,2 phosphorylation in response to subsequent challenge with Ec LPS (TLR4 homotolerance), while restimulation of the same pretreated cells with Pg LPS permits weak activation of these kinases (TLR4/TLR2 heterotolerance). Pretreatment of macrophages with Pg LPS completely blocked JNK 1,2 activation in response to subsequent treatment.
with Ec LPS (TLR2/TLR4 heterotolerance) and Pg LPS (TLR2 homotolerance). However, residual activation of ERK 1,2 in TLR2 agonist-pretreated cells was observed after restimulation with either Ec LPS or Pg LPS (TLR2/TLR4 heterotolerance and TLR2 homotolerance, respectively). Similar data were obtained when Pam3Cys was used as a TLR2 agonist (data not shown).

**NF-κB DNA binding is suppressed in TLR2 and TLR4 homo- and heterotolerance**

To analyze the effects of TLR2 and TLR4 pre-exposure of macrophages on NF-κB DNA binding activity, C3H/OuJ macrophages were first treated with medium only or with Ec LPS, Pg LPS, or Pam3Cys. After 24 h, macrophages were washed and restimulated with medium, Ec LPS, Pg LPS, or Pam3Cys and NF-κB DNA binding activity was assessed 1 h later. Fig. 5A shows that cells exposed to medium only (M) in both the pretreatment and “challenge” exhibit barely detectable NF-κB DNA binding in the nuclear fraction, in contrast to the strong NF-κB DNA binding seen in medium-pretreated macrophages challenged with any of the three agonists. Pretreatment with any of the three agonists also resulted in elevated basal levels of NF-κB DNA binding when these cultures were subsequently challenged with medium only (compare pretreatment/challenge stimulation: M/M to Ec/M, Pg/M, and Pam3Cys/M). Both TLR hom- and heterotolerance can be seen in the autoradiography results shown in Fig. 5A. In cells pretreated with Ec LPS, the relative increase in NF-κB DNA binding over a medium “challenged” background is diminished for all three agonists (Fig. 5A). Densitometry of NF-κB-DNA complexes was performed on three independent experiments (Fig. 5C). All three agonists comparably suppressed NF-κB DNA binding induced by Ec LPS (p < 0.05). However, both TLR2 agonists induced stronger tolerance to the two TLR2 agonists than did pretreatment with Ec LPS (TLR4/TLR2 heterotolerance; p < 0.001).

Analysis of corresponding nuclear fractions by Western blot using anti-p50 and anti-p65 Abs confirmed rapid translocation of these proteins from cytosol to nucleus upon stimulation of medium-pretreated macrophages with either TLR4 or TLR2 agonists. However, these experiments revealed an unexpected phenomenon, i.e., in the nuclear fraction of cells tolerized by either TLR4 or TLR2, we observed high levels of both p50 and p65 proteins (Fig. 5B), while EMSA reveals diminished binding of these NF-κB subunits to DNA (Fig. 5A). This indicates that although the p50 and p65 proteins translocate to the nucleus, their binding to DNA is somehow inhibited.
The absence of Ab.

Lane 2 and 4. These data confirm that band no. 1 contains p50 and p65.

Ab directed against the C terminus of the p65 subunit diminishes complex no. 2, and largely displaces complexes no. 1 and 3.

formed.

is composed of C-terminally truncated p65 and p50.

Western blot analysis with Abs that recognize the activated forms of ERK 1,2 or JNK 1,2.

AN F at the level of NF-

previously published observations using Ec LPS or other TLR2 agonists induce homotolerance in macrophages, consistent with collective knowledge of these mutations in TLR2 and TLR4 to confirm the specificity of these agonists. We also found that TLR2 was not required for the induction of TLR4 homotolerance and, conversely, that TLR2 was not required for TLR4 homotolerance (data not shown). Taken collectively, these data demonstrate that both TLR4 and TLR2 agonists induce homotolerance in macrophages, consistent with previous published observations using Ec LPS or other TLR2 agonists (MALP2 and LTA) (19, 20). However, our data also indicate that TLR-induced heterotolerance is more readily detected at the level of NF-κB binding than at the level of TNF-α secretion or IKKβ kinase activity.

