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Species-specific PAMP recognition by TLR2 and evidence for species-restricted interaction with Dectin-1

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ABSTRACT

TLRs mediate recognition of a wide range of microbial products, including LPS, lipoproteins, flagellin, and bacterial DNA, and signaling through TLRs leads to the production of inflammatory mediators. In addition to TLRs, many other surface receptors have been proposed to participate in innate immunity and microbial recognition, and signaling through some of these, for example, C-type lectins, is likely to cooperate with TLR signaling in defining inflammatory responses. In the present study, we examined the importance of the ECD and intracellular TIR domain of boTLR2 and huTLR2 to induce a species-specific response by creating a chimeric TLR2 protein. Our results indicate that the strength of the response to any TLR2 ligand tested was dependent on the extracellular, solenoid structure, but not the intracellular TIR domain. Furthermore, we examined whether the recognition of two PAMPs by Dectin-1, a CLR, depends on the interaction with TLR2 from the same species. TLR2 expression seemed to affect the Dectin-1-dependent production of CXCL8 to β-glucan containing zymosan as well as *Listeria monocytogenes*. Furthermore, the interaction of Dectin-1 with TLR2 seemed to require that both receptors are from the same species. Our data demonstrate that the differences in the TLR2 response seen between the bovine and human system depend on the ECD of TLR2 and that collaborative recognition of distinct microbial components by different classes of innate-immune receptors is crucial in orchestrating inflammatory responses. *J. Leukoc. Biol.* 94: 449–458; 2013.

Introduction

MAMPs are recognized by germ-line-encoded receptors called PRRs. Two important families of PRRs are the TLRs and the CLRs. TLRs are key sensors of MAMPs, as they initiate innate and subsequently prime adaptive cellular immunity to microbial pathogens [1, 2] and are essential components in the pathogenesis of a variety of diseases [3, 4]. At least 13 TLR members, which cover the recognition of a range of pathogens recognizing a variety of ligands, are known in the mammalian system [5]. Rapid progress has been made with regard to our understanding of expression pattern, ligand specificity, signaling pathways, and effector function of TLRs in mice and humans but not in farm animals (for reviews, see refs. [6–8]). It is now becoming increasingly clear that not all microbes induce the same response when binding to a specific PRR, and the repertoire of PRR activated by a given microbe may define the nature of the overall effector response. MAMP recognition by TLRs occurs at the extracellular region, composed of at least 20 LRRs, which can easily be identified based on fixed motifs [9]. In addition to the MAMP/microbe binding to a TLR, the composition, surface-charge distribution, and resulting hydrophobicity of the LRRs may play an important role in pathogen recognition. Indeed, we were recently able to identify areas within the LRR-containing ECD of TLRs that are under selective pressure [10], resulting in potential functional differences [11–13].

TLR2 is thought to recognize a particularly broad spectrum of PAMPs, for example, impurities found in commercial PGN preparations [14, 15], LTA [14], lipoarabinomannan [16], and certain LPS types [17]. Moreover, the binding of synthetic LPs

Abbreviations: bo=bovine, bodn=bovine dominant-negative, CLR=C-type lectin receptor, ECD=extracellular domain, HEK=human embryonic kidney, hu=human, LP=lipopeptide, LRR=leucine-rich repeat, LTA=lipoteichoic acid, MAMP=microbe-associated molecular pattern, MDMs=monozyte-derived macrophages, Pam3CSK4=palmitoyl-3-cysteine-serine-lysine-4, PGN=peptidoglycan, PRR=pattern recognition receptors, qPCR=quantitative real-time PCR, RFP=red fluorescent protein, RLU=relative luciferase units, RPLP0=ribosomal phosphoprotein-large ribosomal protein P0, TIR=Toll/IL-1R

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has been defined in the crystal structure of the huTLR1-TLR2-LP and huTLR2-TLR6 complex, with binding differences being identified for tri- versus diacylated LP [18]. Heterodimerization of TLR2 with TLR1 or TLR6 may have evolved to expand the ligand spectrum to enable the innate-immune system to recognize the numerous, different structures of LP present in various pathogens [19]. TLR2 has been shown to be involved in the recognition of a variety of Gram-positive bacteria and protozoan parasites [2, 20–24].

Complex microbial surfaces present ligands for phagocytic as well as proinflammatory receptors. This, in conjunction with the fact that many intracellular signaling molecules are shared between these different receptor classes, suggests that functionally important cross-signaling occurs. Thus, in addition to the ligands described above, TLR2 can increase its ligand-binding spectrum by forming heterodimers with the CLR Dectin-1 for the recognition of β-glucans. This receptor, initially identified by Brown and coworkers [25, 26], is expressed on monocytes, macrophages, and DCs and functions as a phagocytic receptor for β-glucan-containing MAMPs, including zymosan and *Candida albicans*. Recently, we were able to characterize the same receptor in the ruminant system [27]. The extracellular COOH terminus of this type 2 transmembrane protein contains a single C-type lectin domain [28]. The short intracellular NH_2 terminus contains an ITAM-like signaling motif, a structure found on a variety of proinflammatory signaling receptors, including FcRs, TCRs, and NKRs [29, 30], confirming that Dectin-1 plays an important role in inflammatory responses to β-glucan, in addition to its role in phagocytosis. Our knowledge regarding TLR ligand interaction is mainly based on studies of human or mouse cells in vitro and mice in vivo. The investigation of species of veterinary interest is severely hampered by restricted accessibility of gene deletion technology. Furthermore, the agonist specificity can vary between species, making inferences from other species invalid. For example, the LPS receptor complex encompassing CD14, TLR4, and myeloid differentiation protein-2 is species-specific with regard to stimulation by partial structures of LPS [11, 12, 31, 32]. Likewise, there is species specificity with regard to the recognition of LP by TLR2 [33, 34]. The species specificity of ligand recognition by PRRs from species of veterinary interest, e.g., cattle, is largely unknown; however, some evidence suggests that there is a species-specific discrimination [35].

