Human but Not Murine Toll-like Receptor 2 Discriminates between Tri-palmitoylated and Tri-lauroylated Peptides*

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Toll-like receptors (TLRs) mediate activation of the immune system upon challenge with microbial agonists, components of disintegrating cells of the body, or metabolic intermediates of lipidic nature. Comparison of murine (m) and human (h) TLR2 primary sequences revealed 65% of identical residues within the extracellular domains in contrast to 84% in the intracellular domains. Comparative analysis of TLR2-driven cell activation by various TLR2 agonists showed that the tri-palmitoylated lipopeptide analog (Lau₃CSK₄) is recognized efficiently through mTLR2 but not hTLR2. Genetically complemented human embryonic kidney 293 cells and murine TLR2⁻/⁻ embryonic fibroblasts, as well as human and murine macrophage cells, were used for this analysis. In contrast to cellular activation, which depended on blockable access of the TLR2-ligand to TLR2, cellular uptake of Lau₃CSK₄ and tri-lauroylated peptide (P₃CSK₄) was independent of TLR2. A low-conserved region spanning from leucine-rich repeat (LRR) motif 7 to 10 was found to control TLR2 species-specific cell activation. Exchange of mLRR8 for hLRR8 in mTLR2 abrogated mTLR2-typical cell activation upon cellular challenge with Lau₃CSK₄ but not P₃CSK₄, implicating mLRR8 as a central element of Lau₃CSK₄ recognition. The point mutation L112P within LRR3 abrogated hTLR2-dependent recognition of lipopeptides but merely attenuated mTLR2 function, whereas deletion of the N-terminal third of each LRR-rich domain (LRRs 1 to 7) had the opposite effect on P₃CSK₄ recognition. Despite similar domain structure of both TLR2 molecules, species-specific properties thus exist. Our results imply distinct susceptibilities of humans and mice to challenge with specific TLR2 ligands.

Immediate-early host responses to potentially harmful microbial challenges depend on innate immunity, whereas adaptive immune responses come into play later. Innate immune receptors with specificity for pathogen-derived ligands are expressed constitutively and allow sensing of pathogens when they appear in the host. LPS,1 peptidoglycan, lipoproteins, flagellin, and nucleic acids are examples of microbial and viral products eliciting host responses (1–3). Both Gram-negative and Gram-positive bacterial products induce overactivation of the host immune system and are a major cause of severe sepsis and septic shock (4).

The toll-like receptor (TLR) family includes 10 members in humans. Mice have no TLR10 but carry TLR11 (5). TLRs and other pattern-recognition receptors such as dectin 1 and complement receptor 3 mediate specific host cell activation by components of microorganisms (6, 7) as well as by endogenous cellular products liberated upon disruption (8). In contrast to Drosophila toll and cytokine receptors, which exclusively bind endogenous ligands, TLRs directly interact with exogenous pathogen-derived ligands (9–11). They contain leucine-rich repeats (LRRs) line-ups in the TLR-extracellular domains (ECDs), which resemble known structures of LRR-rich domains (12). In addition, the structures of other microbial ligand receptors such as CD14 may be organized similarly. Structurally unrelated proteins can exert similar receptor functions, as exemplified by the LPS binding capacity of LPS-binding protein (13, 14).

Bacterial species, such as Gram-positive Listeria monocytogenes and Staphylococcus aureus, and Gram-negative Chlamydia pneumoniae elicit host cell activation through TLR2 (3, 15). Bacterial products, for instance lipoteichoic acid, bacterial lipoproteins or their analogs such as di/tri-palmitoyl-cysteiny1-seryl-(lysyl)₃-lysine (P₂₋₃CSK₄), and mycoplasmal macrophage activating lipoprotein are agonists of TLR2 (3). Although further cellular detection mechanisms exist that participate in innate host defense such as TLR2-independent, but detcin-dependent, immune reactions, TLRs seem to be essential for induction of a comprehensive innate immune response (6, 16, 17).

