The Functional Effects of Physical Interactions among Toll-like Receptors 7, 8, and 9*

Received for publication, June 2, 2006, and in revised form, September 29, 2006. Published, JBC Papers in Press, October 13, 2006, DOI 10.1074/jbc.M605311200

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Toll-like receptors (TLRs) TLR1, TLR2, TLR4, and TLR6 are evolutionarily conserved, highly homologous, and localized to plasma membranes of host cells and recognize pathogen-associated molecular patterns (PAMPs) derived from bacterial membranes. These receptors cooperate in a pairwise combination to elicit or inhibit the inflammatory signals in response to certain PAMPs. The other TLRs that are evolutionarily closely related and highly homologous are TLR7, TLR8, and TLR9. They are all confined to the membranes of endosomes and recognize similar molecular structures, the oligonucleotide-based PAMPs. However, the cooperative interactions among these receptors that may modulate the inflammatory signaling in response to their cognate agonists are not reported. We report here for the first time the functional effects of one TLR on the other among TLR7, TLR8, and TLR9. The results indicate that TLR8 inhibits TLR7 and TLR9, and TLR9 inhibits TLR8 but not vice versa in HEK293 cells transfected with TLRs in a pairwise combination. This is concluded by selectively activating one TLR over the other by using small molecule TLR agonists. We also show that these inhibitory interactions are the result of direct or indirect physical interactions between the TLRs. The murine TLR8 that does not respond to any known human TLR8 agonist also inhibits both murine and human TLR7. The implications of the inhibitory interactions among these TLRs in host-pathogen recognition and subsequent inflammatory responses are not obvious. However, given the complexity in expression pattern in a particular cell type and the variation in distribution and response to different pathogens and stress signals in different cell types, the inhibitory physical interactions among these TLRs may play a role in balancing the inflammatory outcome from a given cell type to a specific challenge.

In vertebrates the innate immune system is a first line of defense against invading agents (1). The innate immune system contains components conserved between vertebrates and invertebrates and includes receptors recognizing molecular structures that are highly conserved among pathogens called pathogen-associated molecular patterns (PAMPs). Activation of the innate immune system through pattern recognition receptors by molecules derived from evolutionarily distant pathogens like bacteria, fungi, and viruses provides the necessary signals for the initiation and activation of the adaptive immune response. Thus, the innate immune system plays a critical role in triggering the activation of adaptive immune responses by promoting the presentation of antigens and up-regulation of various cytokines and co-stimulatory molecules (2).

Toll-like receptors (TLRs), mammalian homologs of the Drosophila receptor Toll, are an integral part of the innate immune system (1, 3). So far 10 different human TLRs (TLR1 to 10) have been identified (4). All TLRs are type I transmembrane proteins composed of an N-terminal extracellular domain with leucine-rich repeats involved in ligand (PAMP) recognition, a single transmembrane domain, and a cytoplasmic domain largely made up of a TIR (Toll/IL-1 receptor homology) domain required for downstream signaling (5, 6). While TLR1, -2, and -4–6 are involved in recognizing PAMPs associated with bacterial membranes (1, 3), TLR3 and -7–9 recognize oligonucleotide (RNA and DNA)-based molecular patterns from both bacteria and viruses (7–9). To date, the role of TLR10 has not been established, and its ligand (PAMP) has not been identified. The TLRs that recognize bacterial membrane components are confined to the plasma membrane (10). The other TLRs that recognize oligonucleotide -based ligands are distributed to the membranes of the endosomal compartment (10, 11). Although most TLRs function as homodimers (or homopolymers) upon ligand binding, some ligands require cooperation of more than one TLR in activating the downstream signaling cascade. For example, in vitro studies using cotransfection of different TLRs into cells that normally do not express TLRs have confirmed that heterotypic interactions between TLR1 and TLR2, TLR2 and TLR6, and TLR4 and TLR6 are required for recognition and activation of signaling in response to triacyl lipopolipetides, diacyl lipopeptides, and other bacterial cell wall components (11, 13–17). However, the TLR-TLR heterotypic interactions are not always activating. The heterotypic TLR1-TLR4 interactions were shown to be inhibitory to TLR4 signaling in response to lipopolysaccharide (LPS) (18). Therefore, certain heterotypic TLR-TLR interactions are essential not only for the recognition of a particular PAMP but may also play a regulatory role in controlling the extent of inflammatory response.