In view of the absence of suitable antibodies and knockout technology for studies in ruminant cellular systems, we sought to compare boTLR2 and Dectin-1 with their human orthologues in a well-defined system, HEK293 cells**.** Full-length protein and chimeric proteins consisting of the ECD of one species cloned with the transmembrane/intracellular TIR domain of the second species were also studied. We examined the capacity of the chimeric TLR2 to confer a species-specific response, as well as the ability of TLR2 to interact with Dectin-1 in defining inflammatory responses to a variety of ligands on a species-specific background. With the use of a human-bovine chimeric approach, the present study confirms that some but not all stimulants of huTLR2 also strongly activate boTLR2 in HEK293 cells and shows that the activation of cells stably transfected with TLR2, as well as in primary macrophages stimulated with the same ligand, depends on the ECD of the species. Furthermore, interaction of boTLR2 with Dectin-1 changes the response in a species-dependent manner.

MATERIALS AND METHODS

Constructs

Previous investigations from our group found the coding sequence of bo*TLR2* (Genbank AF368419) to be >99% identical to other sequences reported. bo*TLR2* was cloned initially into pCR 2.1 TOPO (Stratagene, La Jolla, CA, USA) [36], and this construct was used to make the chimeric TLR2 by fusing the ECD of boTLR2 (nucleotides 1–1788) with the intracellular domain of hu*TLR2* (nucleotides 1789–2352) and vice versa. The junction was made within the transmembrane domain. The resulting chimera was cloned into pcDNA3.1-YFP. YFP fluorescence served as an indicator of chimera expression in vitro. The boDectin-1 molecule was cloned recently [27], and both huDectin-1 and boDectin-1 were subsequently expressed in pDsRed-monomer-C vector (Clontech, Mountain View, CA, USA), allowing for the monitoring of expression. All constructs were confirmed by sequencing (Geneservice, Cambridge, UK). The bodnMyD88 adaptor protein construct has been described recently [37].

Confirmation and comparison of TLR2 with Dectin-1 expression levels

qPCR was performed on triplicate samples using an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 1 μg reverse-transcribed RNA as a starting template. Primers and probes for boTLR2, huTLR2, and RPLP0 were designed based on the publically available Genbank sequences NM_174197, NM_003264, and NM_001002, respectively. For boDectin-1 and huDectin-1, Genbank sequences NM_001031852 and NM_197947 were used. Where allowed, primer:probe combinations were designed to cross intron:exon boundaries to prevent detection of contaminating genomic DNA. Amplification consisted of an initial denaturation step of 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The transcript copy number for a given gene was calculated by comparison with plasmid standard curves containing known copy numbers of target genes. Relative mRNA abundance values were then calculated according to the "Relative Quantitation of Gene Expression Experimental Design and Analysis: Relative Standard Curve Method" (Applied Biosystems technical bulletin, *Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR*). For each target gene, transcript levels were normalized to the housekeeping gene RPLP0, according to the following formula (Copy No. Target/Copy No. RPLP0), which has been shown in a number of publications to be very stable [38–40].

In addition to the assessment of mRNA expression, protein expression was monitored by flow cytometry. In the absence of antibodies detecting conserved residues for boTLR2 and huTLR2, expression levels or rTLRs were compared based on expression of YFP. After successful transfection, cells were released from adherence using cell Accutase (Sigma, Dorset, UK). Following washing, flow cytometric acquisition was carried out on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). Data were analyzed using FlowJo (version 7.6; TreeStar, Asland, OR, USA) or FCS Express version 3 (DeNovo Software, Ontario, Canada). A minimum of 30,000 events was collected.

Cell culture conditions

Primary MDMs were used for comparison. PBMCs were isolated from freshly drawn venous blood of Holstein-Friesian cattle, according to home office guidance or healthy human volunteers using a modified Ficoll-Hypaque procedure [36, 41]. PBMCs were cultured in Teflon foil bags, allowing monocytes within PBMCs to mature to MDMs. At Day 7, cells were harvested, and MDMs within the cell culture were purified by an adherence step prior to testing. These cells represent resting MDMs by morphol-

ogy or CD14 expression but respond to various stimuli by TNF production or NO synthesis, as shown previously [42, 43].

Nontransfected HEK293 cells, originally obtained from the American Type Culture Collection (Manassas, VA, USA), were used, as they did not produce CXCL8 in the absence of appropriate stimuli and are relatively easy to transfect. HEK293 cells and stably transfected cell lines were generated as described recently [11] and were serially passaged twice/week. Cells were cultured in Eagle's MEM with Earle's salts containing 10% FCS, 2 mM glutamax, and 1 mM sodium pyruvate. They were slightly adherent and could be dislodged by repeated aspiration and expiration of the medium using a pipette. Plasmids were prepared using the PureYield Midiprep system (Promega, Hampshire, UK) and transfected into HEK293 (80% confluency) using Lipofectamine 2000 (Invitrogen, Paisley, UK), according to the manufacturer's instructions.

Cell stimulation

To characterize their function, several different types of ligands were used to stimulate TLR2 and Dectin-1 when expressed by HEK293 cells or by in vitro-derived MDM. *Staphylococcus aureus*-derived LTA, *S. aureus*-derived PGN, and Pam3CSK4 were purchased from Invivogen (Source BioScience UK, Nottingham, UK); heat-inactivated *Listeria monocytogenes* was prepared as described [44]. Alexa Fluor 594-labeled and unlabeled forms of zymosan, derived from *Saccharomyces cerevisiae*, were used (Molecular Probes, Invitrogen; Invivogen, Source BioScience UK). The ligands were at the following concentrations unless stated otherwise: PGN (10 μg ml^{-1}); Pam3CSK4 (300 ng ml^{-1}); LTA (10 μg ml^{-1}); *L. monocytogenes* (multiplicity of infection of 10); and zymosan (10 μg ml^{-1}). All ligands were tested for LPS contamination using an Endosafe Portable Test System (PTS) logger (Charles River Laboratories, Wilmington, MA, USA) and 0.01–1 European Units ml^{-1} cartridges.

