Unique Properties of the Chicken TLR4/MD-2 Complex: Selective Lipopolysaccharide Activation of the MyD88-Dependent Pathway[1]

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During evolution, mammals have evolved a powerful innate immune response to LPS. Chickens are much more resistant to LPS-induced septic shock. Herein we report that chickens sense LPS via orthologs of mammalian TLR4 and myeloid differentiation protein-2 (MD-2) rather than the previously implicated chicken TLR2 isoform type 2 (chTLR2t2) receptor. Cloning and expression of recombinant chTLR4 and chMD-2 in HeLa 57A cells activated NF-κB at concentrations of LPS as low as 100 pg/ml. Differential pairing of chicken and mammalian TLR4 and MD-2 indicated that the protein interaction was species-specific in contrast to the formation of functional human and murine chimeric complexes. The chicken LPS receptor responded to a wide variety of LPS derivatives and to the synthetic lipid A compounds 406 and 506. The LPS specificity resembled the functionality of the murine rather than the human TLR4/MD-2 complex. Polymorphism in chTLR4 (Tyr^{383}His and Gln^{611}Arg) did not influence the LPS response. Interestingly, LPS consistently failed to activate the MyD88-independent induction of IFN-β in chicken cells, in contrast to the TLR3 agonist poly(I:C) that yielded a potent IFN-β response. These results suggest that chicken lack a functional LPS-specific TRAM-TRIF (TRIF-related adapter molecule/TIR-domain-containing adapter-inducing IFN-β) signaling pathway, which may explain their aberrant response to LPS compared with the mammalian species. ***The Journal of Immunology,*** **2008, 181: 4354–4362.**

Toll-like receptors are components of the innate immune system that sense conserved microbial patterns and endogenous danger signals (1). One of the molecules that is sensed by TLRs is the lipid backbone (lipid A) of the principal bacterial surface glycolipid, known as LPS. In mammals the sensing of LPS involves the LPS-binding protein (LBP)[3]-mediated transfer of LPS monomers to CD14, which commonly delivers the LPS to myeloid differentiation protein-2 (MD-2) (2). MD-2 binds LPS in its hydrophobic pocket, which supposedly causes a conformational change that is transferred upon the complexed TLR4 molecule (3, 4). This triggers the recruitment and modification of intracellular adaptor molecules at the Toll/IL-1R (TIR) domain of the TLR4 receptor and initiates a cascade of signaling events that ultimately drive the innate immune response to LPS. The central role of TLR4 and MD-2 in the mammalian LPS response is evident from the resistance to LPS of MD-2 null mice (5) and C3H/HeJ mice, which carry a point mutation in TLR4 (6).

TLR4 is unique among the TLR family in that upon activation it recruits several different adaptor molecules including MyD88 and TIR-domain containing adapter protein (TIRAP) (MyD88-dependent pathway) and TRIF-related adapter molecule (TRAM) and TIR-domain-containing adapter-inducing IFN-β (TRIF) (MyD88-independent pathway). All other TLRs, except TLR3, solely activate the MyD88-dependent signaling route. TLR3 specifically makes use of the TRIF-signaling pathway but without the use of TRAM. The combined use of TRIF and TRAM is specific for the TLR4 signaling pathway (7). The type of signaling route that is activated by TLRs is important for the nature of the innate immune response. The TIRAP/MyD88 signaling pathway is activated from the plasma membrane and causes nuclear translocation of the transcription factor NF-κB, whereas the TRAM/TRIF-dependent pathway appears to be active in endosomes and induces the phosphorylation of IFN regulatory factor 3 (IRF3) and a delayed NF-κB response (7–10). Activation of IRF3 ultimately results in the production of IFN-β, IP-10, and RANTES (2). This response is not observed in TRIF$^{-/-}$ and TRAM$^{-/-}$ mice (7, 10).

TLR4 and MD-2 are highly conserved during evolution with orthologs found in mammals, birds, and some fish species (11, 12). However, the avian species and fish appear much more resistant to the toxic effects of LPS than do the mammalian species (13–15). Analysis of the TLR repertoire of chicken indicates both common and unique features compared with mammalian TLRs (16–26). Chickens express TLR4 and MD-2 orthologs, and polymorphisms in the proteins have been associated with variable susceptibility to *Salmonella* infection (27, 28). Furthermore, chicken cells respond to LPS with the induction of inducible NO synthase, IL-1β, IL-6, and IL-18 mRNA (29–31). Involvement of TLR4 and CD14 was suggested by the effects of polyclonal Abs directed against human TLR4 and rat CD14 (29, 30, 32). However, Fukui et al. (17) noted that recombinant chicken TLR2 isoform type 2 (chTLR2t2) can

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; chTLR2t2, chicken TLR2 isoform type 2; IRF3, IFN regulatory factor 3; MD-2, myeloid differentiation protein-2; MPL, monophosphoryl lipid A; SE, *S.* Enteritidis; *S.* Enteritidis, *Salmonella enterica* serovar Enteritidis; SG, *S.* Gallinarum; *S.* Gallinarum, *Salmonella enterica* serovar Gallinarum strain 9R; TIR, Toll/IL-1R; TIRAP, TIR-domain containing adapter protein; TRAM, TRIF-related adapter molecule; TRIF, TIR-domain-containing adapter-inducing IFN-β.

Table I. *Primers used in this study for cloning*[a]

Product	Forward (5′ to 3′)	Reverse (5′ to 3′)
chTLR4	GCGGCCGCGCCACATGGCCAGCAGAGCGGCTCCCACC	TCTAGATTACATGAGTTTTATCTCCTCGT
Exon 1	GCGGCCGCGCCACATGGCCAGCAGAGCGGCTCCCACC	AGGGGATGACCTCCAGGCACGGACTGGGGATGC
Exon 2	TGCCTGGAGGTCATCCCCAGCACAGCTTTCAGAT	GTGGCACCTTGAAAGATCCAGAAACTGCAGTTC
Exon 3	TGGATCTTTCAAGGTGCCACATCCATACAATAGAAG	TCTAGATTACATGAGTTTTATCTCCTCGT
chMD-2	GGTACCGCCACCATGGTTGAGTTTGTCTTTTTC	GAATTCTCAGAAAGCGTCTTGTTT
mTLR4	GGTACCGCCACCATGGTGCCTCCCTGGCTCCTGGCTA	GCGGCCGCTCAGGTCCAAGTTGCCGTTTCTTG
mCD14	GAATTCGCCACCATGGAGCGTGTGCTT	TCTAGATTAAACAAAGAGGCGATC

[a] The underlined sequences represent restriction sites used in cloning of the PCR products.

respond to LPS and hypothesized that this receptor covers the function of TLR4 in chicken. We recently demonstrated that chTLR2t2 in conjunction with chTLR16 responds to both di- and triacylated peptides combining the function of the mammalian TLR2/1 and TLR2/6 receptor complexes (22). Thus, the exact nature of the LPS receptor(s) and the basis of the apparent relative resistance of chickens toward the toxic effect of LPS remain to be defined.