Formation of a NF-κB complex composed of p50 and C-terminally truncated p65

A NF-κB complex composed of p50 and C-terminally truncated p65 is formed in medium-pretreated macrophages stimulated with TLR2 and TLR4 agonists, but not in cells subjected to TLR homotolerance or heterotolerance. In addition to the effects of pre-stimulation of macrophages by TLR2 and TLR4 agonists described above, we noted a difference in the composition of the NF-κB complexes induced in medium-pretreated vs agonist-pretreated cells. Three discrete bands were resolved in nuclear extracts from cells that were medium-pretreated, agonist-stimulated cells. In contrast, in all agonist-pretreated samples, band no. 3 was no longer detected (Fig. 5A). To establish the composition of these complexes, supershift analyses of LPS-induced NF-κB were performed. Lane 1 of Fig. 5D illustrates the three major species in the absence of Ab. Lane 2 demonstrates that anti-p50 Ab completely displaces complex no. 2, and largely displaces complexes no. 1 and no. 3. This is consistent with the complex being composed primarily of p50 homodimers as has been reported elsewhere (29). An Ab directed against the C terminus of the p65 subunit diminishes the intensity of band no. 1, with no apparent effect on band nos. 3 and 4. These data confirm that band no. 1 contains p50 and p65 subunits of NF-κB predominantly. Conversely, an Ab directed against the N terminus of p65 results in a supershift of band no. 3 and diminished band no. 1 intensity. This indicates that band no. 3 is composed of C-terminally truncated p65 and p50. C-terminally truncated p65 was also detected by Western analysis in the cytosol of medium-pretreated, agonist-challenged cells (data not shown).

The effect of anti-c-Rel Ab was less striking. A minor amount of c-Rel protein is suggested by the appearance of a slower migrating band in position no. 4 that lacks the intensity observed when using anti-p50 and anti-p65 Abs. Anti-RelB and anti-p52 antisera behaved similarly to anti-c-Rel (data not shown). Using combinations of Abs, the data suggest that all three bands (1–3) are markedly diminished by the combination of anti-p50 and anti-p65N, while anti-p50 + anti-c-Rel and anti-p65N + anti-c-Rel were no more efficacious that anti-p50 or anti-p65N alone. These complexes were confirmed in medium-pretreated cells stimulated with either of the two TLR2 agonists (data not shown). Thus, our data reveal the presence of a C-terminally truncated p65 subunit of NF-κB only in medium-pretreated, agonist-stimulated cells, but not macrophages rendered tolerant by either TLR agonist and re-stimulated with Ec LPS, Pg LPS, or Pam3Cys (data not shown).

Differential regulation of AP-1 and Oct-1 DNA binding

We conducted a series of additional experiments in which the same nuclear extracts described in Fig. 1A were analyzed by EMSA with Oct-1- and AP-1-specific oligonucleotides. Oct-1 is an abundantly expressed DNA binding protein present in the majority of cells and, as such, is often used as a loading control for EMSA. However, the results in Fig. 5E revealed much stronger Oct-1 DNA binding in agonist-pretreated cells compared with medium-pretreated controls. When AP-1 was similarly analyzed by EMSA in these same preparations, we found that in contrast to the comparability of NF-κB DNA binding induced by the three agonists in medium-pretreated cells, the level of AP-1 was significantly greater in response to Ec LPS than in response to either TLR2 agonist. Despite the mitigation of IRAK1, ERK, and JNK phosphorylation in TLR2 and TLR4 tolerance, AP-1 binding to DNA was nonetheless detectable in the nuclear fractions of macrophages pretreated with either TLR4 or TLR2 agonists (Fig. 5E; compare lanes M/M with Ec/M, Pg/M or P3C/M). Subsequent treatments with Ec LPS, Pg LPS, or Pam3Cys resulted in differential modulation of AP-1 DNA binding activity. Thus, the pattern of NF-κB DNA binding in homo- and heterotolerance is distinct from that of AP-1 and Oct-1, demonstrating compositional differences in the pool of nuclear transacting factors in macrophages pretreated with the three agonists.