Transient transfection of stable cell lines and NF-κB gene reporter assay

HEK293 cells, at 5×10^4 cells/well of a 96-well, flat-bottom microtiter plate, were allowed to adhere for 24 h in the absence of antibiotics. After this period, when cells were 70–80% confluent, they were transfected using 0.5 μl Lipofectamine 2000 (Invitrogen) and 50 ng of each reporter vector NF-κB luciferase and pRL-TK renilla (Promega). To obtain the highest transfection efficiency and low nonspecific effects, transfection conditions were optimized using varying concentrations of DNA, Lipofectamine, and cell density. Depending on the assay, wells received 10 ng of the plasmids coding for huTLR2, boTLR, chimeric protein, huDectin-1, or boDectin-1. Unless stated, the plasmid backbone for all of these molecules was pcDNA3.1 (Invitrogen). Cells were left to recover for 24 h and were then stimulated with different ligands (50 μl/well) or left untreated (addition of 50 μl medium/well) for 5 h. Thereafter, cells were lysed and luciferase and renilla activity quantified using a Dual-Luciferase Reporter Assay System (Promega) in a MicroLumat Plus LB96V luminometer (Berthold Technologies, Bad Wildbad, Germany), according to the manufacturer's instructions. Transfection efficiencies were normalized to luciferase activity derived from *Renilla reniformis* (ratio of relative firefly luciferase units to relative renilla units).

CXCL8 ELISA

To assess whether potential effects seen at the level of NF-κB activity may also affect other parameters, CXCL8 production was analyzed in supernatants harvested from the experiments described above, using a commercially available ELISA, according to the manufacturer's instructions (Quantikine Human CXCL8/IL-8 ELISA; R&D Systems, Abingdon, UK), similar to that described [11, 45].

Molecular modeling of boTLR2 and huTLR2 TIR and ECD

huTLR2 (NP_776622) and boTLR2 (NP_003255) protein sequences were used to generate homology models for the ECD (Residues 26–548) and TIR domain (Residues 639–794). Sequences were aligned using CLC Workbench to Protein Data Bank structures for TLR2 huECD and muECD (2Z7X, 2Z80, 2Z82, 3A7B) and the huTIR domain (1O77). Alignments were manually refined and ECD LRR positioning confirmed, using LRRfinder [9]. The ligand Pam3CSK4 and two shared glycans (Residues 199–202 and 442–445) were included from the TLR2 ECD structure 2Z7X. Modeller was used to generate 100 and 50 models/sequence for the ECD and TIR domains, respectively [46]. The top five models were selected by the lowest discrete optimized protein energy score and validated using Procheck, Verify3D, ERRAT, and ProSA-web [47–49]. Optimal models were selected, with >99% residues within core and allowed regions, goodness factors >−0.5, >90% residues with an averaged three-/one-dimensional score >0.2, and an overall ERRAT quality factor of >70%, which are within the accepted range for high-quality models. Models were visualized in PyMOL (PyMOL Molecular Graphics System, Version 1.5; Schrödinger, Portland, OR, USA) and electrostatic surface potential calculated using Adaptive Poisson-Boltzmann SolverTools 2.1.

Statistical analysis

Each transfection was performed in triplicate, and each experiment was repeated at least three times. Data are presented as percentage of NF-κB activity or fold increase related to untransfected cells to enable display on the same axis. For CXCL8 production, the absolute values are given. All ELISA tests were performed in duplicate, and experiments were performed at least twice. Statistical values are shown for pooled data obtained from at least two experiments. Initially, data were assessed for normal distribution using the D'Agostino and Pearson omnibus normality test. Thereafter, an unpaired *t*-test with Welch's correction was performed for data (see Fig. 1C) and a Bonferroni's *t*-test was performed after significant ANOVA for data (see Figs. 5B and C, as well as 7A and B).

RESULTS

Species-specific response to TLR2 ligands in primary cells and transfected HEK cells

In an initial experiment, we compared the CXCL8 response of primary huMDM and boMDM with known TLR2 ligands (**Fig. 1A** and **B**, respectively). In general, a lower response to all ligands tested was observed in bovine cells compared with human cells. All ligands induced a time-dependent release of CXCL8 from boMDM or huMDM, with LTA and *L. monocytogenes* inducing the highest response in both species (Fig. 1A and B, respectively). In relation to other ligands tested, boMDM responded relatively weakly to PGN and Pam3CSK4 stimulation compared with huMDM, which is in line with observations published recently [35]. Figure 1C shows the combined data sets from three individuals at 48 h after stimulation. Whereas clear, individual differences can be seen, the overall response pattern is the same as shown in Fig. 1A and B. Interestingly, for some ligands, the concentration could be increased 100-fold before a similar response was observed on boMDM when compared with huMDM (data not shown). However, it has to be mentioned that the CXCL8 response of boMDM was also lower to LPS when compared with huMDM, fitting to differences in TLR4, described recently [11].

To determine whether these observations were purely based on differences in the CXCL8 ELISA sensitivity, as suggested [50], and to assess the particular impact of TLR2 on the responses observed in huMDM and boMDM, HEK cells were stably transfected with huTLR2 or boTLR2, tagged with YFP. To assess the specific response induced by the TLR2 ligand inter-

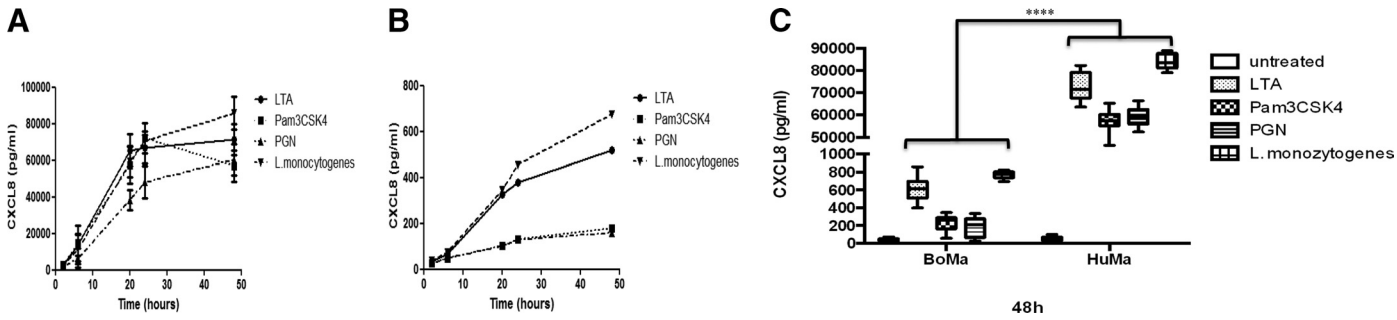

Figure 1. Response of primary macrophages to TLR2 ligands. huMDM (A) and boMDM (B) were generated as described and stimulated with LTA, Pam3CSK4, PGN, and *L.monocytogenes* in the concentrations described for the time-points indicated. CXCL8 concentration was analyzed in the supernatants by ELISA. Values are shown as mean ± SD of samples analyzed in triplicates and represent the results of at least three independent experiments. (C) The response of the pooled data is shown for 48 h after stimulation; $****P < 0.0001$. Ma, MDM.