Species-specific differences in cellular pattern recognition have been found for certain LPS variants and Taxol; this may reflect the variant use of TLR4 and MD2 (18–20). Similarly, TLR9 has been recognized as the mediator of species-specific DNA sequence recognition (21, 22). Here, we used complementation of human embryonic kidney (HEK) 293 cells or murine embryonic TLR2⁻/⁻ fibroblasts with murine and human TLR2 to identify and analyze species specificity of pattern recognition through TLR2 ortholog products. In addition, we analyzed the murine RAW264.7 cell line, as well as human MonoMac6 and THP1 macrophage cell lines. Our results reveal species-specific recognition of a tri-lauroylated peptide through murine TLR2.

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¶ The abbreviations used are: LPS, lipopolysaccharide; TLR, toll-like receptor; LRR, leucine-rich repeat; ECD, extracellular domain; ICD, intracellular domain; P₃CSK₄, tri-palmitoyl-cysteiny1-seryl-(lysyl)₃-lysine; Lau, lauroyl; Myr, myristoyl; MEF, murine embryonic fibroblast; PBS, phosphate-buffered saline; NF-κB, nuclear factor-κB; mAb, monoclonal antibody; IL, interleukin.
and involvement of a relatively low conserved LRR-rich subdomain of the TLR2ECD in this process.

**MATERIALS AND METHODS**

**Reagents—** Purified bacterial components applied were LPS from *Escherichia coli* 0111:B4 (Sigma), soluble peptidoglycan from *Staphylococcus aureus* prepared by pronase extraction (Sigma). Synthetic mycoplasmal lipopeptide Lau3CSK4 (Tri-myr-Cys-Ser-Lys3Lys) was designated h1–10m/m or m1–10h/h, respectively; h or m K19/Q25 from the primary sequences of both immature proteins. Human (h) or Amsterdam, Netherlands). Chimera constructs were generated as deduced chain reaction-based mutagenesis (Quick Change kit, Stratagene, Amsterdam, Netherlands). 

**Cell Culture—** HEK293 cells (ATCC305) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (Invitrogen, Auckland, Scotland), whereas culture of primary murine embryonic fibroblasts (MEFs), 10 μM monothioglycerol (Sigma) was added to these medium components. MEFs were prepared from TLR2−/− mice (Tularak, South San Francisco, CA) as described (26). Murine RAW264.7 (ATCC No.TIB71) cells, as well as human MonoMac6 (ATCC No.TIB208) cells, were cultured in RPMI 1640 medium supplemented as described for Dulbecco's modified Eagle's medium (Invitrogen), whereas nonessential amino acids and media supplement (OPI, Sigma) were added for culture of MonoMac6 cells specifically.

**Mutagenesis—** A human and a murine TLR2 expression plasmid (pFlag-CMV, Sigma) (27) were used as templates in splice polymerase chain reaction-based mutagenesis (Quick Change kit, Stratagene, Amsterdam, Netherlands). 

**Analysis of Supernatants by Enzyme-linked Immunosorbent Assay—** Transfected HEK293 cells and TLR2−/− MEFs, as well as RAW264.7 and MonoMac6 cells, were cultured on 96-well plates (1 × 103, 5 × 103, 1 × 104, and 1 × 105 cells per well, respectively) and challenged as indicated for 24 h. Culture supernatants were analyzed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) for measurement of human IL-8, as well as human and murine IL-6 and tumor necrosis factor-α concentrations by enzyme-mediated colorimetry (Meggell, Tecan, Crailsheim, Germany), according to enzyme-linked immunosorbent assay material supplier protocols.

**Immunoblot Analysis—** Transfected HEK293 cells and TLR2−/− MEFs were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England). Western blots were carried out on nitrocellulose membranes, as recommended by the supplier protocol before incubation with antibodies. 

**Sample Preparation for Electrophoresis—** RAW264.7 and MonoMac6 cells (0.5 × 106 cells/ml) were rinsed from the culture plates, whereas THP1 cells differentiated by incubation with 50 ng/ml phorbol 12-myristate 13-acetate overnight were detached by incubation with PBS on ice for 5 min. Cells were incubated first with 2% fetal calf serum, 5% normal goat serum, and anti-murine Fc γRII/III Receptor mAb (BD PharMingen, Heidelberg, Germany) or Endobulin S/D (Baxter, Unterschleissheim, Germany) for murine or human cells, respectively.