In the present study, we cloned the full-length chicken TLR4 and chicken MD-2 gene and assessed the function of the chTLR4/chMD-2 complex, the species specificity of the interaction of TLR4 and MD-2 using coexpression of the different mammalian and chicken proteins, and the LPS specificity of the chTLR4/chMD-2 complex. Strikingly, our results suggest that the chicken lacks the MyD88-independent signaling route that underlies the LPS-induced production of IFN-β in the mammalian species.

Materials and Methods

Cells and reagents

The HD11 chicken macrophage cell line (33) was maintained in DMEM supplemented with 5% FCS. The C3H/HeJ murine macrophage cell line stably transfected with mTLR4 (34) was maintained in DMEM/10% FCS. The HeLa 57A cell line stably transfected with a NF-κB luciferase reporter construct (35) and HEK293 cells was generously provided by Dr. R. T. Hay (Institute of Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland, U.K.) and Dr. B. van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands), respectively, and maintained in DMEM/5% FCS. All cell lines were grown at 37°C in a CO_2-rich atmosphere. *Salmonella enterica* serovar Enteritidis (herein referred to as *S.* Enteritidis or SE) strain 90-13-706 and *Salmonella enterica* serovar Gallinarum strain 9R (herein referred to as *S.* Gallinarum) (Animal Science Group, Lelystad, the Netherlands) were grown on Luria-Bertani agar plates or in Luria-Bertani broth at 37°C.

LPS of *Pasteurella multocida* (*P. multocida*) strains PBA885 and AL251 was generously provided by Dr. B. Adler (Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Australia). Synthetic lipid A 406 and 506 were a kind gift from Dr. K. Fukase (Osaka University Graduate School of Sciences, Osaka, Japan). LPS of *Neisseria meningitidis* (*N. meningitidis*) H44/76 and its LpxL1 mutant was generously provided by Dr. L. Steeghs from our department. LPS of *Rhodobacter sphaeroides* (*R. sphaeroides*) and monophosphoryl lipid A (MPL) were purchased from List Biological Laboratories and Sigma-Aldrich, respectively. The synthetic lipoprotein Pam_3CSK_4 and the expression vectors pUNO-hTLR4, pUNO-hMD-2, pUNO-mMD-2, and pUNO-hTRAM were purchased from InvivoGen.

Construction of expression plasmids

Full-length ch*tlr4* and full-length ch*md-2* were PCR amplified from cDNA derived from whole blood of a Lohmann Brown chicken. Briefly, 2.5 ml of chicken blood was diluted 1/2 in 5 ml of PBS and layered on top of 5 ml of Ficoll SG (density = 1.078 g/ml) in a 50 ml Falcon tube. After centrifugation (1000 × *g*, 30 min, 20°C) using a swing-out rotor, the leukocyte-containing interphase was carefully isolated and extracted using RNA-Bee (Bio-Connect). Isolated RNA was treated with DNase (Invitrogen) to remove any DNA contamination. Purified mRNA (1 μg) was reverse transcribed with the ImProm-II reverse transcriptase kit (Promega) using a poly-T primer according to the manufacturer's instructions. PCR amplification of cDNA was performed using *pfu* polymerase (Promega) and the primers listed in Table I. PCR products were purified and cloned into the pGEM-T Easy vector (Promega). The resulting plasmids were digested with the appropriate restriction enzymes (Table I), and isolated inserts were ligated in the digested mammalian expression vector pTracer-CMV2 (Invitrogen), yielding pTracer-chTLR4 and pTracer-chMD-2.

The gene encoding chMD-2 of HD11 cells was cloned essentially as described above, yielding pTracer-chMD-2H. The gene encoding chTLR4 of HD11 cells was constructed by overlap extension PCR (36) using isolated chromosomal DNA as a template. In this procedure the three exons from ch*tlr4* were PCR amplified with *pfu* polymerase using the primers listed in Table I. The resulting three PCR products were purified and fused in one PCR reaction using the chTLR4 forward and reverse primers (Table I). This PCR product was cloned into the pGEM-T Easy vector and was subsequently cloned into pTracer-CMV2, yielding pTracer-chTLR4H.

Murine TLR4 (mTLR4) and CD14 (mCD14) were PCR amplified with *pfu* polymerase with the primers listed in Table I using chromosomal DNA of C3H/HeJ cells stably transfected with mTLR4 (32) as a template. Purified PCR products were cloned into the pGEM-T Easy vector and subcloned into pTracer-CMV2 using the appropriate restriction enzymes (Table I), yielding pTracer-mTLR4 and pTracer-mCD14.

The construction of the expression vectors containing chTLR2t2, chTLR16, hTLR2, hTLR1, and hCD14 has been described previously (21). All constructs were verified by DNA sequencing (BaseClear).

LPS isolation

LPS of *S.* Enteritidis strain 706 and of *S.* Gallinarum strain 9R was extracted and purified using the hot phenol extraction procedure as described (37). Briefly, bacteria grown in 500 ml of Luria-Bertani broth for 16 h were collected by centrifugation (6800 × *g*, 20 min, 4°C) and suspended in 10 volumes of distilled water and an equal volume of hot phenol (70°C) for 2 h at 70°C. After centrifugation (18,000 × *g*, 20 min, 4°C), LPS was precipitated from the water phase by the addition of sodium acetate (0.1 g/g wet weight of bacteria) and two volumes of acetone (−20°C). After 16 h (−20°C), the LPS was collected by centrifugation (18,000 × *g*, 30 min, −20°C), dissolved in distilled water, and again collected (100,000 × *g*, 2 h, 4°C). After treatment with DNase and proteinase K, the LPS was centrifuged again (100,000 × *g*, 2 h, 4°C) and dissolved in distilled water up to a final concentration of 1.5 mg/ml. Then LPS was repurified to remove contaminating endotoxin protein (38). Approximately 700 μg LPS was suspended in endotoxin-free water containing 0.2% triethylamine and 0.5% deoxycholate and an equal volume of water-saturated phenol. The mixture was vortexed intermittently for 5 min and centrifuged (10,000 × *g*, 2 min, 4°C). The upper, aqueous phase was transferred to a new tube and the phenol phase reextracted with 500 μl water containing 0.2% triethylamine and 0.5% deoxycholate. The aqueous phases were pooled and reextracted with 1 ml of water-saturated phenol, vortexed, and centrifuged. The aqueous phase was adjusted to 75% ethanol, and 30 mM sodium acetate was added. After precipitation (16 h, −20°C), the pellet was collected (10,000 × *g*, 10 min, 4°C), rinsed in 100% cold ethanol, and air-dried.