action, we applied a similar system as described [51, 52]. In this system, heterodimerization with TLR1/TLR6 does not seem to be necessary, as this has recently been shown to impact the ligand-binding spectrum only but not on the subsequent signal generated [19]. A temporal profile of the NF-κB response was analyzed at several time-points after exposure to each ligand. Data are presented as fold increase compared with RLU values obtained from unstimulated cells. In accordance with the above data, the response of huTLR2-YFP cells to each ligand was significantly greater than that of the boTLR2-YFP HEK293 cells (**Fig. 2A** and **B**, respectively), despite similar expression levels of both receptors in transfected cells (Supplemental Fig. 1A–F). Whereas the NF-κB activity of huTLR2-YFP HEK293 cells exhibited a much more rapid increase in the first 20 h (Fig. 2A), the response of boTLR2-YFP HEK293 cells gradually increased and plateaued at ~24 h post-treatment (Fig. 2B). Following stimulation with LTA and *L. monocytogenes*, the magnitude of the response was different between the species, but for PGN and Pam3CSK4 we observed that these stimuli did not lead to a significant NF-κB induction in cells transfected with boTLR2 in line with our CXCL8 observations, although the overall pattern of response seemed to be somewhat similar. In both species, *L. monocytogenes* induced the strongest response, followed by LTA, Pam3CSK4, and PGN. For some of the ligands, such as *L. monocytogenes*, a 100-fold-higher concentration could be used in the bovine system before reaching the same value as obtained for huTLR2 (Supplemental Fig. 1G–I). In both systems, LPS did not induce NF-κB activation over time (data not shown). A direct comparison of the response pattern for TLR2 of both species with *L. monocytogenes* and LTA is shown in Supplemental Fig. 2.

In addition to measuring NF-κB activation as an indicator of intracellular signaling and activation, CXCL8 was analyzed by ELISA in supernatants taken from the above experiments (Fig.

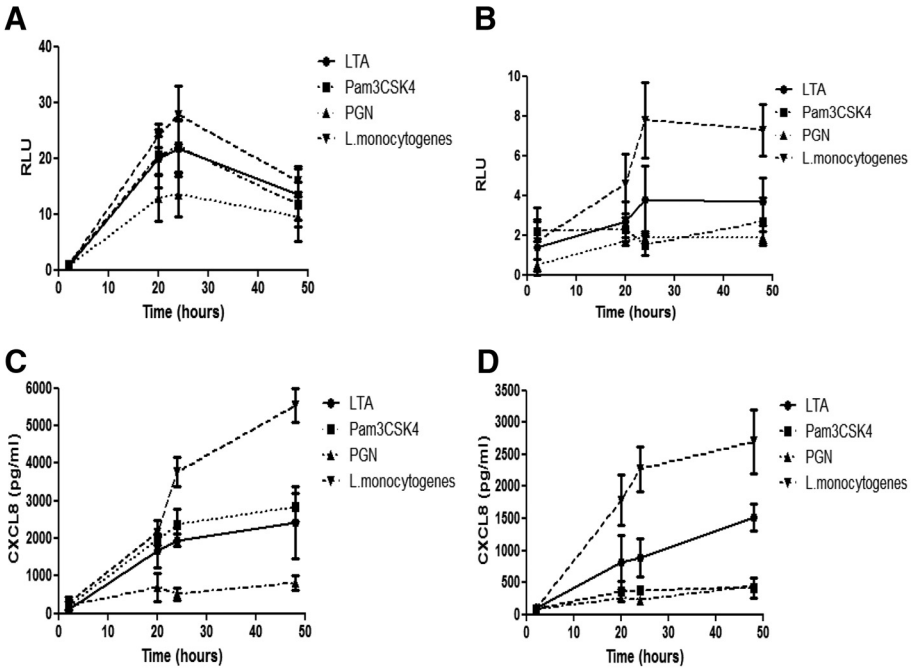

Figure 2. HEK293 cells transfected with huTLR2-YFP or boTLR2-YFP show species-specific responses to known TLR2 ligands. HEK293 cells were transfected with huTLR2-YFP (A and C) or boTLR2-YFP (B and D) and stimulated with LTA, Pam3CSK4, PGN, and *L. monocytogenes* in the concentrations described. At the time-points indicated, NF-κB activity was analyzed by a gene reporter assay, as described, and expressed as RLU (A and B) and CXCL8 concentrations (C and D) in the corresponding supernatants analyzed by ELISA. Values are shown as mean ± SD of samples analyzed in triplicates and represent the results of at least three independent experiments.

2C and D, respectively). As seen with NF-κB activation, CXCL8 production was highest in response to *L. monocytogenes* stimulation, followed by LTA and then Pam3CSK4 and PGN for both cell lines, although stimulation of huTLR2 induced a relatively higher response to Pam3CSK4 than LTA. Overall, the kinetics of CXCL8 release was similar in both systems. The patterns generally concur with that of NF-κB activation. As above, the response of the huTLR2-YFP HEK293 cells (Fig. 2C) was significantly greater than that observed with the boTLR2-YFP HEK293 cells (Fig. 2D).