NF-κB transactivating activity is more strongly suppressed in TLR homotolerance than in TLR heterotolerance

In the previous series of experiments, TLR tolerance was analyzed at the level of NF-κB DNA binding. Therefore, NF-κB-dependent transduction of the luciferase reporter gene was next measured following transient transfection of the RAW 264.7 mouse macrophage cell line with a NF-κB reporter plasmid, pELAM-Luc. Ec LPS induced a dose-dependent increase in NF-κB-mediated luciferase activity in medium-pretreated cells with maximal stimulation observed at 10 ng/ml LPS (data not shown). Fig. 6 illustrates that Ec LPS induces a 5-fold stimulation over medium-pretreated cells, whereas NF-κB-dependent transactivation observed in response to 1 μg/ml Pg LPS was somewhat lower (i.e., 3.2-fold stimulation over medium-pretreated cells). Pretreatment of RAW 264.7 macrophages with Ec LPS resulted in a 46% inhibition of Ec LPS-mediated transactivation, whereas pretreatment with Pg LPS suppressed the response by 20%. Conversely, pretreatment of macrophages with Pg LPS inhibited the capacity of cells to respond to subsequent homologous stimulation by 40%, while Ec LPS pretreatment resulted in only 12.5% inhibition of Pg LPS-mediated NF-κB-dependent transduction. These data were subjected to statistical analysis, which confirmed acquisition of TLR2 and TLR4 homo- and heterotolerance at the level of NF-κB transactivation (p ≤ 0.05) and established that...
FIGURE 5. NF-κB DNA binding activity is inhibited in TLR2 and TLR4 homotolerance and heterotolerance. Peritoneal macrophages derived from C3H/OuJ mice were pretreated with medium only, Ec LPS (10 ng/ml), Pg LPS (1000 ng/ml), or Pam3Cys (100 ng/ml) for 24 h. After that, cells were washed three times with warm medium and restimulated with medium, Ec LPS (10 ng/ml), Pg LPS (100 ng/ml), or Pam3Cys (100 ng/ml). A, NF-κB DNA binding activity was analyzed by EMSA as described in Materials and Methods. B, Western blot of corresponding nuclear extracts was (Figure continues)
TLR homotolerance is statistically significantly greater than TLR heterotolerance at the level of NF-κB transactivation (p < 0.001).

Expression of IκBα protein is elevated in the macrophages, rendered tolerant by TLR2 and TLR4 agonists

NF-κB activation is preceded by degradation of inhibitor proteins including IκBα, IκBβ, and IκBε. Previous studies have demonstrated inhibition of IκBα and IκBβ in cells pretreated with TLR agonists and then challenged homologously (18, 40) or heterologously (18). However, to date, the potential role of IκBε in tolerance has never been evaluated. Therefore, protein levels of IκBα and IκBε were measured in total lysates of cells challenged with either TLR4 or TLR2 agonists. In medium-pretreated macrophages, both TLR2 and TLR4 agonists induced degradation of these inhibitor proteins, although with different kinetics, i.e., IκBα degradation is very fast and clearly detected by 30 min, while degradation of IκBε is slower, yet clearly observed after 1 h of treatment (Fig. 7). In cells pretreated with Ec LPS, degradation of IκBα in response to the same agonist (homotolerance) was perturbed, i.e., it was not observed after 30 min of challenge and was very weak after 1 h. If Ec LPS-treated cells were challenged with TLR2 agonists, IκBα degradation was inhibited, but to a lesser extent. A similar pattern was observed in cells pretreated with TLR2 agonists and challenged with either TLR2 or TLR4 agonists. These data confirm and extend previous observations (18, 40) and also demonstrate that homotolerance is stronger than heterotolerance at the level of IκBα degradation.

In contrast to the results obtained with IκBα, pretreatment of macrophages with either TLR2 or TLR4 agonist results in a striking and sustained increase in the level of IκBε (Fig. 7; compare lanes Ec LPS/M, Pg LPS/M, and with M/M for IκBε). Macrophages pretreated with TLR agonists and restimulated (either homotolerance or heterotolerance) exhibited levels of IκBε that were well above those observed following initial exposure to any of the agonists tested. To determine whether overexpression of IκBε might contribute to tolerance by inhibiting NF-κB DNA binding induced by TLR2 and TLR4 agonists, we performed experiments with the HEK 293 cell line (Fig. 8). In contrast to the LPS-unresponsive HEK 293T cell line or other HEK 293 clones that have been reported to be LPS-unresponsive (e.g., Refs. 32 and 44), the cells used in this study were obtained from the American Type Culture Collection, express TLR2 and TLR4 mRNA (data not shown), and respond to Ec LPS, Pg LPS, and Pam3Cys within 1 h. We transfected these cells with a reporter expression vector that carries either no cDNA or cDNA that encoded human IκBε, stimulated the transfectants with Ec LPS, Pg LPS, or Pam3Cys, and measured NF-κB DNA binding by EMSA. Fig. 8A illustrates that in empty vector transfectants, all agonists induce NF-κB DNA binding, while overexpression of IκBε (confirmed by Western blot, Fig. 8B) results in significant inhibition of activity. These data support the hypothesis that elevated IκBε induced by TLR agonists may contribute to suppressed NF-κB activity observed in tolerized murine macrophages.