**Flow Cytometry—** RAW264.7 and MonoMac6 cells (0.5 × 106 cells/ml) were rinsed from the culture plates, whereas THP1 cells differentiated by incubation with 50 ng/ml phorbol 12-myristate 13-acetate overnight were detached by incubation with PBS on ice for 5 min. Cells were incubated first with 2% fetal calf serum, 5% normal goat serum, and anti-murine Fc γRII/III Receptor mAb (BD PharMingen, Heidelberg, Germany) or Endobulin S/D (Baxter, Unterschleissheim, Germany) for murine or human cells, respectively.

**Immunocytochemical Staining and Uptake of Biotinylated Lipopeptide—** THP1 cells, differentiated with phorbol 12-myristate 13-acetate overnight were detached by incubation with PBS on ice for 5 min. Cells were incubated first with 2% fetal calf serum, 5% normal goat serum, and anti-murine Fc γRII/III Receptor mAb (BD PharMingen, Heidelberg, Germany) or Endobulin S/D (Baxter, Unterschleissheim, Germany) for murine or human cells, respectively.

**Electrophoretic Mobility Shift Analysis—** RAW264.7 and MonoMac6 cells were challenged in RPMI 1640 serum containing 2% fetal calf serum for 2 h, and nuclear extracts were prepared. Cells were lysed, and nuclear proteins analyzed as described previously (26). 

**Preparation of Inactivated Bacteria Suspensions—** Bacteria of the species *S. aureus* (DSMZ 20231), *S. pyogenes* (DSMZ 20055), as well as of *Enterococcus faecalis* (DSMZ 20474) were cultured at 37 °C in standard brain-heart medium overnight and plated on standard blood agar plates for determination of culture purity and colony forming unit titers. 

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RESULTS

Comparative Mutagenesis of Human and Murine TLR2—Comparative amino acid sequence analysis of human and murine TLR2 revealed 70% overall identity. In contrast, extracellular and intracellular domain sequences show 65 and 84% identity, respectively. We subdivided the ECD sequences of human and murine TLR2 into 20 LRR/LRR-like motifs (32) and refer to these as LRRs. Alignment of both TLR2 sequences showed identical localization of these motifs and thus enabled the individual comparison of each of the LRRs (Fig. 1A). The intracellular domain was exchanged between human and murine TLR2 to control for their potential species-specific activity. No evidence for species-specific properties was apparent from analysis in human (HEK293) or murine (TLR2−/− MEF) cells (Fig. 1B, constructs h/m and m/h, and data not shown). To search for species specificity through non-conserved regions of the two TLR2ECD sequences, we swapped the first half of the LRR-rich domains because LRR motifs 1 to 10 displayed lower interspecies similarity than motifs 11 to 20 (Fig. 1B, h1–10m/m and m1–10h/h). We also exchanged a region encompassing the four LRR motifs 7 to 10 because conservation of their sequences is particularly low (Fig. 1, A and B, mh1–10m/m and hm1–10h/h). For fine-mapping of this block of LRRs, LRRs 8 to 10, 9 to 10, 8 to 9, as well as single motifs 8, 9, and 10 were swapped (constructs hm8–9h/h, hm9–10h/h, hm8–9h/h, hm1–10h/h, mh8–9m/m, hm1–10m/m, hm8–9m/m, and hm8–9h/h). To analyze a potential role of another low-conserved region within the TLR2ECD, we additionally exchanged the LRR motifs 19 and 20 (Fig. 1B, constructs mh1–10m/m and hm1–10h/h) and exchanged LRRs 19 and 20 only (hm19–20/h). Another relatively small conserved region is the LRR block encompassing motifs 14 to 20 which was analyzed similarly (mh14–19m/m and hm14–19h/h). A mutant “H” lacking the N-terminal third of the LRR-rich domain of human TLR2 has been described previously (26), and an analogous murine TLR2 construct was generated (Fig. 1B, mMutH). Two constructs of human and murine TLR2 carrying the point mutation L112P (this affects a consensus leucine residue within the respective third LRR motifs) were also prepared.