A bodnMyD88 molecule effectively blocks TLR2 signaling in both species

As we tested a bovine receptor cloned into a human cell system, we investigated alternative reasons for the observed differences by comparing the structures of the TIR domains of huTLR with boTLR2, as well as assessing the effect of the main TLR adaptor protein MyD88, in a bovine dominant-negative (bodn) form, in both systems. With the comparison of a homology model of the boTLR2 TIR domain with the huTLR2 TIR domain crystal structure, we detected neither structural alterations nor significant differences in surface-charge distribution (**Fig. 3**). It thus appears likely that coupling of the boTLR2 TIR to the MyD88 TIR domain will be indistinguishable compared with huTLR2. To test this hypothesis, a bodnMyD88 construct was used.

Cotransfection of HEK293 cells stably expressing huTLR2- or boTLR2-YFP with bodnMyD88 significantly reduced the activation of NF-κB after stimulation with *L. monocytogenes*, LTA, and PGN in both systems and for Pam3CSK4 in the human system. As an example, the data for stimulation with LTA are shown (**Fig. 4A**), with the percentage of reduction over time shown in Fig. 4B, indicating a very similar trend. To assess whether this reduction in NF-κB activation had functional consequences, supernatants from the above experiments were also analyzed for CXCL8 content. As with the NF-κB data, bodnMyD88-treated TLR2-YFP HEK293 cells had a reduced response to LTA, *L. monocytogenes*, and PGN stimulation and Pam3CSK4 in the human system. As before, CXCL8 production in response to LTA is shown (Fig. 4C). To ensure that these effects were specifically a result of the bodnMyD88 plasmid, boTLR2 and huTLR2-YFP HEK293 cells were also transfected with the control vector pBluescript (Fig. 4D), which produced a similar NF-κB response to LTA compared with transfection with no plasmid. This response was reduced significantly when the cells were transfected with bodnMyD88.

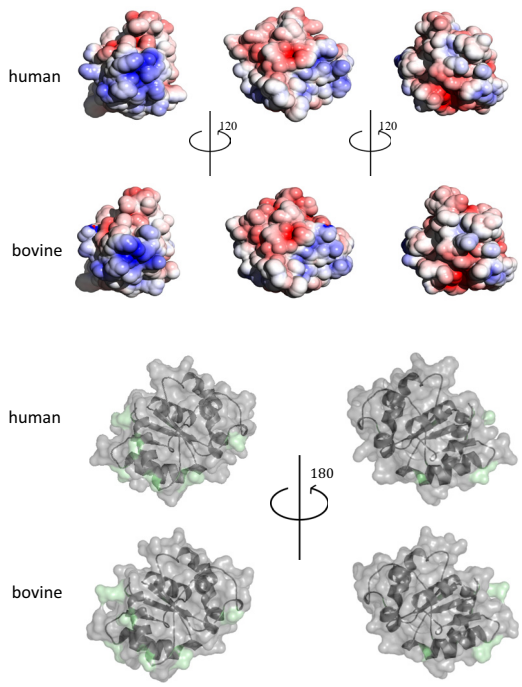

Figure 3. Comparison of the intracellular huTLR2 (upper panels) and boTLR2 (lower panels) TIR domain. Comparative models of huTLR2 and boTLR2 TIR surface-electrostatic potential. Surface-potential gradient was generated to span −5.0 (red) and 5.0 (blue). Species-specific residues within the modeled domains are highlighted in pale green.

The ECD of TLR2 confers a species-specific response

To verify that the observed differences are conferred by the ECD, a chimeric TLR2, consisting of the boECD fused onto the human transmembrane/intracellular TIR domain or vice versa, was created and its gene and protein expression in HEK cells assessed by qPCR and flow cytometry for YFP, in the absence of suitable TLR2 antibodies (**Fig. 5A** and Supplemental Fig. 3). As a similar level of expression was achieved, cells were stimulated as above with LTA, and NF-κB activity (Fig. 5B) and CXCL8 production (Fig. 5C) were analyzed. Stimulation of either of the chimeric TLR2 molecules with LTA resulted in values similar to those obtained for the corresponding full-length TLR2 molecule, with PMA used as positive control. Having analyzed the NF-κB and CXCL8 following stimulation of the chimeric proteins, we next compared the ECD of huTLR2 with boTLR2. The ECD of TLR2 from both species shows clear structural and surface-charge distribution differences (**Fig. 6**).

Species-specific coexpression of TLR2 and Dectin-1 in HEK cells results in further modulation of a NF-κB and CXCL8 response

To investigate whether boTLR2 acts synergistically when coexpressed with Dectin-1, boTLR2-YFP HEK293 cells were transiently transfected with boDectin-1-RFP and cells stimulated with zymosan particles (Supplemental Fig. 4A). To analyze the potential, functional consequences, supernatants were analyzed for CXCL8 production. HEK293 cells transfected with huDectin-1-RFP or boDectin-1-RFP alone responded to zymosan in a dose-dependent manner but not to LTA or *L. monocytogenes* (**Fig. 7A** and **B**). In contrast, HEK293 cells transfected with huTLR2-YFP or boTLR2-YFP responded to *L. monocytogenes* and LTA but not to any concentration of zymosan (Fig. 7A and B). Coexpression of TLR2 with Dectin-1 in both species did not enhance the response to challenge with receptor-specific ligands. Coexpression of TLR2 and Dectin-1 of either species seemed to dampen the response to the TLR2 ligands LTA and *L. monocytogenes* (Fig. 7A and B). CXCL8 production, in response to zymosan in the double-transfected cells, was unaf-