The factors that may facilitate either homo- or heterotypic interactions among TLR1, -2, -4, and -6 appear to be their spa-

mDCs, myeloid dendritic cells; pDCs, plasmacytoid dendritic cells; PMA, phorbol ester 12-myristate 13-acetate; m, murine; h, human; LPS, lipopolysaccharide.

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The abbreviations used are: PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; NF-κB, nuclear factor-κB; IP, immunoprecipitation; EGFP, enhanced green fluorescent protein; CFP, cyan fluorescent protein; HA, hemagglutinin; PBMCs, peripheral blood mononuclear cells;
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tional antibody from U. S. Biological (Swampscott, MA), anti-obtained from different sources as follows: anti-TLR7 poly-Biotec (High Point, NC). Antibodies used in this study were GTCGTTTTGTCGTT-3
chem. TLR9 agonist, CpG-2059 (5'glyceryl-cystein (Pam-3-Cys), was purchased from Calbio-
was purchased from Sigma, and TLR2 agonist, tripalmitoyl-
respectively, were made by 3M chemists (9). TLR4 agonist, LPS,

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Plasmids—HEK293 cells stably trans-
fected with NF-κB-luciferase reporter construct (HEK293-NF-
kB) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. TLR7- and TLR8-selective agonists, 3M-001 and 3M-002, respectively, were made by 3M chemists (9). TLR4 agonist, LPS, was purchased from Sigma, and TLR2 agonist, tripalmitoyl-S-glyceryl-cystein (Pam-3-Cys), was purchased from Calbio-
chem. TLR9 agonist, CpG-2059 (5’-TCGTGCTTTTT-GTCGTTTTTGTCGTT-3’) was custom-synthesized by MWG Biotec (High Point, NC). Antibodies used in this study were obtained from different sources as follows: anti-TLR7 polyclonal antibody from U. S. Biological (Swampscott, MA), anti-
TLR9 antibodies from InvivoGen (San Diego), anti-HA antibod-
ies from Novus Biologicals (Littleton, CO), and anti-EGFP antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All the antibodies were validated for their selectivity by using total cell extracts made from HEK293 cells transfected with corresponding TLRs and tagged TLR. Luciferase reporter gene assay kits (LucLite®) were obtained from PerkinElmer Life Sciences.

The human TLR2 and TLR4-MD2 (the DNA sequence correspond-

ting to TLR4 and MD2 was separated by the internal ribosomal entry site), murine TLR7 (mTLR7), human TLR7, murine TLR8 (mTLR8), and human TLR8 (isoform 1), and human TLR9 expression vectors were made by ATG Laborato-
ries (Coon Rapids, MN) for 3M Co., by cloning human or murine full-length cDNA corresponding to TLR into pCI-Neo vector (Promega Corp., Madison, WI). The TLR8 expression vector with N-terminal HA tag was constructed by inserting the HA tag at the C-terminal end of the predicted signal peptide sequence (after 45 residues from the N-terminal end or TLR8 isoform 1) in pCI-Neo-hTLR8 clone. TLR2-CFP and TLR7-
EGFP expression plasmids were a kind gift from Dr. Golenbock (University of Massachusetts, Worcester).