N-Glycosylation is critical for TLR4 function (33) and for TLR2 surface expression (34), suggesting that distinct N-glycosylation might lead to species specificity of pattern recognition through TLR2. Application of the NetNGlyc 1.0 algorithm2 revealed presence of four N-residues that are putatively glycosylated in hTLR2ECD (amino acid residues 114, 199, 414, and 442, respectively), whereas in mTLR2ECD only three sites (amino acid residues 147, 414, and 442) were considered glycosylation of a putative fourth site as unlikely (residue 296) because of conformational constraints induced by a proline following the

2 Internet address: www.cbs.dtu.dk/services/NetNGlyc.

FIG. 1. Illustration of human and murine TLR2ECD homology and the TLR2 constructs generated. A, alignment and comparative analysis of human and murine extracellular TLR2 primary sequences and definition of 20 LRR/LRR-like motifs (boxes numbered according to the order from the N terminus) revealed similarities illustrated as identities within each of the sequence motifs; low-conserved regions (bold and italics) and high-conserved regions (italics) are highlighted within the N-terminal and the C-terminal half of the LRR-rich domain. B, fusion constructs of human (h, black) and murine (m, white) TLR2 are aligned schematically with wild-type proteins, as well as deletion constructs (MutH, AS4-V220) to illustrate relative localization of LRR (unmarked) and LRR-like (asterisks) motifs (rectangles). ●, localizations of putative N-glycosylation sites; ECD, ECD encompasses LRRs symbolized as boxes, N-terminal domain as rectangles, and C-terminal domain as small half-oval; TM, trans-membrane domain depicted as small rectangles; N terminus pictured as large rectangles and Toll-IL-1 receptor/TIR-domain as large ovals. Nomenclature in combination with the graphic code illustrates the order (N toward C terminus) of subdomains from both receptors within the fusion constructs; numbers correspond to the LRR motifs exchanged. +*, symbolizes the trans-membrane domain; symbols beside each construct cartoon represent the relative cellular activity upon challenge with LaunCSK, mediated through the respective TLR2 construct: ++, high; +, intermediate; +, low; (−), very low; −, no detectable signal upon LaunCSK challenge; −, no detectable signal upon TLR2-specific challenge.
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A. HEK293 cells overexpressing human (●) or murine (■) TLR2 were challenged with suspensions of heat-inactivated bacteria at the concentrations indicated before measurement of NF-κB-dependent reporter gene activation. Unstim., unstimulated; CFU, colony-forming unit. B, human wild-type TLR2 (▲), murine wild-type TLR2 (■), human TLR2 ECD fused to complete C-terminal portion of murine TLR2 (●), or murine TLR2 ECD fused to complete C-terminal portion of human TLR2 construct (□) mediated reporter gene activation upon challenge with the defined microbial products indicated. OspA, 4.5 μg/ml of outer surface protein A of B. burgdorferi; LTA, 5 μg/ml of lipoteichoic acid of S. aureus; MALP, 100 ng/ml of mycoplasmal monocyte activating lipoprotein; PGN, 5 μg/ml of peptidoglycan of S. aureus; HSP60, 8 μg/ml of recombinant heat shock protein 60 of C. pneumoniae; 50 μg/ml of Zymosan, resuspended lipolysaccharide of yeast. Rel. lucif. activity, relative luciferase activity.

Fig. 2. Human and murine TLR2-specific responsiveness of transfected HEK293 cells to bacterial challenge. A, HEK293 cells overexpressing human (●) or murine (■) TLR2 were challenged with suspensions of heat-inactivated bacteria at the concentrations indicated before measurement of NF-κB-dependent reporter gene activation. Unstim., unstimulated; CFU, colony-forming unit. B, human wild-type TLR2 (▲), murine wild-type TLR2 (■), human TLR2 ECD fused to complete C-terminal portion of murine TLR2 (●), or murine TLR2 ECD fused to complete C-terminal portion of human TLR2 construct (□) mediated reporter gene activation upon challenge with the defined microbial products indicated. OspA, 4.5 μg/ml of outer surface protein A of B. burgdorferi; LTA, 5 μg/ml of lipoteichoic acid of S. aureus; MALP, 100 ng/ml of mycoplasmal monocyte activating lipoprotein; PGN, 5 μg/ml of peptidoglycan of S. aureus; HSP60, 8 μg/ml of recombinant heat shock protein 60 of C. pneumoniae; 50 μg/ml of Zymosan, resuspended lipolysaccharide of yeast. Rel. lucif. activity, relative luciferase activity.