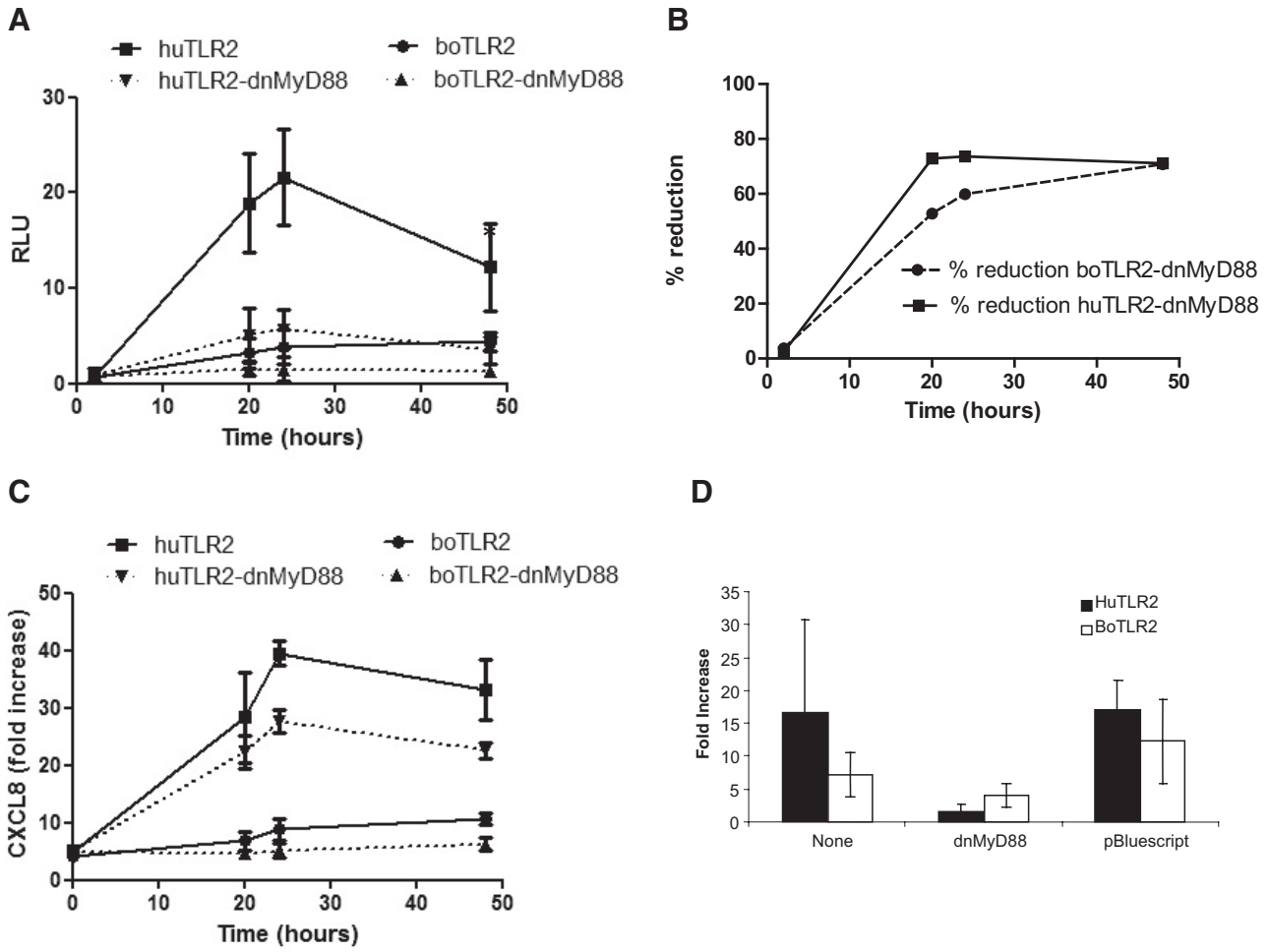

Figure 4. A bodnMyD88 construct inhibits TLR2-dependent responses in HEK293 cells transfected with huTLR2 or boTLR2 to the same extent. HEK293 cells were transfected with huTLR2-YFP or boTLR2-YFP and cotransfected with bodnMyD88 (dnMyD88) before stimulation with LTA for the time-points indicated (A). (B) Inhibition by bodnMyD88 in both systems over time is shown, and (C) CXCL8 response in the corresponding supernatants was analyzed by ELISA. The inhibition was specific to bodnMyD88, as transfection with the control plasmid did not reduce the response to LTA (D). Values are shown as mean ± SD of samples analyzed in triplicates and represent the results of at least three independent experiments.

fected (bovine; Fig. 7A) or slightly reduced (human; Fig. 7B), compared with the cells expressing Dectin-1 alone. Furthermore, when HEK293 cells transfected with TLR2-YFP of either species were cotransfected with Dectin-1-RFP of the other species, the response to the zymosan response was reduced significantly (Fig. 7A and B, last set of columns). These effects were not based on differences in mRNA expression levels (Supplemental Fig. 4G).

DISCUSSION

Recent evidence suggests species and potentially, even breed-specific, genetic-based functional differences in TLR sequences [53]. Some of these differences have already been suggested to play an important role in how different hosts recognize and respond to the same TLR ligand [12, 35, 54, 55]. The importance of specific LRRs in the structure of muTLR2 and huTLR2 for species-specific ligand recognition has already been shown [34] In the present study, we extended this work to show that the extracellular, solenoid part of the boTLR2 plays a key role in the shaping of the species-specific response to TLR2 ligands. The data presented in the current work, together with earlier observations [10], may also indicate that the same LRRs as identified in the human/murine systems are important for this effect.

Despite the fact that the response to all ligands tested followed a similar trend in both species, the response was far more pronounced in the human system. Whereas we do not have a clear answer for this observation, we hypothesize that these differences are not the result of intracellular incompatibility problems due to the use of a bovine receptor expressed in a human cell system, as comparison of the TIR domains with the use of a bodnMyD88 adaptor protein did not indicate species differences on the intracellular level. Furthermore, the data obtained using transfected HEK cells were confirmed using MDMs. Recently, it was shown by us and others that posi-