Transient Transfections and Luciferase Reporter Assay—

HEK293-NF-κB cells were seeded at 5 × 10^4 cells/well in 10-cm dishes containing Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C and 5% CO2. After 4 h, the cells were transiently transfected with a mammalian expression vector encoding human or murine TLRs at 1 μg of total DNA/10^6 cells, and FuGENE 6 (Roche Applied Science) was used as transfection reagent at 1:3 DNA to FuGENE 6 (w/w ratio). In experiments where individual TLRs were transfected, 0.5 μg of the TLR DNA and 0.5 μg of control DNA (pCI-Neo vector or pCI-Neo-TLR2 plasmid DNA) were used, and in pairwise cotransfection experiments 0.5 μg of each TLR DNA was used. Twenty four hours post-transfection, 5 × 10^4 cells/well were transferred to 96-well flat-bottom plates. After 2 h, the cells were stimulated for 24 h with TLR agonists. TLR7- and TLR8-selective agonists, 3M-001 and 3M-002, respectively, were added from MeSO stock solutions, and TLR2, TLR4, and TLR9 agonists were added from the stocks solutions made in the culture media. The cells were lysed and analyzed for lucif-
erase activity using LucLite® by measuring luminescence intens-
ity on an LMax lumimeter (Molecular Devices, Sunnyvale, CA). The luminometer was calibrated to read relative luciferase units.

Differentiation of THP1 Cells—THP1 cells were grown at 1.5 × 10^5 cells/ml in RPMI containing 10% fetal bovine serum (all media reagents from BIOSOURCE), 1 mM sodium pyruvate, 2 mM glucose, 10 mM Hepes, pH 7.4, with 10 μg/ml streptomycin. The day before phorbol ester 12-myristate 13-acetate (PMA) treatment, cells were split into fresh media to ensure they were in log phase growth. The following day the cells were adjusted to 5 × 10^5 cells/ml in fresh medium containing 30 mM PMA (Sigma) and incubated at 37 °C and 5% CO2 for 48 or 72 h. Cells were washed twice with 1 ml of phosphate-buffered saline and replaced with fresh media. After resting for 48 or 72 h, the cells
were challenged with TLR7 or TLR8 selective agonists in fresh media for 24 h. The cells were centrifuged for 10 min at 2000 rpm and supernatants withdrawn for cytokine measurements.

**Western Blotting and Coimmunoprecipitations**—Equal amounts of DNA (1 μg of total DNA/10^6 cells) for each TLR were cotransfected into HEK293-NF-κB cells and harvested after 24 h. Cells were washed once with phosphate-buffered saline and lysed in cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged (10,000 × g, 30 min at 4 °C) to remove the insoluble material. For immunoblotting, cell lysates were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblot analysis was performed with rabbit polyclonal antibodies and visualized with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin, using ECL method (Amersham Biosciences). For immunoprecipitation experiments, cell lysates were pre-cleared with protein A-agarose beads, and then protein concentration was determined. Cell extracts (1 mg of protein in 1 ml) were incubated with 1 μg of the antibody for 2 h at 4 °C followed by the addition of 50 μl (packed volume) of protein A-Sepharose beads. After 1 h at room temperature, the beads were washed three times with 1 ml of lysis buffer. The protein bound beads were boiled for 1 min in 2× sample buffer and subjected to Western blot analysis as described earlier.

**RESULTS**

The immune cells that express multiple TLRs at varying levels are not amenable to the study of specific TLR-TRL functional interactions selectively. It adds more complexity to study functional interactions among TLR7, TLR8, and TLR9 as they are all localized to the same intracellular compartment (10) and expressed in different combinations in different immune cells (25, 26, 28). Also, use of immune cells lacks the control for comparison of the function of one TLR in the absence and presence of the other TLR. As the specific protein-protein interaction studies require control over the stoichiometry of the proteins, we have used HEK293 cells, which provide an opportunity to manipulate the expression levels of one TLR over the other as they do not express any of the TLRs used in this study and are also widely used for studying the function of TLRs in general.