Transfection of eukaryotic cells

HeLa 57A cells were grown in 48-well tissue culture plates in DMEM/5% FCS until 50% confluence was reached (~24 h). Then cells were transiently transfected in DMEM/5% FCS using FuGENE 6 (Roche-Diagnostics) at a lipid-to-DNA ratio of 3:1 following the instructions of the manufacturer. Plasmids carrying the desired inserts were added at concentrations of 62.5 ng/plasmid. Variable amounts of empty vector were included to equalize the total amount of transfected plasmid DNA (250 ng) added to the cells. In all transfections, the pTK-LacZ vector was used for normalization of transfection efficiency. After 48 h of incubation (37°C) of

Table II. *RT-PCR primers and probes used in this study*

Gene	Primer/Probe	Sequence (5′ to 3′)
GAPDH	Forward	GCCGTCCTCTCTGGCAAAG
	Reverse	TGTAAACCATGTAGTTCAGATCGATGA
	Probe	(FAM)-AGTGGTGGCCATCAATGATCCC-(TAMRA)
IL-8	Forward	GCCCTCCTCCTGGTTTCAG
	Reverse	CGCAGCTCATTCCCCATCT
	Probe	(FAM)-TGCTCTGTCGCAAGGTAGGACGCTG-(TAMRA)
IL-1β	Forward	GCTCTACATGTCGTGTGTGATGAG
	Reverse	TGTCGATGTCCCGCATGA
	Probe	(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)
IFN-β	Forward	ACAACTTCCTACAGCACAACAACTA
	Reverse	GCCTGGAGGCGGACATG
	Probe	(FAM)-TCCCAGGTACAAGCACTG-(TAMRA)

the cells in the presence of the added DNA, the medium was replaced with fresh medium and stimulated with the different ligands.

Luciferase assays

TLR signaling was assessed using the NF-κB-luciferase reporter system as described (39). Cells were stimulated (5 h) with the TLR ligands, rinsed twice with 0.5 ml of Dulbecco's PBS (pH 7.4), and immediately lysed in 0.1 ml of reporter lysis buffer (Promega). Firefly luciferase activity was measured with the luciferase assay system (Promega) using a luminometer (TD-20/20, Turner Designs). For normalization of the efficiency of transfection, luciferase values were adjusted to β-galactosidase values as determined with the β-galactosidase assay (Promega). Statistical significance was confirmed by a paired *t* test. A two-tailed p of <0.05 was taken to be significant.

RNA isolation and quantitative RT-PCR

HD11 macrophages were grown in DMEM/5% FCS in 12-well tissue culture plates until confluence was reached. Cells were then stimulated by the addition of 5 μg/ml of LPS or incubated in the same medium with 3 μl of FuGENE in the presence and absence of 500 ng/ml of poly(I:C). After 2 h of incubation, the cells were rinsed once with Dulbecco's PBS and extracted with RNA-Bee. Isolated RNA was stored at −80°C until further use.

Fresh chicken (Ross broilers) leukocytes were isolated with a Ficoll density gradient as described above and washed once in 10 ml RPMI 1640 without serum. Isolated cells were seeded ($\sim 250 \times 10^4$ cells/well) in a 12-well plate in 2 ml of RPMI 1640 medium without serum. After attachment to the well (1.5 h, 37°C), cells were stimulated with 5 μg/ml of *S.* Enteritidis LPS or with 50 μg/ml of poly(I:C). After 2 h of stimulation, cells were rinsed once with Dulbecco's PBS and total RNA was isolated with RNAeasy Mini kit (Qiagen).

RNA transcript levels were determined by quantitative RT-PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems). Primers and probes (Table II) were designed as described (40, 41) or using the Primer Express software (Applied Biosystems). Probes (Isogen Life Science) were labeled with the reporter dye carboxyfluorescein (FAM) and the quencher tetramethyl-6-carboxyrhodamine (TAMRA). RT-PCR was performed on 50 ng of DNase I (Fermentas) treated RNA with the One Step RT-PCR MasterMix kit for probe assays (Eurogentec). Real-time cycler conditions were 30 min at 48°C, followed by 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The mRNA levels for the target gene corrected to those for the housekeeping gene GAPDH were calculated by subtracting their corresponding C_t before and after treatment using the following formulae: 1) before treatment, $\Delta C_{t\ control} = C_{t\ target\ gene\ control} - C_{t\ GAPDH\ control}$, and 2) after treatment $\Delta C_{t\ treated} = C_{t\ target\ gene\ treated} - C_{t\ GAPDH\ treated}$. The fold change in mRNA was determined by: Fold change $= 2^{(\Delta Ct(treated) - \Delta Ct(control))}$. Experiments were performed at least twice, and one representative experiment is shown.

Results

LPS responsiveness in transfected HeLa 57A cells

LPS has been reported to activate the NF-κB response via chTLR2t2 (17); therefore, we determined the LPS response of HeLa 57A cells transfected with chTLR2t2 using a NF-κB-sensitive luciferase reporter system (22). No activation of NF-κB was observed (data not shown). We have recently shown that chTLR16 acts as a coreceptor for chTLR2t2 for the recognition of di-and triacylated lipoproteins (22). However, HeLa 57A cells transfected with chTLR2t2, chTLR16, and the LPS scavenger molecule hCD14 still failed to respond to LPS (Fig. 1). Control experiments with these cells showed that the TLR2 agonist Pam_3CSK_4 stimulated NF-κB activity (Fig. 1), indicating that the reconstituted TLR pathway was functional. Similarly, stimulation of HeLa 57A transfected with hTLR4/hMD-2/hCD14 yielded a strong response to 100 ng of SE LPS (Fig. 1), while no response to LPS was obtained for hTLR2/hTLR1/hCD14-transfected cells (Fig. 1). These results indicate that SE LPS was able to activate NF-κB via hTLR4/hMD-2 but not via the chicken or the human TLR2 pathway.

Cloning and analysis of chTLR4

Inspection of the chicken genome indicated the presence of an ortholog of mammalian TLR4. Sequence analysis of the ch*tlr4* gene has indicated gene polymorphisms among chicken breeds that have been linked to resistance or susceptibility to *Salmonella* infections (27). However, direct evidence that chTLR4 is functional is lacking. We cloned the *tlr4* gene of the HD11 macrophage cell line (33), as well as the same gene from blood cells of Lohmann Brown chickens. Determination of both *tlr4* sequences revealed several nucleotide substitutions compared with the published chTLR4 (accession number AY064697). The two changes in HD11 *tlr4* (A219G and T2487G) did not cause alterations in protein sequence. However, the three substitutions in the *tlr4* gene of the Lohmann Brown chickens (T1147C, A1644G, and A1832G)

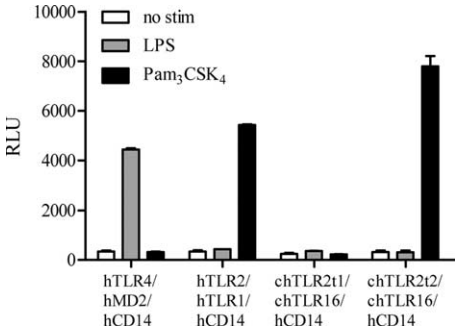

FIGURE 1. LPS response of HeLa 57A cells expressing chTLR2t2. Cells transfected with the indicated TLR receptors and hCD14 were incubated (5 h) with 100 ng/ml of *S.* Enteritidis LPS or 100 ng/ml of the TLR2 agonist Pam_3CSK_4. NF-κB luciferase activity was measured after 5 h of stimulation. Data represent the means of duplicate values of one of three independent experiments. Values are given in relative light units (RLU).

