Cutting Edge: Functional Interactions Between Toll-Like Receptor (TLR) 2 and TLR1 or TLR6 in Response to Phenol-Soluble Modulin

Adeline M. Hajjar, D. Shane O'Mahony, Adrian Ozinsky, David M. Underhill, Alan Aderem, Seymour J. Klebanoff and Christopher B. Wilson

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Toll-like receptors (TLRs) are type 1 transmembrane receptors that are involved in the early immune response to pathogens (1–4). Nine mammalian TLRs, defined by sequence similarity, have been cloned (5–11). TLR2 appears to mediate responses to lipoteichoic acid, lipopeptides, and peptidoglycan from Gram-positive bacteria and mycobacteria (12–22). TLR2 and TLR4 have both been reported to function as LPS receptors in vitro (23–26). However, TLR4 is the predominant receptor mediating the response to LPS in vivo, because TLR4 knockout mice respond normally to LPS (27, 28). Furthermore, TLR4 mutations have been identified in mice that are genetically hyporesponsive to LPS (29, 30). To date, no specific ligand recognition has been attributed to TLRs other than TLR2 or TLR4, although a role for TLR5 in the response to Salmonella has been hypothesized (9). We have been studying phenol-soluble modulin (PSM), a factor secreted by Staphylococcus epidermidis and other species of staphylococci (31). Here we report that TLR2 is sufficient to confer PSM responsiveness, but that the response is enhanced by TLR6 and impeded by TLR1, indicating that TLR2 functionally interacts with the latter two proteins in the response to PSM. Furthermore, we demonstrate that a PSM response mediated by TLR2 and TLR6 is less efficiently blocked by TLR1 than one mediated by TLR2 alone.

Materials and Methods

Cloning of murine TLR1

Total RAW 264.7 cell RNA was isolated using RNA exol (BioChain Institute, San Leandro, CA). Superscript II (Life Technologies) was used for reverse transcription of 1 μg of RNA using the primer 5′-GCAGCAA-CATCATATTGAGGGTG-3′. PCR was performed with the antisense primer 5′-GGTGGATATTTCAAACTG-3′ (stop codon underlined) and the sense primer 5′-GGCACGTTAGCACTGAGACTC-3′. The predicted 1.8-kb product was cloned using the TA cloning kit (Invitrogen), and multiple clones were sequenced to determine the consensus sequence. Two rounds of 5′ rapid amplification of cDNA end (Life Technologies) were used for generating the remaining coding sequence. Both strands of at least three clones of each PCR product were sequenced to obtain a consensus sequence.

Plasmids used in transfections were purified using the Endo-free plasmid kit (Qiagen, Chatsworth, CA). HA epitope-tagged TLR constructs were

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generated using a pDisplay vector (Invitrogen) that had previously been modified by deleting the myc epitope tag and the PDGFR transmembrane domain (Sfu/Fxiol 200-bp deletion). The modified vector provides a signal peptide and an amino-terminal HA tag. HA-TLR1, HA-TLR4, and HA-TLR6 were constructed by introducing an Xhol restriction site by PCR) 3’ of the TLR signal peptide to permit in-frame ligation into the Display vector. HA-TLR2 has been described previously (32). Chimeric TLR1-TLR6 proteins were generated by exchanging the 1-kb PstI-SacII fragment of TLR1 (aa 480–795) and TLR6 (aa 485–806; SacII was introduced 3’ of the stop codon by PCR). The C-terminal V5 epitope-tagged TLR2 construct was obtained by cloning a PCR product of the full-length open reading frame into pEF6/V5-His-TOPO (Invitrogen). The Pro-to-His dominant-negative (dn) TLR constructs were generated by PCR. All constructs were verified by sequencing. The mouse CD14 expression construct has been previously described (22). Mouse MD-2 was generously provided by Ken- suke Miyake (Saga Medical School, Saga, Japan) (33).

Luciferase assays

HEK 293 cells were plated at 2 × 10⁴ cells per well in 24-well plates the day before transfection. Cells were transfected by calcium phosphate precipitation (34), washed 3 h after transfection, and stimulated 20–24 h later (as indicated) in medium containing 10% FCS. After a 5-h incubation, the cells were washed once in PBS and lysed in Passive Lysis Buffer (Promega, Madison, WI). The Dual-Luciferase reporter assay system (Promega) was used to quantitate both reporter genes in each lysate.