Sites (DG3). All TLR2 constructs were expressed at similar levels upon transfection in HEK293 cells as analyzed by immunoblot analysis (data not shown).

Comparative Analysis of TLR2 Construct Activities by Genetic Complementation of HEK293 Cells—To compare responsiveness to whole bacterial cells, HEK293 cells expressing either human or murine TLR2 were challenged with suspensions of heat inactivated bacteria at increasing concentrations. Results revealed a similar trend in respect to NF-κB-dependent reporter gene activation and IL-8 release through both wild-type receptors. Cell activation by heat-inactivated bacteria (Fig. 2A) or defined microbial products such as lipoteichoic acid and peptidoglycan through TLR2 was species-independent (Fig. 2B). Exchange of the extracellular domains between human and murine TLR2 did not alter cellular responsiveness, indicating full function of the murine TLR2CD in human embryonic fibroblasts (Fig. 2B and data not shown). However, application of lipopeptide analogs (Fig. 3A) carrying acyl chains of reduced length (as compared with the classical P3CSK4, which contains 16 C-atoms in its palmitoyl chain) revealed TLR2 species specificity. Although P3CSK4 (tri-palmitoylated peptide) and MYR3CSK4 (tri-myristoylated) induced cell activation to similar degrees, Lau4CSK4 (tri-lauroylated) was recognized specifically through mTLR2ECD but not hTLR2ECD unless more than one hundred-fold-increased ligand-concentrations were used (Fig. 3B, D and data not shown).

Constructs containing cross-species subdomains were next tested to identify the subdomain responsible for this species specificity (Fig. 1B). Replacement of the mTLR2ECD domain containing the first 10 LRRs with the corresponding hTLR2 domain did not abrogate signal transduction. The reverse change (introducing mTLR2 LRR 1 to 10 into hTLR2) conferred Lau4CSK4 responsiveness through this otherwise human construct. Responsiveness mediated by both constructs was intermediate as compared with both wild-type receptors (data not shown). Exchange of a subdomain encompassing LRRs 7 to 10 had a similar effect, not only in HEK293 cells but also in TLR2−/− MEFs (Figs. 1B and 3C, B to E, and data not shown) mapping species specificity to this narrowed subdomain. For detailed analysis of the LRR 7 to 10-block constructs carrying blocks of three, two, or only single murine LRRs within the otherwise human receptor were prepared. Although not enhancing cellular Lau4CSK4 recognition to a degree similar to wild-type mTLR2, all constructs containing blocks of three or two cross-species LRRs conferred enhanced activity to hTLR2. The only exception was construct hm7–10h/h, which did not confer detectable responsiveness. Exchange of single LRRs within the LRR 7 to 10 subdomain in hTLR2 did not promote increased cell activation as compared with wild-type hTLR2 activity. The most active construct was hm7–9h/h, the activity of which was intermediate as compared with mTLR2 (high) and hTLR2 (low) (Figs. 1B and 3C, B to E, and data not shown). Analysis of a cross-specific LRR8 construct (mh7–10m/m, carrying the murine motif in the otherwise human receptor) indicated a central role of LRR8 in recognition of Lau4CSK4. The activation level mediated through this construct upon Lau4CSK4 challenge was nearly as low as wild-type hTLR2-dependent cell activation (Fig. 3B to D, and data not shown). In conclusion, these results indicate that the murine LRR block from motif 7 to 10 is involved in mouse-specific Lau4CSK4 recognition, and LRR8 plays a prominent role within this block.