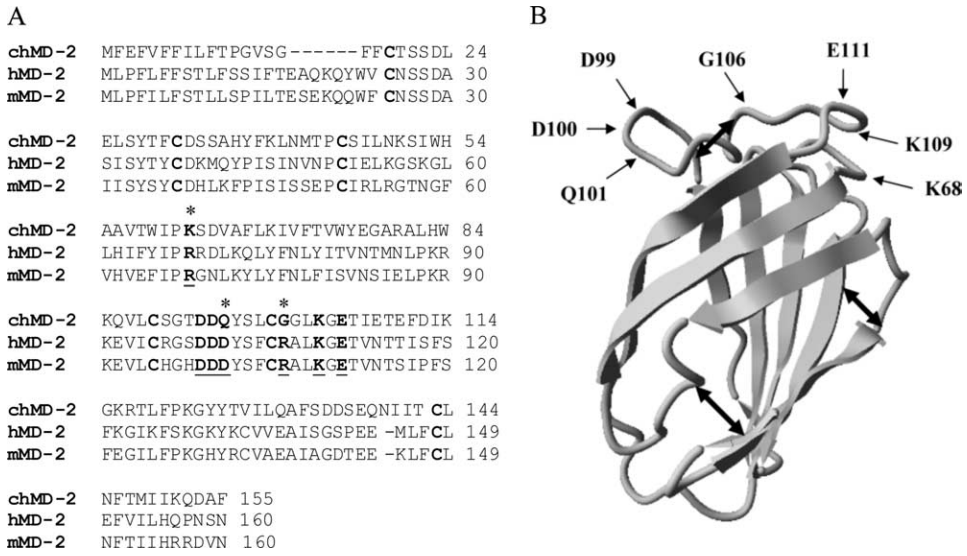

FIGURE 2. *A*, Alignment of chMD-2 with human and murine MD-2. The chMD-2 protein sequence was aligned with human and murine MD-2 using the ClustalW program. Conserved cysteine residues that form disulfide bridges are given in bold (*A*) or indicated with arrows (*B*). *B*, Predicted structural model of chMD-2. The putative chMD-2 structure was generated using the CPH-model 2.0 server (http://www.cbs.dtu.dk/services/CPHmodels/). Underlined amino acids in *A* or indicated with arrows in *B* are essential for binding of MD-2 to TLR4. The amino acids of mammalian MD-2 indicated with an asterisk are not conserved in chMD-2.

resulted in two amino acid changes (Tyr^{383}His and Gln^{611}Arg). These changes were similar to those associated with resistance to *Salmonella* infection (27).

N-glycosylation of the extracellular domain of hTLR4 has been shown to be important for surface expression and thus for the functionality of the receptor (42). Interrogation of the extracellular domain of chTLR4 for potential glycosylation sites using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) predicted the presence 10 putative glycosylation sites. Comparison of these sites with the nine motifs predicted for hTLR4 (42) indicated Asn^{177}, Asn^{209}, and Asn^{313} of chTLR4 as conserved glycosylation sites. Notably, the asparagine residues at positions 526 and 575 in hTLR4, which appear absent in chTLR4, are crucial in the trafficking of hTLR4 to the cell membrane (42, 43). However, it cannot be excluded that other asparagine residues may be important for the translocation of chTLR4 to the cell surface.

Analysis of chicken MD-2

As MD-2 is a key component of the mammalian TLR4 receptor complex, we also cloned the chicken *md-2* gene of HD11 macrophages and freshly isolated chicken blood cells. Nucleotide sequencing yielded for both genes identical sequences that fully matched the ch*md-2* gene deposited in the NCBI database (accession number XM_418301). The cloned genes are predicted to encode a protein of 160 amino acids that is slightly larger than human and murine MD-2 (155 aa). Modeling of chMD-2 on the crystal structure of its human ortholog revealed a similar overall protein architecture (Fig. 2). The chicken protein also adopts a β cup with two antiparallel β sheets that contain three and six β strands. Mammalian MD-2 contains seven conserved cysteine residues of which six form disulfide bridges (Cys^{25}–Cys^{51}, Cys^{37}–Cys^{148}, and Cys^{95}–Cys^{105}) (Fig. 2) (3, 4, 44). The cysteine at position 133 is located deep in the cavity and does not form a disulfide bridge. Chicken MD-2 contains six of these seven conserved cysteine residues but lacks the cysteine at position 133.

For activation of the human TLR4/MD-2 receptor complex, the *N*-glycosylated asparagine residues at positions 26 and 114 of the MD-2 protein are essential (45). In addition to these residues, murine MD-2 is predicted to contain a third glycosylation site at position 150. Interestingly, chMD-2 does not contain the two conserved Asn^{26} and Asn^{114} residues. Instead, it has two putative glycosylation sites at the positions 41 and 49, in addition to the Asn-X-Ser/Thr motif around position Asn^{150} as in mMD-2. These unique characteristics of chMD-2 may influence the function and/or specificity of the chTLR4/MD-2 complex (see below).

Functional analysis of chicken TLR4 and MD-2

To assess the function of chicken TLR4 and MD-2 proteins as a putative LPS receptor complex, we transfected HeLa 57A cells with chTLR4 and/or chMD-2. In some experiments we also cotransfected human (hCD14) or murine CD14 (mCD14), as a functional CD14 ortholog in chicken has thus far not been identified. Stimulation of the cells transfected with either chTLR4, chMD-2, or hCD14 with 10 ng/ml of SE LPS did not result in activation of NF-κB (Fig. 3). Similarly, expression of the combinations chTLR4/chMD-2, chTLR4/hCD14, and chMD-2/hCD14 did not elicit an innate response to LPS. However, when HeLa 57A cells were transfected with chTLR4, chMD-2, and hCD14 strong NF-κB activation was seen after stimulation with LPS (Fig. 3).