Intracellular TNF-α staining

The RAW-TT10 single cell assay has been previously described (22, 32). Briefly, RAW-TT10 cells were transiently transfected by electroporation with constructs expressing dnTLRs. The cells were washed 3 h posttransfection and were allowed to recover for 20 h before stimulation with 25 ng/ml PSM or 2 ng/ml human IL-1β. Intracellular TNF-α was analyzed on a FACScan using CellQuest (Becton Dickinson, Mountain View, CA).

Results and Discussion

Cloning of marine TLR1

Two mouse expressed sequence tags (ESTs) (AA177549 and AA175009) that display homology to human TLR1 were identified using TBLASTN. The first EST, which contains the translational start site and 3’ flanking sequences, was used to design a antisense oligonucleotide primer for reverse transcription of RNA from RAW 264.7 cells. A second antisense primer encompassing the stop codon was used in PCR with a sense primer designed from the sequence of the second EST, and 5’ rapid amplification of the cDNA end was used to isolate the remainder of the coding sequence, which included an in-frame stop codon upstream of the initiating methionine. RT-PCR using primers spanning the entire coding region was used to confirm that a full-length open reading frame had been cloned. Mouse TLR1 shows 74% identity to human TLR1 and 65% identity to mouse TLR6 (data not shown; TLR1 GenBank accession no. AY009154).

TLR2 confers responsiveness to PSM

The recent identification of PSM as a factor that activates monocyteic cells and is secreted from Staphylococci led us to investigate the role of TLRs in mediating the response to PSM (31). HEK 293 cells, which do not respond to PSM, were transiently transfected with constructs expressing murine TLR1, TLR2, TLR4, or TLR6 together with reporter constructs ELAM-1-Luc (35) to measure NF-κB activation, and β-actin Renilla-Luc (36) as a transfection control (Fig. 1A). Full-length TLRs were expressed in each transfection (Fig. 1B). CD14 and MD-2 were included in the transfections shown in Fig. 1A because they facilitate or are required, respectively, for responses to LPS, which was tested in parallel with PSM. IL-1 was used as a positive control for activation because the parental HEK 293 cells respond to IL-1. IL-1 induced NF-κB in all transfectants tested (Fig. 1A). PSM induced the ELAM reporter 12-fold in cells transfected with TLR2, but <1.5-fold in cells transfected with TLR1, TLR4, or TLR6. CD14 enhanced the TLR2-mediated PSM response (Fig. 1C), whereas MD-2 had no effect on the TLR2-mediated PSM response (data not shown), but was required for the LPS response as was reported previously (26, 33). In contrast, LPS responsiveness was clearly detected in cells transfected with TLR4. We did not detect a TLR4-mediated response to PSM, confirming that PSM does not contain trace amounts of LPS. These results demonstrate that expression of TLR2 in HEK 293 cells is sufficient to render them PSM responsive.

Functional interactions between TLR2 and TLR1 or TLR6 in response to PSM

To determine whether other TLR family members might contribute to the TLR2-mediated PSM response, we cotransfected HEK
293 cells with nonsaturating amounts of TLR2 and each of the other TLR clones. CD14 was also included in each transfection. Fig. 2A shows the NF-κB response of cotransfected cells. All of the transfectants expressed equivalent amounts of V5 epitope-tagged TLR2 as judged by Western blots (Fig. 2B), and each of the cotransfected HA-tagged TLR proteins was readily detected (Fig. 2B). IL-1 stimulation resulted in a 4- to 5-fold induction of the reporter construct in all cotransfectants tested. Cells transfected with V5 epitope-tagged TLR2 alone showed a 6-fold induction of the reporter construct in all cotransfectants tested. Cells transfected with HA-TLR2 (0.05 μg), 0.05 μg HA-TLR6, 0.6 μg HA-TLR1, 0.05 μg HA-TLR4, 0.6 μg HA-TLR1-6, and 0.1 μg HA-TLR6-1 were transfected as indicated for each well. We found that the expression patterns of the protein is responsible for its decreased expression with respect to TLR1. dnTLR1, in conjunction with the cytoplasmic domain of either TLR1 or TLR6, interferes with the TLR2-mediated response to PSM. The converse chimeric receptor, TLR6-1, was expressed at similar levels to TLR6 and TLR1-6 (Fig. 2B) but had no effect on the TLR2-mediated PSM response (Fig. 2A). This suggests that both the extracellular and cytoplasmic domains of TLR6 are required to facilitate the response to PSM, although we cannot exclude the possibility that TLR6-1 could be improperly localized within the cell or could be misfolded.