Peritoneal macrophages rendered tolerant by both TLR2 and TLR4 agonists reveal significant expression of IκBε mRNA that is not detected in control cells

Recently, Yamazaki et al. (45) described another related protein, IκBζ, whose maximum mRNA expression is induced by LPS and IL-1β within 1 h in RAW 264.7 cells and is maintained at a low, but elevated, level as late as 48 h after LPS stimulation. They also demonstrated that IκBζ is able to bind to the p50 subunit of NF-κB and prevent binding of the p50/p65 heterodimer to DNA. Fig. 9 shows that both TLR4 and TLR2 agonists strongly induce expression of IκBζ mRNA. These data suggest that induced IκBζ may also contribute to inhibition of NF-κB DNA binding by TLR2 and TLR4 agonists that we observed in peritoneal macrophages rendered TLR homotolerant or heterotolerant.

Discussion

The phenomenon of “endotoxin tolerance” was first formally described by Favorite and Morgan (46). Later, macrophages were identified as pivotal cellular participants in the acquisition of the tolerant phenotype (47). Endotoxin tolerance is not limited to the potent TLR4 agonist, enterobacterial LPS, but also targets signaling initialized by agonists that are recognized by other members of the Toll/IL-1R family (18–20, 28). The major signaling components leading to NF-κB translocation, i.e., IRAK-1, IκBα, and IκBβ, have been reported to be perturbed in LPS-tolerized macrophages (18, 25, 27). In vitro tolerance has also been attributed to diminished cell surface expression of the TLR4/MD2 complex (27); however, the failure to observe this at low, but tolerizing, doses of LPS, coupled with the observation that TLR4/MD2 overexpression does not block tolerance (28), would argue against this as a general mechanism of inducible hyporesponsiveness.
FIGURE 7. Western blot analysis of IκB proteins from control macrophages or cells rendered tolerant by TLR2 or TLR4 agonists. Peritoneal macrophages derived from C3H/OuJ mice were pretreated with medium only, Ec LPS (10 ng/ml), Pg LPS (1000 ng/ml), or Pam3Cys (100 ng/ml) for 24 h. After that, cells were washed three times with warm medium and re-stimulated with medium, Ec LPS (10 ng/ml), Pg LPS (100 ng/ml), or Pam3Cys (100 ng/ml) for 1 h. IκBα and IκBe proteins were analyzed by Western blot as described in Materials and Methods. The results are representative of three independent experiments.

FIGURE 8. Overexpression of IκBe protein in HEK 293 cells suppresses the NF-κB DNA binding activity induced by TLR2 or TLR4 agonists. HEK 293 cells were transiently transfected with either empty vector or vector carrying IκBe cDNA. Twenty-four hours after transfection, cells were treated with medium only, Ec LPS (10 ng/ml), Pg LPS (1000 ng/ml), or Pam3Cys (10 ng/ml) for 1 h. A, NF-κB DNA binding activity was measured by EMSA as described in Materials and Methods. B, Western blot analysis of whole cell lysates with anti-IκBe Ab was performed to confirm expression of this inhibitory protein. The results are representative of two independent experiments.

FIGURE 9. TLR2 and TLR4 agonists induce expression of IκBε mRNA in tolerant macrophages. Peritoneal macrophages derived from C3H/OuJ mice were treated with medium only, Ec LPS (10 ng/ml), Pg LPS (1000 ng/ml), or Pam3Cys (100 ng/ml) for 24 h. After that, total RNA was extracted and analyzed by Northern blot hybridization as described in Materials and Methods. Equal amounts of loaded RNA was confirmed by methylene blue staining of the membrane before hybridization. To confirm that at this time period macrophages demonstrate a tolerant phenotype, cells from control plates were restimulated with agonists and NF-κB DNA binding activity was measured by EMSA. The results are representative of two independent experiments.