Dose-response experiments indicated that SE LPS concentrations of as low as 1 ng/ml were sufficient to activate the TLR4 receptor complex (Fig. 4). Activation of NF-κB was observed (Fig. 3) for chTLR4 cloned from the Lohmann Brown chickens, which are susceptible to *Salmonella* infection, as well as for chTLR4 of

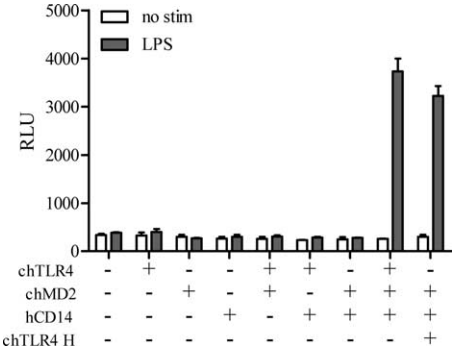

FIGURE 3. Functionality of the chTLR4/chMD-2 complex. HeLa 57A cells transfected with chTLR4, chMD-2, and/or hCD14, or with vector only, were stimulated with 10 ng/ml of *S*. Enteritidis LPS. NF-κB luciferase activity was measured after 5 h of stimulation. ChTLR4 was cloned from freshly isolated chicken blood cells, while chTLR4H was derived from HD11 macrophages. Data represent the means of duplicate values of one of three independent experiments. Values are given in relative light units (RLU).

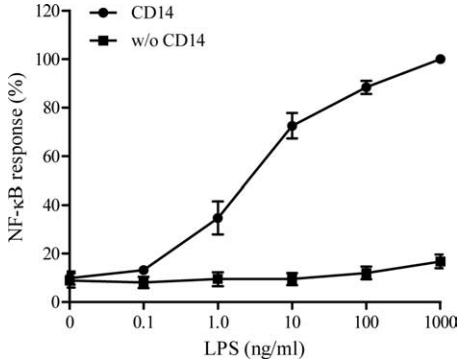

FIGURE 4. Effect of CD14 on the function of chTLR4/chMD2. HeLa 57A cells transfected with chTLR4/chMD-2 with or without CD14 were stimulated with the indicated concentrations of *S*. Enteritidis LPS. Data are expressed as percentage NF-κB luciferase activity measured after 5 h of stimulation. The maximal response for the chTLR4/chMD-2/CD14 (1 μg/ml of SE LPS)-expressing cells was set as 100% value. Values are the means ± SEM of five independent experiments.

the HD11 macrophage cell line (chTLR4H) that originates from chickens that are more resistant to *Salmonella* infection (27). At all concentrations of LPS tested, the addition of hCD14 was needed to elicit an immune response in chTLR4/chMD-2 transfected HeLa cells (Fig. 4). Overall, these results provide the first direct evidence that the chTLR/chMD-2 complex responds to LPS.

Species-specific formation of the TLR4 and MD-2 receptor complex

Resolution of the structure of the TLR4/MD-2 complex indicates multiple points of interaction between TLR4 and MD-2 (3). Functional studies indicate that the complex formation is not species-specific, that is, hTLR4 can act with murine MD-2, while mTLR4 can use human MD-2 as a coreceptor (46). Considering the predicted structural differences in chTLR4 and chMD-2 compared with their mammalian orthologs, we assessed the formation of a functional TLR4 complex for different combinations of mammalian and chicken TLR4 and MD-2 (Fig. 5). LPS stimulation assays using HeLa 57A cells expressing chTLR4 in combination with hMD-2 or mMD-2 did not cause activation of NF-κB in contrast to the homologous chTLR4/chMD-2 complex (Fig. 5*A*). The latter complex was functional with both hCD14 and mCD14 (Fig. 5*A*). Coexpression of hTLR4 or mTLR4 in combination with chMD-2 did not yield LPS-responsive cells either (Fig. 5, *B* and *C*). These receptors were functional when expressed in combination with human and murine MD-2 (Fig. 5, *B* and *C*), irrespective whether human or murine CD14 was present. These results indicate clear species specificity in the interaction between chTLR4 and chMD-2 in contrast to the formation of the mammalian TLR4/MD-2 complex.

LPS specificity of the chTLR4/chMD-2 complex

The apparent unique interaction of chTLR4 and chMD-2 led us to investigate the LPS specificity of the receptor complex. Heretofore, HeLa 57A cells were transfected with chTLR4/chMD-2/hCD14 and stimulated with a large repertoire of LPS (derivatives) of different microbial origin or synthetic lipid A molecules. LPS responses were measured for a concentration range of 0.1–1000 ng/ml. As shown in Fig. 6, purified LPS derived from *S*. Gallinarum, *N. meningitidis, P. multocida*, and its *waaQ*-negative derivative significantly activated NF-κB in the chTLR4/chMD-2 transfected cells, with half-maximal stimulation at <1 ng/ml of LPS ($p < 0.05$ at 0.1 ng/ml of LPS) (Fig. 6). *N. meningitidis* LpxL1

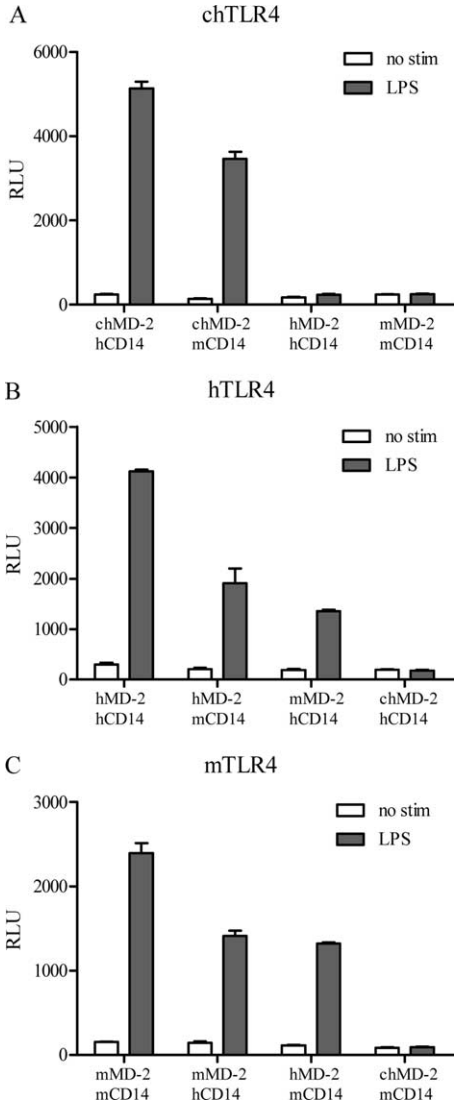

FIGURE 5. Species specificity of the formation of a functional chTLR4/chMD-2 complex. HeLa 57A cells were transfected with chTLR4 (*A*), hTLR4 (*B*), or mTLR4 (*C*) and MD-2 and CD14 from the indicated species. Cells were stimulated (5 h) with 10 ng/ml of *S*. Enteritidis LPS, after which the NF-κB luciferase activity was measured. Data are duplicate values from one of three experiments. Values are given in relative light units (RLU).