dnTLR2 or dnTLR6 also inhibits the PSM response

To extend our analysis, we compared the ability of TLR1 and of dn forms of MyD88, TLR1, TLR2, TLR4, or TLR6 to inhibit the response to PSM mediated by TLR2 or by TLR2 + TLR6 (Fig. 3A). C3H/HeJ mice express dnTLR4 encoding a single missense mutation that converts a cytoplasmic proline residue to histidine (P712H) (29, 30). The analogous mutation was engineered in TLR2 (P681H), as was described previously (32), and in TLR1 (P678H) and TLR6 (P691H). To better evaluate the inhibition by the dn proteins, we adjusted our transfection conditions such that the PSM response was similar in cells cotransfected with TLR2 and TLR6 to cells expressing TLR2 alone (Fig. 3A). dnMyD88 completely blocked the PSM response in both transfectants (>90% inhibition). dnTLR2 and dnTLR6 blocked the response in TLR2-expressing cells (80% inhibition) but impeded the TLR2 + TLR6-mediated response less efficiently (65 and 50% inhibition, respectively). dnTLR1 was expressed much more poorly than the other dnTLRs (Fig. 3B) but, like wild-type TLR1, dnTLR1 impeded the TLR2-mediated PSM response (55% inhibition). Surprisingly, neither of the TLR1 proteins inhibited the TLR2 + TLR6-mediated PSM response (Fig. 3A). These results indicate that a functional complex between TLR2 and TLR6 is more resistant to inhibition by TLR1, and to a lesser extent by dnTLR6 or dnTLR2, than is a signaling complex by TLR2 alone. This suggests that the relative abundance of these TLRs within a cell is likely to play a critical role in the response to PSM. As a complementary approach, we next expressed dnMyD88, dnTLR2, dnTLR4, or dnTLR6 in the PSM-responsive RAW 264.7 cell clone TT10 (22, 32), which expresses TLR1, TLR2, TLR4, and TLR6 as determined by RT-PCR (data not shown). The construct encoding each dn protein also expresses green fluorescence protein (GFP), permitting transfected cells to be identified by fluorescence. Expression of GFP alone did not inhibit the response to PSM or LPS (Fig. 3C), whereas expression of dnMyD88 blocked both responses. The PSM response was blocked by dnTLR2 and dnTLR6, whereas greater than 80% of cells expressing dnTLR4 responded to PSM. We were unable to test dnTLR1 in this assay because this construct could not be expressed in RAW cells (data not shown). Conversely, the LPS response was completely inhibited by dnTLR4 in RAW cells, whereas dnTLR2 and dnTLR6 blocked the LPS response in <15% of cells. Thus, the TLR signaling complex that recognizes PSM in RAW cells can be inhibited by dnTLR2 and dnTLR6.

The experiments described above indicate that PSM signals through TLR2. Although the transmembrane and cytoplasmic domains of TLR6 and TLR1 are highly conserved, these TLRs enhanced or impeded, respectively, the TLR2-dependent response to PSM. These divergent effects appear to result from differences in

The extracellular domain of TLR1 is sufficient to inhibit the PSM response of TLR2