Selective activation of either TLR7 or TLR8 is the most important aspect of this study to understand the effect of TLR-TRL physical interactions on signaling by either of the receptors while they are coexpressed. Previously, it has been shown that 3M proprietary imidazoquinolines, 3M-001 and 3M-002, selectively activate TLR7 and TLR8, respectively, as measured by NF-κB-reporter activation in TLR7 or TLR8 transiently transfected HEK293 cells and selective cytokine induction in human PBMCs (9). Here we have used 3M-001 and 3M-002 to selectively activate TLR7 and TLR8, respectively, and CpG-2059 to activate TLR9, and we studied the effect of one TLR on the signaling (NF-κB activation) of the other TLR.
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The inhibitory effects of TLR8 and TLR9 are dose-dependent. HEK293-NF-κB cells were transfected with increasing amounts of DNA (0 to 5 μg/10⁶ cells) corresponding to the inhibitory TLR and fixed amount of DNA (5 μg/10⁶ cells) corresponding to the TLR that is inhibited, while the total DNA was kept constant (10 μg/10⁶ cell) using a control vector DNA (pCI-Neo-TLR2). The activation of NF-κB in terms of luciferase activity was measured in response to 10 μM of corresponding TLR-selective agonists. The plots represent fold increase over vehicle control (CTRL) (MeSO for 3M-001 and 3M-002 and media for CpG-2059) and plotted as a function of increasing DNA corresponding to the inhibitory TLR that is transfected. Each data point represents mean ± S.E. of three measurements (triplicate), and the plots are representative of at least two to three separate experiments (note different y axis scale in B and C but not A to represent fold increase in response to different agonists). Protein plots were developed from total cell extracts made from the same batch of transfected cells used for the functional studies. The lane corresponding to no TLR (right-most lane) was loaded with total cell extracts made from HEK293-NF-κB cells transfected with control vector (10 μg/10⁶ cells) alone. Note that in these experiments, HA-tagged TLR8 was used for the purpose of protein detection using anti-HA antibody, as there was no anti-TLR8 antibody available commercially.

and TLR9 inhibits TLR7 (Fig. 1C). On the other hand, TLR7 had no inhibitory effects on either TLR8 (Fig. 1B) or TLR9 (Fig. 1D), and TLR9 did not inhibit TLR8 (Fig. 1E). The stimulatory effects of TLR7 on the TLR9 response to CpG-2059 (Fig. 1D) were consistent across all the experiments. Day to day variations in the magnitude of fold increase in response to the activation of TLRs were common. Therefore, we always performed a given set of experiments (e.g. Fig. 1, A and B, as one set, C and D, as one set, and E and F as another set) together for a better comparison. The inhibition of TLR7 by TLR8 was consistently more complete compared with the inhibition of either TLR7 by TLR9 or TLR9 by TLR8 (Fig. 1, A versus C and F).

The molecular basis for the inhibitory effects of TLR8 and TLR9 may be 2-fold as follows: 1) competition for downstream signaling proteins as all these TLRs share a common adaptor protein, MyD88 (31, 32), and the kinases, IRAK1 and IRAK4 (33); and 2) direct physical interaction as all these TLRs confined to the same cellular compartment, the endosomes. If these inhibitory effects are because of the competition for the downstream signaling proteins, then the coexpression of either TLR8 or TLR9 with other TLRs of different compartment should also have similar inhibitory effects. To test this hypothesis, TLR8 or TLR9 were cotransfected with either TLR2 or TLR4-MD2 in HEK293-NF-κB cells, and the activation in response to either Pam-3-Cys (TLR2) or LPS (TLR4) was measured. The results show that TLR8 or TLR9 does not inhibit either TLR2 (Fig. 1G) or TLR4 (Fig. 1H). Previously, we showed that TLR2 (used as control plasmid in single TLR transfections to keep the amount of DNA levels similar to cotransfections) does not have any functional effects on TLR7, TLR8, or TLR9 (Fig. 1, A–F). Therefore, it is unlikely that the inhibitory effects of TLR8 (on TLR7 and TLR9) and TLR9 (on TLR7) are because of a direct competition for the downstream signaling components. Next, the adaptor proteins and the kinases are recruited to the receptor only after agonist binding, and in these experiments only one of the TLRs was selectively stimulated.