LPS, which specifically activates the murine but not human TLR4/MD2 complex (47), also activated the chTLR4/chMD-2 complex, although the maximum response was reduced compared with the LPS of the parent strain (Fig. 6). LPS derived from *S*. Enteritidis significantly ($p < 0.05$) activated the chTLR4/chMD-2 complex at a concentration of 1 ng/ml LPS. Similarly, the synthetic lipid A derivative 406 (48) yielded a significant response at 1 ng/ml, but this response did not reach the high activation levels observed for, for example, *S*. Enteritidis LPS. Interestingly, the synthetic lipid A compound 506 yielded a very potent response, although this was only evident at concentrations of >10 ng/ml ($p < 0.05$ at 10 ng/ml). A similar response was observed for MPL, which is a known vaccine adjuvant with immunostimulatory properties but reduced reactogenicity (49). Finally, *R. sphaeroides* LPS, which is antagonistic toward hTLR4 and mTLR4 but acts as an agonist for hamster and bovine TLR4 (50–52), barely activated NF-κB in chTLR4/chMD-2 transfected cells (Fig. 6). For comparison, the same lipid A derivatives as used above were also tested (at 1 μg/

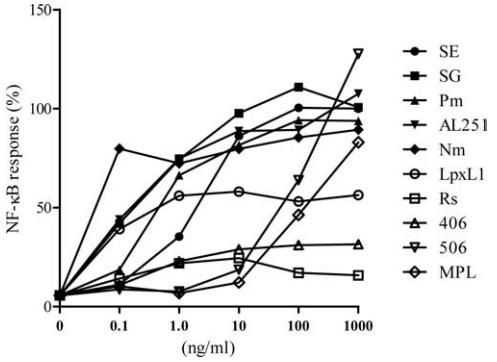

FIGURE 6. LPS specificity of chTLR4/chMD-2. HeLa 57A cells transfected with chTLR4/chMD-2/hCD14 were stimulated (5 h) with the indicated concentrations of LPS from *S*. Enteritidis (SE), *S*. Gallinarum (SG), *P. multocida* strain PBA885 (Pm), *P. multocida* ΔwaaQ (AL251), *N. meningitidis* strain H44/76 (Nm), *N. meningitidis* ΔLpxL1 (LpxL1), *R. sphaeroides* (Rs), lipid A 406 (406), lipid A 506 (506), or MPL. Values are presented as the percentage NF-κB luciferase activity, where 100% is set to be the response at 1 μg/ml of SE LPS. Values are the means of three independent experiments. Statistical analysis (paired *t* tests) indicated that the responses to LPS from SG, Pm, AL251, Nm, and LpxL1 were already significant at a concentration of 0.1 ng/ml. SE LPS, Rs LPS, and lipid A 406 stimulations were significant at 1 ng/ml; lipid A 506 gave a significant stimulation at 10 ng/ml; and MPL significantly increased NF-κB activation at 100 ng/ml. Values were considered significant for p of <0.05.

ml) for their reactivity toward HeLa 57A cells transfected with the empty vector (Fig. 7*A*) or the genes encoding the human (Fig. 7*C*) or murine (Fig. 7*D*) TLR4/MD-2 complex. The cells that lacked TLR4/MD-2 displayed no activation of NF-κB with any of the LPS derivatives tested (Fig. 7*A*). In contrast, HeLa 57A cells transfected with mTLR4/mMD-2 responded to all TLR4 ligands ($p < 0.05$) (Fig. 7*D*), including the LPS from *R. sphaeroides*, which was previously reported to have antagonistic activity toward murine TLR4 (53, 54). In cells expressing the human TLR4/MD-2 complex, the LPS derived from *S*. Enteritidis, *S*. Gallinarum, *N. meningitidis, P. multocida*, and its *waaQ*-mutant as well as compound 506 yielded significant ($p < 0.05$) increases in NF-κB response (Fig. 7*C*) as found for the chicken in murine TLR4/MD-2 complexes. However, *N. meningitidis* LpxL1 LPS, *R. sphaeroides* LPS, MPL, and compound 406 yielded virtually no response in the hTLR4/MD-2 cells ($p > 0.05$) (Fig. 7*C*). Overall, the data indicate that, despite the species-specific interaction of TLR4 and MD-2, the functional specificity of the chTLR4/MD-2 complex toward LPS resembles that of the murine but not the human TLR4/MD-2 complex.

Lack of MyD88-independent LPS response in chicken cells

In mammalian cells LPS stimulation of the TLR4 pathway results in activation the MyD88/TIRAP-dependent and TRAM/TRIF-dependent signaling routes. Activation of the MyD88-dependent pathway induces enhanced transcription of a number of NF-κB regulated genes, including IL-8 and IL-1β. On the other hand, stimulation of the TRAM-TRIF pathway, which is unique for TLR4, results in the transcription of IRF3-dependent cytokines such as IFN-β (55). To assess the activity of both signaling routes in chicken cells, we stimulated HD11 cells with LPS and measured the mRNA levels for IL-8, IL-1β, and IFN-β. As shown in Fig. 8, SE LPS induced a strong increase in IL-8 and IL-1β transcripts, while virtually no IFN-β transcript was measured. Similar results were obtained for LPS derived from the chicken pathogens *S*. Gallinarum, *P. multocida*, and the attenuated *waaQ*-mutant strain of *P. multocida* (Fig. 8, *A* and *B*). Stimulation of the cells with the TLR3 agonist poly(I:C), which specifically activates the TRIF-dependent (but TRAM-independent) pathway, showed strong up-regulation of the level of IFN-β transcript, while, as expected, no up-regulation of IL-8 and IL-1β mRNA was observed (Fig. 8*C*). To ensure that the apparent defect in TRAM/TRIF signaling in response to LPS was not a specific trait of the HD11 cell line, we repeated these experiments with freshly isolated leukocytes from chicken blood. Similar results were obtained (Fig. 8, *D–F*). Inspection of the chicken genome for components of the TRAM/TRIF-dependent pathway using the basic local alignment search tool (BLAST) algorithm (http://www.ensembl.org/Gallus_gallus/index.html) yielded no ortholog of human or murine TRAM. Moreover, introduction of human TRAM into chicken HD11 cells did not restore the MyD88-independent response to LPS (data not