Murine TLR1 is 65% identical with murine TLR6, and the C-terminal halves of TLR1 and TLR6, consisting of one-fourth of the extracellular domain and the entire cytoplasmic domain, are nearly 90% identical. Because TLR6 enhanced the response of TLR2 to PSM, whereas TLR1 inhibited this response, we wished to determine whether the different responses to the two receptors could be attributed to their divergent extracellular domains. Therefore, we constructed chimeric receptors that fused the extracellular domain of TLR1 to the cytoplasmic domain of TLR6 (TLR1-6) or joined the extracellular domain of TLR6 to the cytoplasmic domain of TLR1 (TLR6-1). The response to PSM was inhibited in cells expressing TLR1-6 and TLR2-V5 (Fig. 2A). TLR1-6 and TLR1 inhibited the TLR2-mediated response to PSM to a similar extent, demonstrating that TLR1 and TLR1-6 are functionally equivalent in these assays. This indicates that the extracellular domain of TLR1, in conjunction with the cytoplasmic domain of either TLR1 or TLR6, interferes with the TLR2-mediated response to PSM. The converse chimeric receptor, TLR6-1, was expressed at similar levels to TLR6 and TLR1-6 (Fig. 2B) but had no effect on the TLR2-mediated PSM response (Fig. 2A). This suggests that both the extracellular and cytoplasmic domains of TLR6 are required to facilitate the response to PSM, although we cannot exclude the possibility that TLR6-1 could be improperly localized within the cell or could be misfolded.

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FIGURE 2. Functional interaction between TLR2 and TLR6 or TLR1. A. HEK293 cells were transiently transfected and stimulated as described in Fig. 1. TLR2-V5 (0.025 μg) and 0.1 μg CD14 were included in each well. HA-TLR2 (0.05 μg), 0.05 μg HA-TLR6, 0.6 μg HA-TLR1, 0.05 μg HA-TLR4, 0.6 μg HA-TLR1-6, and 0.1 μg HA-TLR6-1 were transfected as indicated for each well. We found that the expression patterns of the chimeric proteins reflected the levels of the amino-terminal donor. TLR1-6 was poorly expressed, like TLR1, indicating that the extracellular domain of the protein is responsible for its decreased expression with respect to TLR6. Similarly, TLR6-1 was expressed as a doublet, characteristic of TLR6. RLU represents the mean ± SD of triplicate wells. Similar results were obtained in four separate experiments. B. Western blots of the lysates shown in A. Equal volumes of each lysate were loaded on separate gels and immunoblotted with anti-V5 or anti-HA Abs. The positions of prestained molecular mass markers are indicated.

As a complementary approach, we next expressed dnMyD88, dnTLR2, dnTLR4, or dnTLR6 in the PSM-responsive RAW 264.7 cell clone TT10 (22, 32), which expresses TLR1, TLR2, TLR4, and TLR6 as determined by RT-PCR (data not shown). The construct encoding each dn protein also expresses green fluorescence protein (GFP), permitting transfected cells to be identified by fluorescence. Expression of GFP alone did not inhibit the response to PSM or LPS (Fig. 3C), whereas expression of dnMyD88 blocked both responses. The PSM response was blocked by dnTLR2 and dnTLR6, whereas greater than 80% of cells expressing dnTLR4 responded to PSM. We were unable to test dnTLR1 in this assay because this construct could not be expressed in RAW cells (data not shown). Conversely, the LPS response was completely inhibited by dnTLR4 in RAW cells, whereas dnTLR2 and dnTLR6 blocked the LPS response in <15% of cells. Thus, the TLR signaling complex that recognizes PSM in RAW cells can be inhibited by dnTLR2 and dnTLR6.

The experiments described above indicate that PSM signals through TLR2. Although the transmembrane and cytoplasmic domains of TLR6 and TLR1 are highly conserved, these TLRs enhanced or impeded, respectively, the TLR2-dependent response to PSM. These divergent effects appear to result from differences in
their extracellular domains, which may reflect differences in interaction between these receptors and TLR2, in ligand binding, or in both. We have also found that dnTLR6 impedes the TLR2-mediated response to peptidoglycan, intact Gram-positive bacteria, and yeast, but not the TLR2-mediated response to lipopolipides in RAW cells, suggesting that TLR6 may interact functionally with TLR2 in response to certain ligands but not to others (37). Furthermore, our results suggest that the ratio of different TLRs within a cell may modify the response to a given ligand.

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References