Using highly purified or synthetic TLR4 or TLR2 agonists, we found that TNF-α secretion is strongly suppressed in TLR2 and TLR4 homotolerance (Fig. 1), consistent with many examples of in vitro tolerance induced by other serotypes of enterobacterial LPS or TLR2 agonists such as MALP2 and LTA (19, 20). However, our finding that Pg LPS induces homotolerance in murine peritoneal macrophages contrasts with previous findings by Martin et al. (40), who demonstrated enhanced TNF-α production in response to Pg LPS in PMA-differentiated THP-1 cells, pretreated with the same agonist. This discrepancy may be due to the fact that PMA alone may modulate expression of a variety of genes including those examined in the context of TLR homotolerance. Although previous studies using MALP2 and LTA for cross-tolerance with LPS showed clear induction of TLR heterotolerance at the level of TNF-α secretion (19, 20), production of TNF-α by macrophages pretreated with the TLR4 agonist and restimulated with TLR2 agonists (i.e., TLR4/TLR2 heterotolerance) was not affected in our study (Fig. 1, A and B). TLR2/TLR4 heterotolerance was induced by Pg LPS, but not by the other TLR2 agonist, Pam3Cys, despite the fact that they both tolerate against each other (Fig. 1C).

Several factors may contribute to the observed differences between Pg LPS and Pam3Cys effects on subsequent challenge with Ec LPS at the level of TNF-α secretion. For example, MALP2 was recently shown to require TLR6 in addition to TLR2, while Pam3Cys was found to require both TLR1 and TLR2 (41, 48). LTA was initially reported to require both TLR2 and TLR4; however, recent studies using highly purified LTA indicate that it uses TLR2 and not TLR4 (3, 49). It is possible that unlike Pam3Cys, Pg LPS uses a TLR complex other than TLR2 and TLR1 and, thus, elicits a distinct signaling pattern revealed only in the context of Ec LPS challenge. Both Pg LPS and Ec LPS also share a common coreceptor, CD14 (50); therefore, Pg LPS may cross-tolerize against Ec LPS by modifying the expression and/or function of this component of the receptor complex. The findings presented herein indicate that for TNF-α secretion, TLR homotolerance (i.e., the tolerizing stimulus and the challenge stimulus use the same TLR) is substantially stronger than TLR heterotolerance (i.e., the tolerizing and secondary stimuli activate different TLRS).

It has been previously shown that MyD88 is essential for TNF-α secretion, in response to both TLR2 and TLR4 agonists (51), and more recently, it has been shown that TLR4 homotolerance is associated with a failure of MyD88 to be recruited to TLR4 (52). In contrast, IFN-β gene expression is normal in LPS-stimulated MyD88 null macrophages, but inhibited by expression of a TIRAP/Mal dominant negative construct or by a TIRAP/Mal inhibitory peptide (36). Here we show that expression of the IFN-β gene was completely inhibited in TLR4 homotolerance, but not by prior exposure to either TLR2 agonist. Thus, engagement of the
MyD88-dependent arm of the TLR2 signaling pathway is not sufficient to induce tolerance to the TIRAP/Mal-dependent pathway.

Certain cytokines produced by LPS-stimulated macrophages have been suggested as potential mediators of the tolerant phenotype. However, the participation of IL-10 and TNF have been excluded because macrophages derived from IL-10-deficient mice or TNFR-I/TNFRII double knockout mice were readily tolerized by LPS and/or MALP2 (18, 19). IL-1 has also been implicated as a mediator of tolerance in vivo (16, 53) and has been shown to induce a state of cross-tolerance to LPS in vitro (18). However, we observed no difference in the acquisition of the TLR4 and TLR2 tolerant phenotype between IL-1R⁻/⁻ and wild-type macrophages at the level of TNF-α secretion and NF-κB DNA binding (data not shown). Thus, IL-1 signaling does not contribute to the tolerance induction in vitro.