To analyze additional subdomains for involvement in species-specific pattern recognition, further fusion constructs were generated and analyzed (Fig. 1B and data not shown). We found that neither the region encompassing LRRs 14 to 18, nor LRR 19 and 20 within the C-terminal portion of TLR2, contributed to species-specific pattern recognition because their cross-species exchange did not alter characteristics of the respective constructs. Notably, exchange of human motifs 19 and 20 for its murine counterpart abrogated recognition of known TLR2 agonists (Fig. 1B, hm19–20/h). In contrast, the respective “human-in-murine” exchange had no detectable effect (Fig. 1B, mh19–20m/h vs. mh19–20m/h and data not shown).

Comparative Analysis of TLR2 Construct Activities by Genetic Complementation of TLR2−/− MEFs—Next, TLR2-deficient mouse fibroblasts genetically complemented with TLR2 constructs were analyzed. Although TLR2−/− fibroblasts gained responsiveness to both lipopeptide analogs upon transfection of mTLR2, hTLR2 did confer detectable responsiveness to P3CSK4 but not to Lau4CSK4 (Fig. 3E). The finding that Lau4CSK4 was recognized exclusively through murine TLR2ECD was confirmed through analysis of ECD-ICD exchange constructs (data not shown). Results obtained by transient overexpression of TLR2 murine-human fusion constructs...
TLR2 expression of murine and human monocyte/macrophage cell lines and species-specific responsiveness to distinct TLR ligands — We extended our analysis on species-specific cell activation through TLR2 to macrophage cell lines of human or murine origin. Using a cross-reactive TLR2 mAb recognizing both human and murine TLR2, we analyzed TLR2 expression of RAW264.7, MonoMac6, and THP1 cells. Intracellular TLR2 expression was higher than cell surface expression, but all three cell lines expressed detectable amounts of TLR2 on the cell surface (Fig. 5, A and B). Both human and murine macrophages responded to P3CSK4 or P2CSK4 and LPS. In contrast, Lau3CSK4 activated murine RAW264.7 but not human macrophage-like cells unless the ligand was applied at one hundred- to thousand-fold higher concentrations (Fig. 5C). In line with these results, nuclear translocation and DNA binding of NF-κB, as well as the phosphorylation of mitogen-activated protein kinases p38 and extracellular signal-regulated kinase 1/2 (all of which play central roles in TLR-mediated signal transduction (3)), were different. The measured events of signal transduction did not differ between TLR2+/− MEFs and TLR2−/− MEFs.
transduction upon Lau$_3$CSK$_4$ challenge were not detectable or strongly decreased in human as compared with murine macrophages (Fig. 6, A and B).

**Immunocytochemical Analysis of Lipopeptide Uptake by Human Macrophage Cell Lines—**Uptake of P$3$CSK$_4$ and Lau$_3$CSK$_4$ in human and murine macrophages was compared to determine whether cellular uptake was also species specific. Weak lipopeptide-specific cell surface staining after 5 min of incubation but significant intracellular staining after 30 or 45 min indicated time-dependent cellular uptake by human wild-type macrophages (Fig. 7) and also by murine TLR2$^+$/H$2$O$2^+$/H$2$O$2^+$/H$2$O$2$ macrophages (data not shown). Similar kinetics of internalization indicated TLR2-independent binding of the lipopeptide analogs P$3$CSK$_4$ and Lau$_3$CSK$_4$ to both human and murine macrophages (Fig. 7 and data not shown). Free biotin was not detectable within cells after application under the same conditions used for biotinylated lipopeptides (data not shown), indicating lipopeptide specificity of the cellular uptake observed.

**Deletion- and Point Mutagenesis-based Analysis of Structural Requirements for Species-specific TLR2 Function—**A hTLR2 construct, lacking the N-terminal third of its LRR-rich domain (mutant H), mediated di- or tri-palmitoylated peptide-induced cell activation but not to other TLR2 agonists (26). We generated an analogous mTLR2 construct. This mutant failed to mediate P$3$CSK$_4$-induced cell activation (Fig. 8A, right panel), indicating a species-specific difference of TLR2 structure. Neither H$^+$/H$2$O$2$-mutant mediated a Lau$_3$CSK$_4$ signal (Fig. 8A). Although diminished, a mTLR2 construct containing a point mutation in its ECD mediated a P$3$CSK$_4$ signal, whereas the analogous human construct did not (Fig. 8B). All mutant constructs were expressed at similar levels, and molecular
weights were as expected (Fig. 8C and data not shown). As mentioned above, both h and mTLR2 primary sequences contain putative N-glycosylation sites, the localization of which within h and mTLR2ECD sequences differs. N-specific deglycosylation of both wild-type TLR2 proteins attenuated their apparent molecular weights to that of the mutant TLR2, in which all three glycosylation sites had been mutated (Fig. 8C).