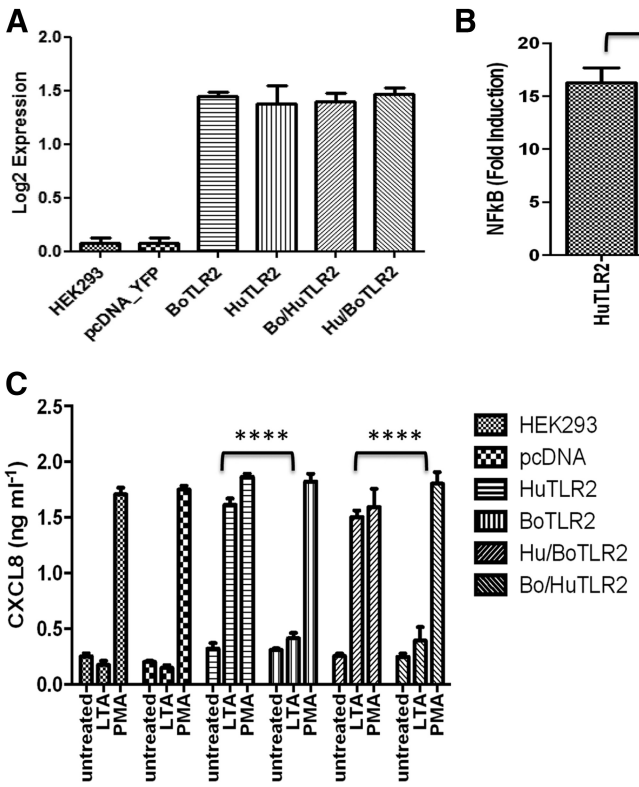

Figure 5. HEK293 cells, transfected with full-length TLR2 or chimeric TLR2, recognize TLR2 ligands in a species-specific manner. Expression levels of full-length huTLR2 and boTLR2 and their corresponding chimeric constructs were assessed by qPCR as described (A). NF-κB induction in transfected HEK293 cells was analyzed by a gene reporter assay, 24 h after stimulation with LTA (B), and CXCL8 response in the supernatants of transfected HEK293 cells to PMA or LTA was analyzed by ELISA (C). Values are shown as mean ± SD of pooled values obtained in three independent experiments of samples analyzed in duplicates. Significant differences between cells transfected with full-length or chimeric TLR2 are shown as $****P < 0.0001$.

tive selective, evolutionary pressure exists on specific amino acids within the LRR region and that this selective pressure was highest in the area of ligand-binding/dimerization [10, 56]. Our data, using chimeric proteins, clearly confirm the hypothesis that it is the ECD that is responsible for the observed differences in response. In particular, our protein modeling demonstrates that most of the differences in secondary structure occurs in the region of the ECD thought to form the binding groove for ligand interaction. Our data regarding the similarity of boTLR2 and huTLR2 TIR domains, in conjunction with the differences seen in the ECD, are supported further by the recent, extensive analysis of evolutionary differences between LRRs and the TIR domain [53, 57]. In both publications, the authors identified a higher degree of homology between TIR domains of different TLRs and within the same TLR of different species compared with their solenoid ECD.

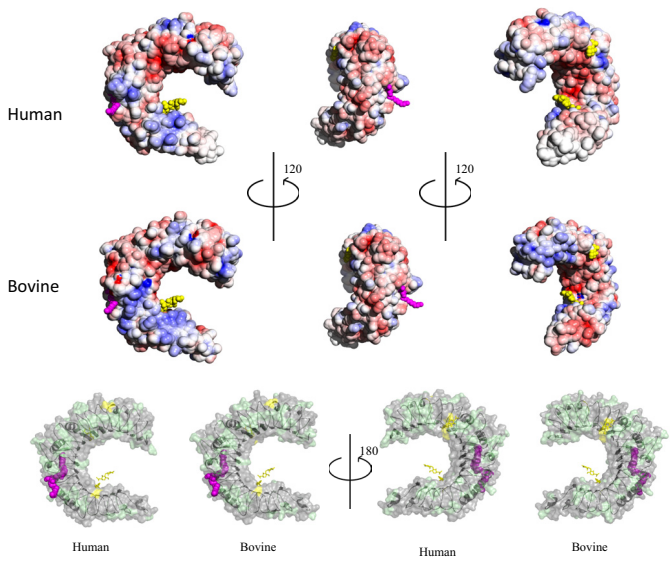

Figure 6. Comparative models of huTLR2 (top row) and boTLR2 (bottom row) ECD with the ligand Pam3CSK4 (magenta) and glycosylated side-chains (yellow) derived from the huTLR2 structure 2Z7X. Surface potential gradient was generated to span −5.0 (red) and 5.0 (blue). Species-specific residues within the modeled domains are highlighted in pale green.

A potential explanation for the observed differences might be that the ligands used were not generated from the most appropriate bacterial species for the bovine system. If this were the case, one could assume a host-pathogen coevolution, which would potentially also allow for the detection of ligands inducing a stronger response in the cattle system and potentially a weaker response in the human system. Indeed, some of the observed differences may be explained by the absence of biologically active LP accessible for HEK293-boTLR2 cells. The ability to activate TLR2 heterodimers is determined by the composition of the LP, with the number and length of the fatty acids, as well as the peptide tail, contributing to the immunostimulatory capacity of the LP [58]. It has been shown that these structural prerequisites differ between species. Whereas LPs, with short, ester-bound fatty acids, are able to activate muTLR2, they are not recognized by huTLR2 [33]. Furthermore, differences in the response of boTLR2-trans-

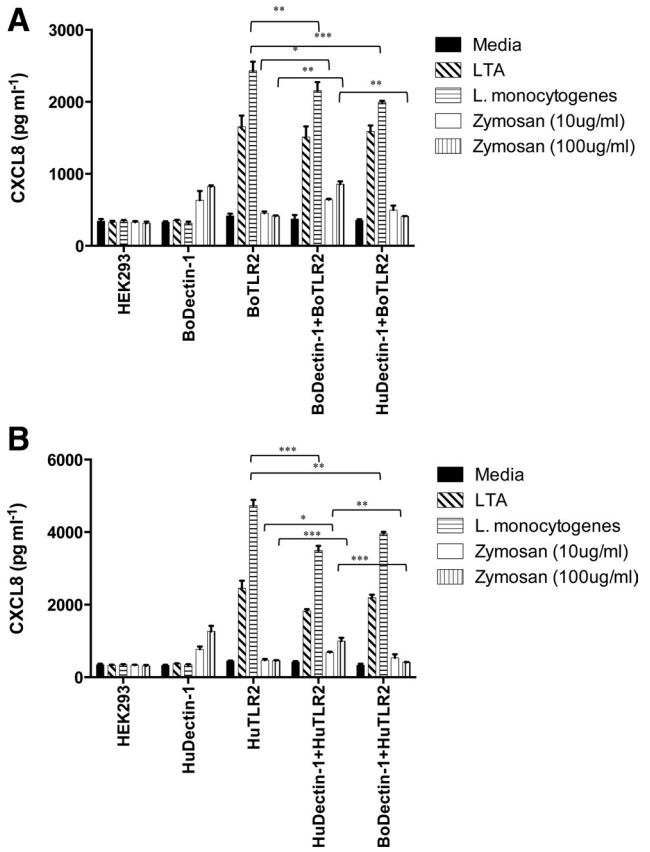

Figure 7. Coexpression of TLR2 and Dectin-1 alters the response of HEK293 cells to TLR2 and Dectin-1 ligands. HEK293 cells were transfected with the bovine receptors in different combinations (A) and the human receptors in the same combinations (B) and stimulated with LTA, *L. monocytogenes*, or zymosan for 24 h. CXCL8 was analyzed in the supernatants by ELISA. Values are shown as mean ± SD of pooled values obtained in three independent experiments of samples analyzed in duplicates. Significant differences between the treatments indicated are shown as $*P < 0.05$; $**P < 0.01$; and $***P < 0.0001$.