To dissect the mechanism(s) that underlie in vitro tolerance induced by TLR2 and TLR4 agonists in murine macrophages, we also analyzed the major shared signaling elements of the NF-κB pathway. We first analyzed IRAK-1 kinase, which acts downstream of the MyD88 adaptor protein upon IL-R/TLR engagement (25, 35). Diminished IRAK-1 kinase activity in LPS-tolerized human and murine macrophages was described previously (25, 27). Our data extend these findings by showing that the kinase activity associated with IRAK-1 immunoprecipitates was completely blocked in TLR4/TLR4 homotolerance and TLR2/TLR4 heterotolerance, while residual activity was detected in cells subjected to TLR4/TLR2 heterotolerance (Fig. 2). It was recently reported that IRAK-1 and IRAK-2 associate differentially with MyD88 vs TIRAP/Mal (12). In addition, recently, two other serine/threonine kinases, IRAK-4 and receptor interacting protein 2, that act downstream of IL-1R, TLR2, TLR3, and TLR4 receptors, but upstream of TNFR-associated factor 6, were demonstrated to be important for cytokine production and NF-κB activation in macrophages (13, 14). Therefore, it is possible that TLR2 and TLR4 agonists activate these kinases and their adaptor proteins differentially. This is supported by the most recent observation of Jacinto et al. (54), in which heterotolerance between highly purified LTA and LPS was not observed at the level of IRAK-1 degradation.

Activation of the IKK complex is the next critical element in this cascade and is absolutely necessary for NF-κB activation. Both IKKβ- and IKKγ-deficient cells fail to activate NF-κB in response to inflammatory stimuli, while IKKα deficiency has no effect on IkB phosphorylation (15). We observed complete inhibition of IKKβ kinase activity in TLR2 and TLR4 homotolerance (Fig. 3), but surprisingly, IKKβ kinase activity was not affected in heterotolerance. This suggests that IKKβ dysregulation may contribute to the observation that TLR homotolerance is consistently stronger than TLR heterotolerance. Recently, two atypical protein kinases (PK), i.e., PKCε and PKCζ, were reported as critical for NF-κB activation by LPS and IL-1 (55, 56) and were associated with IRAK (57). If PKC-mediated signaling overlaps in part with that of IRAK, this may represent an additional signaling arm differentially affected by TLR2 and TLR4 agonists and explain why we observe IKK activation in heterotolerance in the absence of IRAK phosphorylation.

We next analyzed the effect of TLR homo- and heterotolerance on NF-κB DNA binding. Induction of TLR2 and TLR4 homo- and heterotolerance was evidenced by diminished nuclear translocation of the major NF-κB components, p50 and p65. Again, TLR2 homotolerance is stronger than TLR4/TLR2 heterotolerance as evidenced by statistical analysis of NF-κB complexes bound to DNA. In several previous studies, the involvement of p50 and p52 subunits of NF-κB that lack the transactivating domain (and, thus, may serve as a transrepressors) in the acquisition of the tolerant phenotype was described (58–60). However, later data by Wysocka et al. (61) demonstrating that p50 knockout mice and macrophages derived from them are readily tolerized by LPS would suggest otherwise. Our findings support those of Wysocka et al. (61). Analysis of nuclear fractions by Western blot with Abs specific to p50 and p65 subunits of NF-κB, however, revealed the presence of both these proteins at the levels comparable with that observed in medium-pretreated/agonist-stimulated cells (Fig. 5B). This suggests the existence of additional inhibitory factors that prevent NF-κB from binding to DNA. Analysis of the composition of NF-κB DNA binding complexes induced by TLR2 and TLR4 agonists in medium-pretreated macrophages revealed a faster migrating species not observed in macrophages tolerized by TLR2 or TLR4 agonists. This complex contains C-terminally truncated p65. Previous studies by Levkau et al. (62) showed that p65 may be cleaved by activated caspases. LPS-activated caspase activity has been reported (63); however, no connection between p65 cleavage and LPS-induced caspase activation has been demonstrated. A second possible mechanism for the generation of this truncated species is in vitro cleavage during cell fractionation by released caspase G and elastase (64), the serine proteases expressed by monocytes and immature macrophages and replaced during maturation by matrix metalloproteinases (MMP), such as MMP9 (65). Murine macrophages produce and secrete elastase and this ability is inhibited by LPS (66), while induction of MMP9 mRNA by both TLR2 and TLR4 agonists is detected (M. A. Dobrovolskaia, unpublished observation). Therefore, the truncated p65 observed in medium-pretreated, agonist-stimulated macrophages may be the result of degradation of p65 by serine protease(s) and that 24 h pretreatment of cells with either TLR2 or TLR4 agonists induces macrophage differentiation and loss of these proteins.