**DISCUSSION**

The structural requirements for the known species-specific LPS and Taxol recognition through murine and human TLR4-MD2 complexes have been analyzed previously by domain-exchange between the respective TLR4 proteins. These data...
implicated a non-conserved region in species-specific pattern recognition through TLR4-MD2 (35). We aimed at the identification of species-specific ligands for TLR2 and analysis of the respective structure-function relationship. Our results identify lauroylated lipopeptides as a species-specific TLR2-agonist. They further indicate that LRR8 and the surrounding region spanning from LRR7 to LRR10 control species-specific ligand recognition.

Comparative analysis of a variety of distinct microbes for their potential to activate hTLR2 or mTLR2 did not reveal significant differences. This indicates that expression of a set of TLR2 agonists by a given microorganism overcomes species specificity of individual TLR2 ligands. This assumption, however, does not exclude differences in the potential of individual TLR2 ligands. Furthermore, incubation with heat-inactivated bacterial cells is not the same as confrontation of host cells with bacterial products that occurs upon lysis, upon treatment with antibiotics, or inside phagosomes/lysosomes in cells of infected host organisms. TLR2 agonists integrated into the cell wall or residing in the bacterial cytoplasm might reach TLR2 only under specific conditions. Additionally, digestive processes catalyzed either by enzymes released from disintegrating microbial cells themselves or provided by the host organism might change the chemical properties of microbial products and TLR specificity. For instance, lipases might degrade acyl moieties of immunostimulatory lipopeptides as exemplified by neutralization of LPS through deacylation (36). Furthermore, individual bacterial species might produce distinct molecular variants of one microbial product, such as lipopeptide, under specific conditions. For instance, distinct LPS species are synthesized by bacteria under specific growth conditions (35).

Considering such chemical modification of immunostimulatory bacterial products during infection, we analyzed distinct synthetic analogs of lipopeptides. The lipopeptides used contained distinct acylations in respect to palmitoyl-moiety numbers (37) or acyl chain lengths (Fig. 3A). Although analysis of the first did not show species specificity of TLR2 (data not shown), analysis of the latter set of lipopeptides indicated that the analog Lau₄-CSK₄ (Fig. 3A) lacking 4 CH₂-groups (12 C-atoms, lauroyl) in each acyl chain as compared with P₆-CSK₄ carrying 16 C-atoms per acyl chain was recognized specifically through mTLR2. This finding was corroborated by use of HEK293 cells and TLR2⁻/⁻ MEFs transfected with hTLR2 or mTLR2 variants (Fig. 3, B to E).

Comparative analysis of wild-type mTLR2 and a mTLR2ECD-hTLR2ICD construct demonstrated equal potential of both human and murine ICs to mediate intracellular signaling, such as NF-κB-signaling (Figs. 2 and 3). Exchange of a region encompassing the N-terminal half of the LRR-rich domain conferred Lau₄-CSK₄ responsiveness to hTLR2 and diminished but did not abrogate mTLR2 activity. Exchange of LRRs 7 to 10 only was sufficient to mediate activation to a similar degree, whereas exchange of mLRR8 by its human counterpart resulted in “loss of function” of mTLR2. These results attribute a central role to murine LRR8 and its surrounding LRRs in cellular recognition of lauroylated peptides. In contrast, exchange of low-conserved LRRs 14 to 18, or of 19 and 20, did not affect activity to a detectable degree. The exchange of LRR19 and 20 in hTLR2 unexpectedly resulted in complete loss of function. When overexpressed, the construct hm₁₉₋₂₀/h was detectable by surface staining and flow cytometry at similar levels as active TLR2 fusion constructs (data not shown), indicating that disruption of its regular integrity was causative for malfunction of this hTLR2 variant.