fected HEK cells to Gram-positive bacteria, derived from different host species, were described recently [44], suggesting a strong host-pathogen coevolution. A further possibility is that potential coreceptors, such as TLR1 and TLR6, may affect the read-out in our system. Recent analysis of the structure-activity relationship showed that the ester-bound acid chains of these LPs need to consist of at least 12 carbon atoms to activate the bovine heterodimer, showing similarity to the recognition by huTLR2/huTLR1 [35]. In contrast, HEK293 cells, cotransfected with muTLR2 and muTLR1, can be activated by LPs with shorter fatty acids of only six carbon atoms, clearly indicating an involvement of TLR1 in a species-specific recognition of bacterial LP. However, the relative abundance of endogenous TLR1 on the HEK293 cell surface compared with hu/boTLR2 is likely to be negligible [59]. Another possible explanation is that the bovine system has evolved to possess a different threshold level in terms of TLR activation. As the rumen is full with bacteria and protozoa, many of which are commensal, and expresses only a limited set of TLRs [60], the TLR system in ruminants may only respond to PAMPs at a higher concentration. This is akin to the human gut, where inappropriate TLR2 activation is deleterious to the host in the form of hyperinflammatory diseases. Our observation that boTLR2 could be stimulated with up to 1000-fold higher doses before inducing the same NF-κB activation may support this theory. Furthermore, *L. monocytogenes*, which is a true pathogen in cattle-inducing Listeriosis, was readily recognized by boTLR2. However, it also needs to be kept in mind that *L. monocytogenes* differs in several respects from other Gram-positive bacteria, e.g., *S. aureus*. A putative boTLR2 ligand of *L. monocytogenes* is active at considerably (five- to 50-fold) lower concentrations than other Gram-positive bacteria, resulting in higher amounts of CXCL8 produced by HEK293-boTLR2 cells [44]. Despite this observation, *L. monocytogenes* still induced a significantly lower response in boTLR2-transfected cells compared with huTLR2-transfected cells.

In addition, our data show that transfection of HEK293 cells with boDectin-1 leads to colocalization of Dectin-1 with zymosan particles, possibly promoting phagocytosis (Supplemental Fig. 4). As TLR2 has been reported to form heterodimers with Dectin-1 in a synergistic collaboration, we analyzed whether boTLR2 and boDectin-1 colocalize. Following incubation with zymosan particles, boDectin-1 was transiently expressed in boTLR2-YFP HEK293 cells. Both PRRs were shown to colocalize around the zymosan particles, at the cell surface and internally. They also seemed to colocalize when not interacting directly with zymosan. There is seemingly a polarization of the bound zymosan particles at one site on the cell membrane that may represent ligand-induced clustering of the cell-surface receptors (Supplemental Fig. 4).

Coexpression of boDectin-1 in the boTLR2-YFP HEK293 cells resulted in a decrease in response to stimulation to *L. monocytogenes* but not LTA (Fig. 7A). This effect seems even more pronounced in cells transfected with huTLR2 and huDectin-1, where an effect is also seen for LTA (Fig. 7B). These results suggest that for recognition of Gram-positive-related PAMPs, there is an antagonistic relationship between boTLR2 and boDectin-1, in line with data described recently [61]. Interestingly, there is an amino acid mutation at Trp221 and His223 in boDectin-1 compared with its murine orthologue, and these have been described as being essential for enhancement of TLR2-mediated NF-κB activation [62]. We hypothesize that this is indicative of a modulation in the effects of each PRR, potentially avoiding an inappropriate immune response, a hypothesis supported by several published observations. TLR2-deficient mice were shown to have greater resistance to infection by *C. albicans* than WT mice, suggesting a more robust Dectin-1-mediated immune response in the absence of TLR2 [63, 64]. In addition, coinjection of LPS and zymosan resulted in a reduced T cell proliferation compared with injection of zymosan alone [65]. This suggests that TLR4 may similarly antagonize Dectin-1 activity, such as the induction of T cell proliferation. Furthermore, stimulation of regulatory DCs with zymosan induced secretion of more IL-10 than IL-12, promoting a Th2 response in contrast to a Th1 response favored by TLR2 stimulation [65]. Similarly, stimulation of splenic MDM with zymosan induces production of TGF-β, which is

also immunosuppressive and would counteract a proinflammatory response mediated by TLR2 [65]. Despite the similarities described for huDectin-1 and boDectin-1 [27], it seems that this similarity is not enough to render TLR2 and Dectin-1 from different species functional, as cotransfection of boTLR2 with huDectin-1 did not result in a measurable response. As single amino acid substitution in the LRRs within the ECD has been shown to provide species-specific ligand recognition [12, 55], these differences may also explain our results.

In conclusion, the model of species-specific recognition of TLR ligands supported by functional and modeling evidence provides a new insight into the mechanism of TLR2 activation. The additional consequences described for the interaction of boTLR2 and boDectin-1 may account for different sensitivity in sensing microbial patterns in this pathway and may impact on the development of vaccine adjuvants.

AUTHORSHIP

S.W. is the main contributor, provided the study design, and performed experiments. V.O. offered the protein modeling and discussion. H-M.S. provided the study design, technical help, and discussion. T.J.C. is the coprinciple investigator and provided the study conception. D.W. is the principle investigator and provided the study design and conception.

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