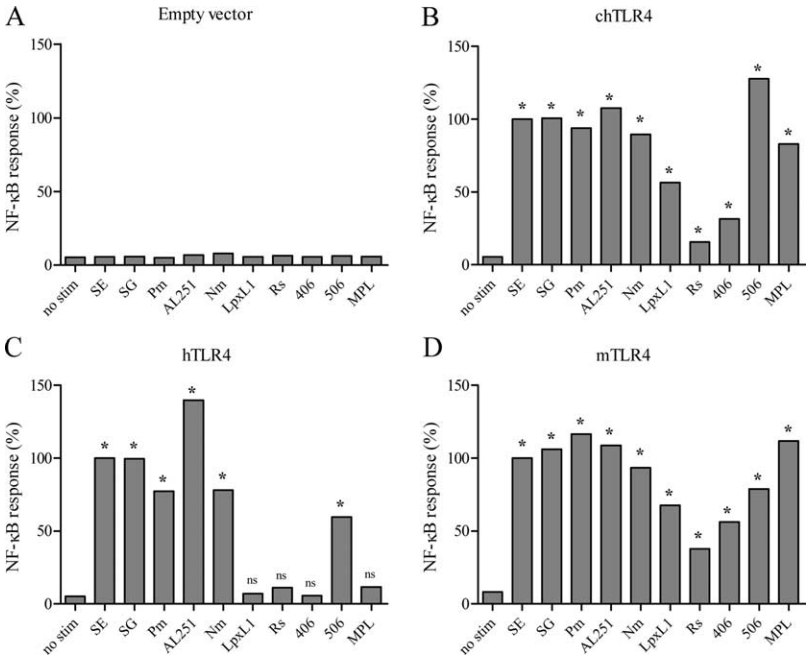

FIGURE 7. Comparison of the LPS specificity of TLR4/MD-2 between species. HeLa 57A cells were transfected with the empty vector (*A*), chTLR4/chMD-2/hCD14 (*B*), hTLR4/hMD-2/hCD14 (*C*), and mTLR4/mMD-2/mCD14 (*D*). Cells were stimulated with 1 μg/ml of LPS from *S.* Enteritidis (SE), *S*. Gallinarum (SG), *P. multocida* strain PBA885 (Pm), *P. multocida* ΔwaaQ (AL251), *N. meningitidis* strain H44/76 (Nm), *N. meningitidis* ΔLpxL1 (LpxL1), *R. sphaeroides* (Rs), lipid A 406 (406), lipid A 506 (506), or MPL. Values are presented as the percentage NF-κB luciferase activity, where 100% is set to be the response at 1 μg/ml of SE LPS. The 100% value for the control experiment (empty vector) was the NF-κB response of the SE LPS-stimulated chTLR4-transfected cells. Values are the means of three independent experiments. Statistically significant differences ($p < 0.05$) in response between stimulated vs nonstimulated cells are indicated by an asterisk on top of the bars. ns, not statistically significant.

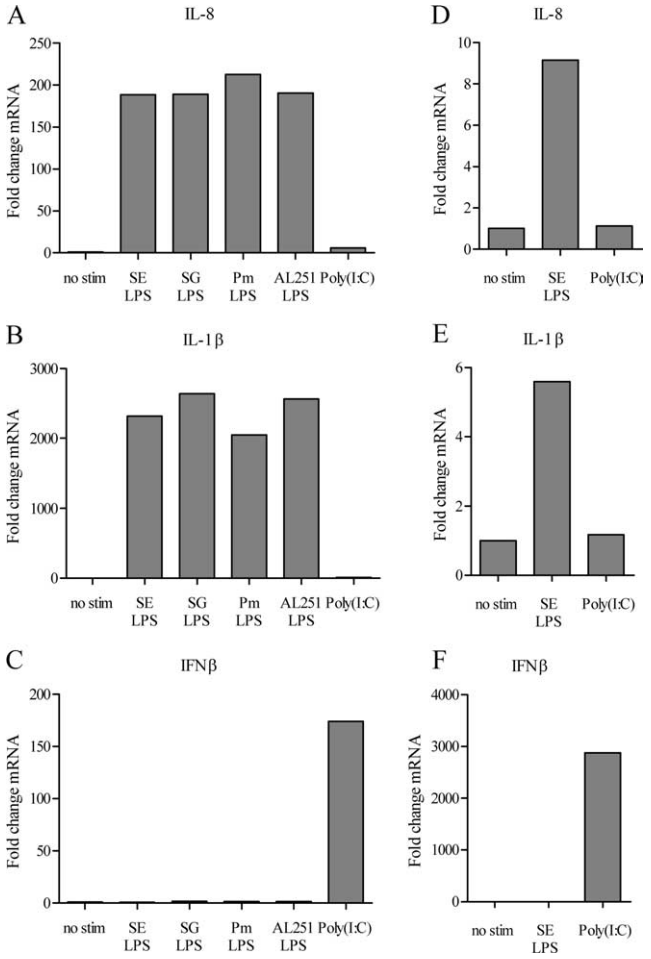

FIGURE 8. LPS stimulation of the MyD88-dependent and MyD88-independent pathway in chicken cells. HD11 macrophages (*A–C*) and leukocytes freshly isolated from chicken blood (*D–F*) were stimulated (2 h) with 5 μg/ml of LPS of *S.* Enteritidis (SE), *S.* Gallinarum (SG), *P. multocida* strain PBA885 (Pm), or *P. multocida* ΔwaaQ (AL251). As control for the TRIF-signaling pathway, 500 ng/ml of poly(I:C) with FuGENE (HD11 cells) or 50 μg/ml of poly(I:C) (leukocytes) was added. Cells treated with FuGENE only yielded no responses (data not shown). Cells were analyzed for IL-8 (*A* and *D*), IL-1β (*B* and *E*), and IFN-β transcripts (*C* and *F*) by real-time RT-PCR. Data are from one representative experiment of at least two assays and are shown as the fold change in mRNA levels after treatment compared with nontreated cells.

shown). The latter result suggests that either species-specific interactions of TRAM or the additional absence of other signaling intermediates of the TRAM/TRIF signaling pathway is responsible for the apparent lack of the MyD88-independent LPS response in chicken cells.

Discussion

The function of the TLR4/MD-2 receptor complex in the recognition of bacterial LPS has been well established for many mammalian systems (2). In the avian species and in fish, which are relatively resistant to endotoxin (13–15), the function of TLR4/MD-2 is less well defined. Herein, we provide for the first time direct evidence that the TLR4/MD-2 complex of chickens confers responsiveness to LPS. However, the interaction between chTLR4 and chMD-2 is species-specific and, importantly, activation of the complex by LPS results in exclusive activation of the MyD88/TIRAP-dependent signaling pathway. To our knowledge, the apparent absence of stimulation of the MyD88-independent route by LPS but not by the TLR3 agonist poly(I:C) has never been reported before and indicates a different evolution of the LPS response in the chicken compared with the mammalian species.