Specific binding of P₆-CSK₄ and TLR2 (31) suggests direct interaction also of TLR2 and Lau₄-CSK₄. In this respect, high exchange rates of ligand molecules at binding sites of the receptor or differences in affinities could explain lack of detectable competition between Lau₄-CSK₄ and P₆-CSK₄ for binding to hTLR2 (Fig. 4A and data not shown). However, mAb-mediated inhibition, not only of Lau₄-CSK₄-dependent activation of mTLR2⁺ cells but also of high-dose Lau₄-CSK₄-induced activation of cells expressing hTLR2, which suggests that both analogs bind to a single site in the TLR2ECD (Fig. 4, C and B, respectively). Alternatively, the mAb used might block several distinct sites in TLR2 involved in lipopeptide recognition either directly or indirectly.

Both murine RAW264.7 and human MonoMac6 macrophages expressed cell surface and intracellular TLR2 and responded equally well to P₆-CSK₄ and LPS. In line with the results discussed above, murine macrophages responded to Lau₄-CSK₄ challenge. Only when Lau₄-CSK₄ was applied at one hundred- or thousand-fold increased concentrations did human macrophages respond, as indicated by detectable cytokine release and intracellular signal transduction (Figs. 5 and 6). These results confirm species specificity of Lau₄-CSK₄ recognition.

Time-limited proximity of TLR2 to zymosan particles bearing TLR2 agonist β-glycan, as well as efficient uptake and processing of a TLR2-specific antibody, have been demonstrated recently (38, 39). However, uptake of P₆-CSK₄ and Lau₄-CSK₄ by human macrophages, as well as murine wild-type and TLR2⁻/⁻ macrophages, was indistinguishable (Fig. 7 and data not shown), implicating a TLR2-independent lipopeptide uptake receptor. Cellular lipid receptors, such as CD14, are candidates for this function (40). Recently, saturated and free lauric acid has been shown to activate TLR2. We propose that free lauric acid activates cells specifically through mTLR2 used in that study (41). Lauric acid might represent a metabolic intermediate resulting from degradation of microbial lipoproteins in the host organism.

Comparison of two different types of mutant TLR2 constructs based on both wild-type hTLR2 and mTLR2 showed functional details of TLR2 species specificity. A hTLR2 mutant lacking an N-terminal portion of the LRR-rich domain conferred responsiveness specifically to P₆-CSK₄, whereas the analogous murine construct did not. Notably, Lau₄-CSK₄ signals were mediated through neither construct, even when this lipopeptide was applied at high concentrations. This suggests a distinctive mechanism of TLR2-dependent P₆-CSK₄ and Lau₄-CSK₄ recognition. In contrast, a point mutation identified by random mutagenesis and localized within the consensus sequence of the third LRR did not abrogate P₆-CSK₄ signaling through mTLR2, whereas cells overexpressing the analogous human construct were unresponsive to P₆-CSK₄ challenge. Results of N-glycosylation analysis indicate similar N-glycosylation of both TLR2 proteins because a constitutively non-N-glycosylated construct resembled the size of the N-deglycosylated wild-type proteins. More pronounced size reduction of wild-type and mutant hTLR2 as compared with mTLR2 through N-deglycosylation might be explained by the potential presence of an additional N-glycosylation site within the seventh LRR in the human TLR2ECD but not in mTLR2. Specific N-glycosylation is involved in TLR2 surface expression (34). Furthermore, N-glycosylation is important for signaling, and sequence-specific differences of TLR2ECDs were also evident from analysis of a variety of monoclonal antibodies raised against mTLR2ECD protein. Only one of the mAbs analyzed recognized TLR2 of both species (31). Our results demonstrate species-specific characteristics of TLR2 and strongly suggest that these differences provide a molecular basis for distinct susceptibilities of humans and mice to specific infections.

3 G. Meng, A. Grabiec, and C. J. Kirschning, unpublished results.
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Human but Not Murine Toll-like Receptor 2 Discriminates between Tri-palmitoylated and Tri-lauroylated Peptides

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