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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 (Tyr383His and Gln611Arg) 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 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 (1.5 g/ml, 30 min, 2°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 reprecipitated with 62.5 ng/μl LPS until 50% confluence was reached (24 h). Then cells were transfected in DMEM/5% FCS using FuGENE 6 (Roche-Fluka) 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 μg/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 I. Primers used in this study for cloning**

<table>
<thead>
<tr>
<th>Product</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
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</thead>
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<tr>
<td>chTLR4</td>
<td>GCGGCGGCCCCACATGGCCACAGGCGGTCCTCCAC</td>
<td>TCTAGATTACATGAGTTTTATCTCCTCGTG</td>
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<tr>
<td>Exon 1</td>
<td>AGGGGATACCCGAGGACGAGTTGGAATGTTTTATCTCCTCGTG</td>
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<tr>
<td>Exon 2</td>
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<td>GGGTACCGCCACATGGTTGAGTTTGTCTTTTTC</td>
</tr>
<tr>
<td>Exon 3</td>
<td>AGATTCTCAGAAAGCGTCTTGTTT</td>
<td>AGATTCTCAGAAAGCGTCTTGTTT</td>
</tr>
<tr>
<td>chMD-2</td>
<td>GAGTACCGCCCACATTGCTGCCACTCCATCAACATAGAAG</td>
<td>GGATTTACGGCCACATGGTTGAGTTTGTCTTTTTC</td>
</tr>
<tr>
<td>mTLR4</td>
<td>GCTGGACCCGACATGGCTGGCTCCTCGGCGCTCTTCTCTTCTTTC</td>
<td>GAGATTCTCAGAAAGCGTCTTGTTT</td>
</tr>
<tr>
<td>mCD14</td>
<td>GACTTTCCCCACGGAGGCGGCTGCTT</td>
<td>TCTAGATTACATGAGTTTTATCTCCTCGTG</td>
</tr>
</tbody>
</table>

* The underlined sequences represent restriction sites used in cloning of the PCR products.
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. For normalization of the efficiency of transfection, luciferase values were adjusted to the well (1.5 h, 37°C), cells were stimulated with 5 g/ml of LPS or incubated in the same medium with 3 g/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 (250 × 10^3 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 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, luciferase activity was measured with a luminometer and firefly luciferase activity was normalized to β-galactosidase activity (Promega). Statistical significance was confirmed by a paired t test. A two-tailed p of <0.05 was taken to be significant.

 Cloning and analysis of chTLR4

Inspection of the chicken genome indicated the presence of an ortholog of mammalian TLR4. Sequence analysis of the chtlr4 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) observed (data not shown). We have recently shown that chTLR16 acts as a coreceptor for chTLR2t2 for the recognition of di- and triacylated lipopolysaccharides (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 Pam3CSK4 stimulated NF-κB activity (Fig. 1), indicating that the reconstituted TLR pathway was functional. Similar 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/Probe</th>
<th>Sequence (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
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<tr>
<td></td>
<td>Reverse</td>
<td>TGTAAACCAGTTGCTGATCGATGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
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</tr>
<tr>
<td>IL-8</td>
<td>Forward</td>
<td>GCCCTCGTCTCGTCTCGTCTGCTGATCGATGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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<td></td>
<td>Probe</td>
<td>(FAM)-TGCTCGTCTGCGAAGTGAGGACCTG(TAMRA)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TGTGCAATGCGCCCAGATGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM)-CCACACTGCAAGCTGGGAAGACCTG(TAMRA)</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Forward</td>
<td>ACAACTTCTCTACAGCAACAAACATCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCGGAAGGGGACATGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM)-TCCAGAAGTCAACGACTG(TAMRA)</td>
</tr>
</tbody>
</table>

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 Pam3CSK4. 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).
resulted in two amino acid changes (Tyr383His and Gln611Arg). 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 of 10 putative glycosylation sites. Comparison of these sites with the nine motifs predicted for hTLR4 (42) indicated Asn177, Asn266, and Asn313 of chTLR4 as conserved glycosylation sites. Notably, the asparagine residues at positions 266 and 313 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 chmd-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 sheet structure 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 (Cys52-Cys85, Cys72-Cys145, and Cys66-Cys105) (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 Asn26 and Asn114 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 Asn160 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 co-transfected 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
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. 5A). The latter complex was functional with both hCD14 and mCD14 (Fig. 5A). 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. Herefore, 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 waqQ-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 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/
In mammalian cells LPS stimulation of the TLR4 pathway results in activation of 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).

Comparison of the LPS specificity of chTLR4/MD-2 complex. HeLa 57A cells transfected with chTLR4/MD-2 were stimulated with compounds of 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. 7A). In contrast, HeLa 57A cells transfected with mTLR4/MD-2 responded to all TLR4 ligands (∼0.05) (Fig. 7D), 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. 7C) 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. 7C). 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

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Discussion

The function of the TLR4/MD-2 receptor complex in the recog-

FIGURE 8. LPS stimulation of the MyD88-dependent and MyD88-in-

dependent pathway in chicken cells. HD11 macrophages (A–C) and leu-

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lated chicken blood cells. This revealed identical sequences for
HD11 chicken macrophage cell line as well as from freshly iso-
these bacterial species, LPS recognition by chTLR4/chMD-2 per
waaQ
is not compatible with the chicken signaling system and/or that
in TRAM/TRIF signaling. This indicates that either human TRAM
transcription of IFN-
Introduction of human TRAM into HD11 cells did not induce the
TRAM (7, 63, 64). Inspection of the chicken genome indicates no
scavenger protein CD14, and the intracellular adaptor molecule
pathway is intact (25, 40). This suggests that the inability of
susceptibility to infection. A similar discrepancy between experi-
implicates a role in the biology of infection with
Salmonella
tlr4
infection
Comparison of the potency of different LPS derivatives in ac-
References
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Disclosures

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References
MD-2 and its complex with antidiabetic lipid A. Science 316: 1632–1634.

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The Journal of Immunology acids 57, 61, and 122 of mMD-2 play a crucial role in the differ-
ential 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 ac-
activating the chTLR4/MD-2/NF-kB signaling pathway revealed vir-
tually no differences in biological activity between LPS derived
from S. Gallinarum, S. Enteritidis, P. multocida, and its harmless
waaQ-negative derivative (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 (Asp299Gly and Thr360Ile) cor-
relates with variable endotoxin hyporesponsiveness (60), and
TLR4 polymorphism in the chicken (Tyr383His and Gln611Arg) 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 iso-
lated chicken blood cells. This revealed identical sequences for
md-2 but several nucleotide differences in tlr4 that resulted in the
two amino acid differences (Tyr383His and Gln611Arg) 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 vari-
able LPS recognition accounts for the observed associations with
susceptibility to infection. A similar discrepancy between experi-
mental 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. Interest-
ingly, IFN-β-null mice show complete resistance to LPS-induced
endotoxin shock (65). Thus, it can be imagined that the absence of
a functional TLR4/TRAM/TRIF pathway in chicken cells contrib-
utes 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 im-
une response in the various species.