NF-κB activation is preceded by degradation of inhibitor proteins including IκBα, IκBβ, and IκBε. Previous studies have demonstrated inhibition of IκBα and IκBβ in cells pretreated with TLR agonists and then challenged homologously (18, 40) or heterologously (18). However, to date, the potential role of IκBε in tolerance has not been evaluated. Our data extend previously published observations for IκBα (18, 40) by demonstrating that homotolerance is stronger than heterotolerance. As was observed for TNF-α secretion, the effect of Pg LPS on IκBε degradation was different from that described in PMA-differentiated THP-1 cells (40). Remarkably, pretreatment of C3H/OU mice macrophages with the either TLR2 or TLR4 agonist results in a striking increase in IκBε in cells that is sustained, regardless of subsequent stimulus. These data suggest that IκBε might well contribute to the induction of TLR homotolerance or heterotolerance, a hypothesis supported by our finding that transfectants that overexpress IκBε exhibit profoundly suppressed NF-κB DNA binding activity (Fig. 8). The original paper, describing IκBε, demonstrated elevated expression of IκBε in murine embryonal fibroblasts from IκBα-deficient mice and suggested that IκBε may substitute for IκBα (31). Our study provides the first evidence for a physiologic effect of such a “substitution.” Recently, Lee demonstrated cytosol-to-nuclear shuttling of IκBε as mechanism to control NF-κB function in the nucleus (67). Therefore, it is possible that when induced by TLR2 and TLR4 agonists, IκBε (Fig. 7) prevents the p50 and p65 that we detect in the nuclear fractions by Western blot (Fig. 5B) from binding to DNA studied by EMSA (Fig. 5A). However, direct evidence for this was not possible as the levels of endogenous IκBε bound to immunoprecipitable nuclear p50/p65 NF-κB complexes were extremely low (data not shown). Expression of the mRNA for a novel IκB species, IκBε, was not detected in medium-pretreated cells and was observed only in cells exposed for 24 h to TLR2.
or TLR4 agonists (Fig. 9), coincident with suppressed responsiveness to restimulation of cells with agonist. Nuclear IkBγ has been shown to inhibit p50/p65 binding to DNA and transactivation. Future studies to establish a role for this molecule in tolerance will be feasible only when specific immunologic and molecular reagents become available. In addition to this, other factors may contribute to the observed inhibition of NF-κB DNA binding. For example, we found that tolerant cells have more Oct-1 bound to DNA. It was recently reported that Oct-1 interacts with both p50 and p65 in vitro and in vivo and leads to suppression of NF-κB DNA binding and transactivation (68). TLR4, but not TLR2, agonists induce NO production by macrophages (37). NO causes nitrosylation of and transactivation (68). TLR4, but not TLR2, agonists induce NO, which may inhibit NF-κB in tolerance.

Our findings revealed that exposure of macrophages to concentrations of TLR4 or TLR2 agonists that led to equivalent NF-κB translocation could be paralleled by unambiguous homotolerance at the level of NF-κB DNA binding or TNF-α secretion. Therefore, for homotolerance, NF-κB is a good surrogate marker. However, it is also clear that restimulation with the opposite TLR agonist can result in augmentation, no change, or inhibition of TNF-α secretion. We believe that this represents “macrophage reprogramming” (70) due to the action of different combinations of transactivating and transpressing factors that are elicited by and accumulate as a consequence of the initial engagement of a specific TLR signaling pathway. A disconnect between the “distal” cytokine data and “proximal” signaling data demonstrated above, may be explained as follows. First, in addition to NF-κB, TNF-α gene expression requires a specific set of transcription factors (TFs), such as activating transcription factor 2, Ets, Elk-1, c-jun, c set of transcription factors (71). Inactivation and inactivation by bacterial lipopolysaccharide. Microbes Infect. 4:903.

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