Therefore, the inhibitory effects of TLR8 and TLR9 may have resulted from direct or indirect (mediated through an unknown protein) physical interactions.

The Inhibitory Effects of TLR8 and TLR9 Result from Direct or Indirect Physical Interactions—If the inhibition of one TLR by the other is not a consequence of direct competition for downstream signaling proteins, then the other obvious reason is the direct or indirect physical interactions between the TLRs. If one TLR inhibits the other TLR through direct or indirect physical interactions, then the inhibition of either TLR7 or TLR9 by TLR8 and TLR7 by TLR9 should be dependent on the protein concentration of the inhibitory TLR. To this end, the cotransfection experiments were performed by systematically varying the amount of DNA corresponding to the inhibitory TLR against a fixed amount of DNA corresponding to the TLR that is inhibited while keeping the total amount of DNA constant by supplementing with a control vector (pCI-Neo-TLR2) DNA (Fig. 2). Both NF-κB activation in response to 10 μM selective TLR agonists and protein expression levels were measured. As the commercial anti-TLR8 antibodies were not of usable quality, as tested in HEK293 cells expressing TLR8, we have used N-terminal HA-tagged TLR8 (see “Experimental Procedures” for details) in these experiments to measure the protein levels using anti-HA antibody. The use of HA-tagged TLR8 did not alter the results as its inhibitory effects on either TLR7 or TLR9 were comparable with untagged TLR8. The results show a good correlation between increased protein levels of either TLR8 or TLR9 resulting from increasing amounts of DNA transfected, while the protein levels of either TLR7 and TLR9, where fixed amount of DNA was transfected, remained qualitatively unchanged (Fig. 2). The increased protein levels of the inhibitory TLRs in turn showed correspondingly increased inhibitory effects (Fig. 2, A–C). The inhibitory effects of TLR8 and TLR9 are independent of the concentration (0.3, 1.0, 3.0, 10, or 30 μM) of agonist used for the selective activation of either TLR7 or TLR9 (only data corresponding to 10 μM agonist is presented in Fig. 2). The inhibitory effects of TLR8 on TLR7 (Fig. 2A) as a function of increasing amounts of TLR8 DNA are much steeper compared with more gradual inhibitory effects of either TLR8 on TLR9 (Fig. 2B) or TLR9 on TLR7 (Fig. 2C). These results are also consistent with previous results (Fig. 1) that the inhibition of TLR7 by TLR8 is more complete than that of TLR9 by TLR8 or TLR7 by TLR9 in cotransfection experiments using equal amount of DNA corresponding to each TLR. It is interesting to note that the response of TLR8 to 3M-002 is qualitatively unchanged with increasing amounts of DNA transfected, even though there is an increase in protein expression levels.
TLR8-dependent NF-κB activation in response to 3M-002 has been reached at very low protein levels of TLR8. On the other hand, in TLR7 and TLR9 coexpression studies, the inhibition of TLR7 or the activation of TLR9 is more typical, because the effects are gradual and reciprocal with increasing expression of protein as a function of increasing amounts of TLR9 DNA. Also, as noted previously (Fig. 1D), in the present DNA titration experiments the increased activation of TLR9 in the presence of TLR7 (Fig. 2C), compared with TLR9 alone, is consistent, indicating that TLR7 and TLR9 interactions are synergistic to TLR9 function.