The response of chicken cells to LPS has been widely investigated and is frequently associated with the presence of TLR4 (29, 30), although at the molecular level chTLR2t2/hMD-2 or hCD14 has been reported to confer a LPS responsiveness (17). The present approach of cloning the chTLR4 ortholog and chMD-2 protein and expressing them either alone or together in eukaryotic cells provides unequivocal evidence that the recombinant chTLR4/chMD-2 complex acts as a LPS receptor. We also expressed the chTLR2t2 receptor alone or in combination with chMD-2 and CD14. These molecules did not confer LPS responsiveness, whereas expression of chTLR2t2 in conjunction with its coreceptor chTLR16 (22) did respond to the TLR2 agonist Pam_3CSK_4 (Fig. 1). For many LPS species, signaling via TLR2 has been attributed to contaminating lipoproteins (38). Thus, the difference between our results and those of Fukui et al. (17) may be caused by differences in impurity of the LPS and/or the use of different concentration of LPS (1 ng/ml of SE LPS vs 10 μg/ml of *E. coli* LPS).

LPS responsiveness required transfection of both chTLR4 and chMD-2, consistent with the recent structural data that provide evidence that TLR4 and MD-2 form a complex (3). The complex formation appears to depend on hydrogen bonding of charged amino acids located in two different positively and negatively charged patches on TLR4 and MD-2. As substitution of a single amino acid of MD-2 (D100G) already results in loss of complex formation (56), we investigated the conservation of the relevant charged amino acids in chTLR4 and chMD-2. Interestingly, several residues were not conserved. In chTLR4, the amino acids G41, T263, and V288 are neutral of charge, whereas in mammalian TLR4 residue D41 is negatively charged, and residues K263 and R288 are located in the positively charged patch. Furthermore, in chMD-2 the residues Q101 and G106 are neutral rather than charged amino acids (Fig. 2), while residue D101 is located in the negatively charged patch and R106 is in the positive region. These differences in amino acid composition suggest that the complex formation between TLR4 and MD-2 of chickens may be different than in the mammalian species. Consistent with this hypothesis is that we were unable to obtain a functional chTLR4/MD-2 complex by transfection of combinations of proteins from chicken and mammalian origin, while chimeric complexes of human and murine TLR4 and MD-2 did respond to LPS. Notably, this species-specific interaction was not observed for CD14, as both human and murine CD14 supported chTLR4/chMD-2 function. This result is consistent with the assumption that CD14 transfers the LPS to the TLR4/MD-2 complex, while the ligand specificity is located on the TLR4/MD2 complex (53, 57, 58). The functional ortholog of CD14 in chicken is unknown. Cloning of a putative CD14-like protein of chicken (GenBank accession number CAP70058) did not yield a functional protein.

The species-specific interaction between chTLR4 and chMD-2 does not necessarily interfere with the LPS binding specificity of the receptor complex, as the LPS responsiveness seems located in a different region of MD-2 that is rich in basic and aromatic residues (56). Comparison of the ability of LPS derived from different microbial sources and synthetic lipid A structures to activate the chTLR4/chMD-2 receptor complex indeed indicate seemingly comparable LPS responses in cells expressing murine and chicken TLR4/MD-2 complexes (Fig. 6). Cells expressing the human TLR4/MD-2 complex displayed different reactivity toward the various LPS derivatives. The basis for this difference in LPS specificity was not studied here, but it has been reported that amino

acids 57, 61, and 122 of mMD-2 play a crucial role in the differential recognition of lipid IVa (compound 406) by human and murine MD-2 (58). Two of these amino acid residues (57 and 122) are conserved in chMD-2, while amino acid 61 is different from both human and murine MD-2. However, it is likely that the LPS specificity is not solely determined by MD-2, but also by TLR4 (58).

Comparison of the potency of different LPS derivatives in activating the chTLR4/MD-2/NF-κB signaling pathway revealed virtually no differences in biological activity between LPS derived from *S*. Gallinarum, *S*. Enteritidis, *P. multocida*, and its harmless *waaQ*-negative derivate (59), despite that these microbes display large diversity in virulence. These data suggest that, at least for these bacterial species, LPS recognition by chTLR4/chMD-2 per se is not correlated with disease. These results were unexpected, as TLR4 allelic variation in humans ($Asp^{299}Gly$ and $Thr^{399}Ile$) correlates with variable endotoxin hyporesponsiveness (60), and TLR4 polymorphism in the chicken ($Tyr^{383}His$ and $Gln^{611}Arg$) has been associated with susceptibility to *Salmonella* infection (27). We cloned and sequenced the *tlr4* and *md-2* genes from the HD11 chicken macrophage cell line as well as from freshly isolated chicken blood cells. This revealed identical sequences for *md-2* but several nucleotide differences in *tlr4* that resulted in the two amino acid differences ($Tyr^{383}His$ and $Gln^{611}Arg$) previously associated with resistance or susceptibility to a *Salmonella* infection (27). However, in our in vitro assay both types of chTLR4 conferred comparable LPS responses. Thus, it is unlikely that variable LPS recognition accounts for the observed associations with susceptibility to infection. A similar discrepancy between experimental and epidemiological data for human TLR4 allelic variation has led to the suggestion that other factors such as alternative TLR4 agonists may be present in vivo (61, 62).

Another striking conclusion from our work is that activation of the chTLR4/chMD-2 complex by LPS exclusively activates the MyD88-dependent signaling route. In mammals, TLR4 is unique among the members of the TLR family in that LPS recognition results in activation of both the MyD88-dependent and the TRAM/TRIF-dependent signaling pathway. Stimulation of HD11 cells with the TLR3 agonist poly(I:C) gives a strong increase in IFN-β transcript (Fig. 8), indicating that the chTLR3/TRIF signaling pathway is intact (25, 40). This suggests that the inability of chicken cells to produce IFN-β in response to LPS is specific for the TLR4 pathway. Key components implicated in mammalian MyD88-independent TLR4 signaling appear to be LBP, the lipid scavenger protein CD14, and the intracellular adaptor molecule TRAM (7, 63, 64). Inspection of the chicken genome indicates no orthologs for these proteins, perhaps with the exception of a CD14-like molecule. Whether the LPS response is different when the full complement of chicken components is utilized awaits future study. Introduction of human TRAM into HD11 cells did not induce the transcription of IFN-β and did not complement the apparent defect in TRAM/TRIF signaling. This indicates that either human TRAM is not compatible with the chicken signaling system and/or that other components of this pathway are lacking in chicken. Interestingly, IFN-β-null mice show complete resistance to LPS-induced endotoxic shock (65). Thus, it can be imagined that the absence of a functional TLR4/TRAM/TRIF pathway in chicken cells contributes to the relative resistance of this species to LPS (14). In support of this hypothesis is that fish are also highly resistant to endotoxin and may also lack a functional TLR4/TRAM/TRIF pathway (15). Collectively, these data may indicate that the key molecules of the MyD88-independent pathway arose later in the evolution and are thus only present in mammals, resulting in a different innate immune response in the various species.

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Disclosures

The authors have no financial conflicts of interest.

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