The correlation between increased protein expression and the increased inhibitory activity suggests that the inhibition of one TLR by the other is because of direct or indirect physical interactions. To further confirm the existence of physical interactions between these TLRs, coimmunoprecipitation (co-IP) experiments were performed on total cell lysates prepared from HEK293-NF-κB cells transfected with DNA corresponding to either TLR7-EGFP and HA-TLR8 or HA-TLR8 and TLR9. As the subsequent lots (compared with the lot used in Fig. 2) of anti-TLR7 antibodies (U. S. Biological) were not of usable quality (as tested against TLR7-transfected HEK293-NF-κB cell lysates), we have used TLR7 with C-terminal EGFP fusion construct (TLR7-EGFP) in co-IP experiments. Similarly, as the vendor (InvivoGen) stopped the supply of anti-TLR9 antibody, we were unable to perform co-IP experiments on lysates prepared from cells cotransfected with TLR7 and TLR9. The results of co-IP experiments show HA-TLR8 pull down using anti-HA antibody co-IPs TLR7-EGFP (Fig. 3B) and TLR9 (Fig. 3C) from the corresponding cell lysates as detected on Western blots using anti-EGFP and anti-TLR9 antibodies, respectively (Fig. 3). These results confirm that the inhibitory effects of either TLR8 or TLR9 may be due to direct or indirect physical interactions between the TLRs. These interactions appear to be selective among TLR7, TLR8, and TLR9 but not TLR2 as evidenced by the lack of TLR2 co-IP with HA-TLR8 (Fig. 3A). Although the TLR7-EGFP was inactive (TLR7 with any tag or fusion protein either at the N or C terminus was inactive), EGFP alone did not show any effect on the pulldown experiments. As noted earlier, the co-IP experiments could not be performed on lysates of cells coexpressing TLR7 and TLR9 because of the limitation on the availability of TLR9 antibodies. However, the physical interactions (as evidenced by colocalization) between TLR7 and TLR9 have been reported earlier using fluorescence imaging methods on HEK293 cells expressing TLR7-YFP and TLR9-CFP.3 The physical association of TLR8 with either TLR7 or TLR9 is agonist-independent, indicating the pre-existence of TLR7, TLR8, and TLR9 hetero-oligomers in their respective compartments. Similarly, it has been shown that plasma membrane-associated TLRs, TLR1 and TLR2, can also pre-exist as hetero-oligomers, and antibody patching of TLR2 can form hetero-oligomers with TLR4 in HEK293 cells (17). Therefore, the homo- and hetero-oligomerization among TLRs appears to be an inherent property essential for agonist recognition and signal activation and, as indicated by our results, may also contribute to the regulatory (synergistic or inhibitory) function.

Murine-TLR8 Also Inhibits Both Murine-TLR7 and Human-TLR7—Both mTLR8 and human TLR8 share significant homology, and yet the mTLR8 does not respond to any of the agonists known to activate human TLR8 (24). However, it is possible that the mTLR8 may still harbor the inhibitory function of human TLR8. Therefore, to test the inhibitory activity of mTLR8, similar coexpression experiments (see “Experimental Procedures” and Fig. 1) were performed by pairwise cotransfection of mTLR7 and mTLR8, hTLR7 and mTLR8, and mTLR7 and hTLR8 in HEK293-NF-κB cells followed by NF-κB activation in response to 3M-001 or 3M-002 as appropriate (3M-001 activates both mTLR7 and hTLR7, and 3M-002 activates mTLR7 and hTLR8). As the results indicate, mTLR8 is inhibitory to both mTLR7 (Fig. 4A) and hTLR7 (Fig. 4B), and hTLR8 is inhibitory to mTLR7 (Fig. 4C). These results also show that mTLR7 can respond to both 3M-001 and 3M-002 (Fig. 4, C and A), confirming that mTLR7 recapitulates human TLR7 and TLR8 function. Note that the fold increase in the mTLR7

3 E. Latz, personal communication.
response is several fold higher than that of hTLR7 and is consistent across all the experiments. The similarities in the inhibitory function of mTLR8 and hTLR8 (both inhibit mTLR7 and hTLR7) demonstrate that the inhibitory function of these TLRs is evolutionarily conserved and also suggest that the molecular interactions that are necessary for the inhibitory interactions are conserved between murine and human TLR7 and TLR8.

**DISCUSSION**

For the first time, our results from carefully controlled pairwise coexpression and selective activation of one TLR over the other indicate that TLR7 and TLR8, TLR7 and TLR9, and TLR8 and TLR9 physically (directly or indirectly through a protein that may bridge two TLRs) interact, and these interactions result in the inhibition of one TLR but not the other (TLR8 inhibits both TLR7 and TLR9, and TLR9 inhibits only TLR7). Therefore, the inhibition is unidirectional and hierarchical. Also, this is the first instance where we show that a functionally active TLR (human TLR8 or TLR9) also play a secondary role, the inhibition of other TLR function. Although this study clearly demonstrates the inhibitory effects of TLR8 on TLR7 and TLR9, and TLR9 on TLR7 in an artificial system, the significance of these inhibitory effects in the regulation of innate immune system response to a given PAMP is not clear. However, there are several lines of indirect evidence that suggest that the inhibitory interactions among these three TLRs may contribute to the regulation of inflammation.

In human immune cells, the expression of both TLR7 and TLR9 in a given cell type is restricted mostly to plasmacytoid dendritic cells (pDC) and B cells (25–28). Both TLR7- and TLR9-selective agonists induce type I interferon in pDCs (9, 34) and B cell proliferation (35–37). If the physical interactions between TLR7 and TLR9 are inhibitory to TLR7, and yet TLR7 agonists are capable of signal activation leading to either B cell proliferation or induction of type I interferon in pDCs, then both these cells may express an excess of TLR7 to overcome the inhibitory effects of TLR9. Alternatively, as the inhibitory effects of TLR9 on TLR7 are more gradual as a function of increasing levels of TLR9 protein (typical dose-dependent inhibition), it is possible that the relative expression of these TLRs is optimized to overcome the inhibitory effects of TLR9. Naive B cells express very low but comparable levels of both TLR7 and TLR9, whereas they proliferate only in response to TLR9 agonists (38, 39). These observations appear to be consistent with our results that TLR9 inhibits TLR7 but not vice versa. The finding that interferon-α selectively up-regulates TLR7 in naive B cells resulting in the proliferation of these cells in response to TLR7 agonists, while the TLR9-dependent proliferation activity remains unchanged (39), also gives credence to our observation that the inhibition of TLR7 is TLR9 dose-dependent.

As both TLR7 and TLR9 agonists induce type I interferon in pDCs, it is presumed that the relative levels of TLR9 may have balanced for an optimal activation of TLR7. Since the coexpression of both TLR8 and TLR9 in a given cell type has not been reported, the physiological significance of TLR9 inhibition by TLR8 is not explicit at this point.

In humans, among TLR7, TLR8, and TLR9, pDCs express TLR7 and TLR9 (25–28), and myeloid DCs (mDCs) express TLR8 but not TLR7 or TLR9 (27). However, the expression of both TLR7 and TLR8 in a given cell type is restricted to only monocytes (25) and neutrophils (40). Although the monocytes express both TLR7 and TLR8, they respond to only the TLR8-selective agonist 3M-002 but not to the TLR7-selective agonist 3M-001 (9). Similarly, a monocytic cell line, THP1 cells, reportedly expresses both TLR7 and TLR8 mRNA (41) (Fig. 5A) and responds to only TLR8-selective agonists resulting in the production of tumor necrosis factor-α and IL-12p40 (Fig. 5C). Although the THP1 cells express very low levels of TLR7.
The expression pattern of different TLRs in different biological conditions. These observations indicate that TLRs may function in concert with other TLRs depending on the type of pathogen (or PAMP) and the physiological state of the cell. The variation in relative levels of TLRs, especially those that interact with each other in recognizing the PAMP or those that inhibit to interact or synergize the activation of the immunological signal in response to any external stress, may play a role in the homeostasis of inflammation.

Acknowledgments—We thank Raj Rajagopal for HA-TLR8 expression vector, Sheila Gibson for cytokine measurements, Mark Tomai for continued support of this work, and Kenneth Lipson for constructive comments and suggestions on the manuscript.

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