Importance of extra- and intracellular domains of TLR1 and TLR2 in NFκB signaling

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Recognition of ligands by toll-like receptor (TLR) 2 requires interactions with other TLRs. TLRs form a combinatorial repertoire to discriminate between the diverse microbial ligands. Diversity results from extracellular and intracellular interactions of different TLRs. This paper demonstrates that TLR1 and TLR2 are required for ara-lipo-arabinomannan– and tripalmitoyl cysteinyl lipopeptide–stimulated cytokine secretion from mononuclear cells. Confocal microscopy revealed that TLR1 and TLR2 co-translationally form heterodimeric complexes on the cell surface and in the cytosol. Simultaneous cross-linking of both receptors resulted in ligand-independent signal transduction. Using chimeric TLRs, we found that expression of the extracellular domains along with simultaneous expression of the intracellular domains of both TLRs was necessary to achieve functional signaling. The domains from each receptor did not need to be contained within a single contiguous protein. Chimeric TLR analysis further defined the toll/IL-1R domains as the area of crucial intracellular TLR1–TLR2 interaction.

Introduction

The toll-like receptor (TLR) family of proteins is an integral part of the human innate immune system (Means et al., 2000; Medzhitov and Janeway, 2000; Akira et al., 2001). The function of the innate immune system is thought to be the recognition of invading pathogens, the activation of inflammation to control pathogen spread, and the subsequent activation of an adaptive immune response specifically directed to the elimination of the pathogen.

To date, at least 10 different human TLRs have been identified (Means et al., 2000; Medzhitov and Janeway, 2000; Akira et al., 2001), and at least two additional mouse TLRs are present in the GenBank/EMBL/DDBJ database. Moreover, according to some authors, more TLRs will be found in the future (Takeda et al., 2003). TLRs are expressed on cells of the immune system, including monocytes, macrophages, dendritic cells, and lymphocytes; however, their expression is also observed in other cells, including vascular endothelial cells, lung and intestinal epithelial cells, cardiac myocytes, and adipocytes (Akira et al., 2001).

TLRs distinguish a great variety of microbial ligands, including molecules conserved within the major groups of pathogens such as Gram-positive and Gram-negative bacteria, mycobacteria, spirochetes, viruses, and fungi (Means et al., 2000; Akira et al., 2001).

All identified TLRs are type I transmembrane proteins. Their extracellular domains contain leucine-rich repeats that may participate in ligand recognition or act as structural scaffolds for ligand recognition sites (Lien et al., 1999, 2000). The intracellular domains contain regions that are highly homologous to the intracellular domain of the IL-1R, and these regions are referred to as toll/IL-1R (TIR) domains (Means et al., 2000; O'Neill, 2000; Akira et al., 2001). The intracellular signaling pathway is known to activate mainly the nuclear factor kappa B (NFκB) transcription factor (Medzhitov et al., 1998; Muzio et al., 1998; Zhang et al., 1999), which in turn triggers the expression of many pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 and leads to maturation of antigen-presenting cells (Akira et al., 2001).

One of the most studied TLRs, TLR2, has been shown to recognize a wide array of microbial molecules (Aliprantis et al., 1999; Brightbill et al., 1999; Lien et al., 1999; Means et al., 1999a; Takeuchi et al., 1999; Underhill et al., 1999; Yoshimura et al., 1999). For example, TLR2 participates in recognition of Gram-positive bacteria, peptidoglycan, lipopeptides, and zymosan from yeast cell wall; moreover, leptospiral lipopolysaccharide (LPS) exerts its activity in a TLR2-dependent manner (Werts et al., 2001). TLR2 is also involved in recognition of viable *Mycobacterium tuberculosis* as well as recognition of lipoarabinomannan from rapidly growing mycobacteria (Means et al., 1999a,b). Moreover, recent reports revealed numerous additional ligands recognized via TLR2 (for review see Lien and Ingalls, 2002).

Recently, it has been shown that TLRs interact with other different coreceptors as well as with each other (Ozinsky et al., 2000; Wyllie et al., 2000; Akira et al., 2001; Hajjar et al., 2001; Imler and Hoffmann, 2001; Takeuchi et al., 2001, 2002; Alexopoulou et al., 2002; Latz et al., 2002). TLR4 interacts with at least two other proteins, CD14, MD-2 in the human macrophage, and human embryonic kidney 293 (HEK293) cell response to LPS (Imler and Hoffmann, 2001; Latz et al., 2002).

In recent analyses of TLR1 and TLR2 knockout mice, these receptors were shown to cooperate in recognizing *Borrelia burgdorferi* outer-surface protein A lipoprotein OspA

(Alexopoulou et al., 2002). Knockout experiments have also suggested that TLR1 and TLR2 cooperate in the recognition of the 19-kD mycobacterial lipopeptide and several synthetic triacylated lipopeptides (Takeuchi et al., 2002).

In this report, we define the nature of TLR1 and TLR2 interaction. Antibody cross-linking analyses suggest that cooperation between TLR1 and TLR2 on the cell surface of normal human peripheral blood mononuclear cells (PBMCs) leads to the activation of pro-inflammatory cytokine secretion. Using fluorescent protein–tagged TLR1 and TLR2, our confocal microscopy results indicate colocalization between TLR1 and TLR2. In addition, we show that the simultaneous expression of the extracellular and intracellular domains of both TLR1 and TLR2 is essential for ara-lipoarabinomannan (araLAM) recognition. Thus, our analyses suggest that TLR1 and TLR2 associate functionally in a signaling complex.

Results

Anti-TLR1 and -TLR2 mAbs block cytokine production in PBMCs stimulated with AraLAM and tripalmitoyl cysteinyl lipopeptide (Pam$_3$CSK$_4$)

Genetic studies suggest that TLR2 is required for the recognition of a diverse group of microbial ligands, including araLAM, Pam$_3$CSK$_4$, and zymosan. The role of TLR1 and

Figure 1. **Anti-TLR1 and -TLR2 mAbs block the IL-6 response of human PBMCs to araLAM and Pam$_3$CSK$_4$.** Fresh human PBMCs were preincubated for 30 min with anti-TLR1 mAb (GD2.F4) or anti-TLR2 mAb (11G7) before adding the stimulants. After 18 h of stimulation with 1 μg/ml araLAM, 100 ng/ml Pam$_3$CSK$_4$ (A), 10 μg/ml zymosan, or 10 ng/ml phenol LPS (B), supernatants were harvested and IL-6 was measured by ELISA.
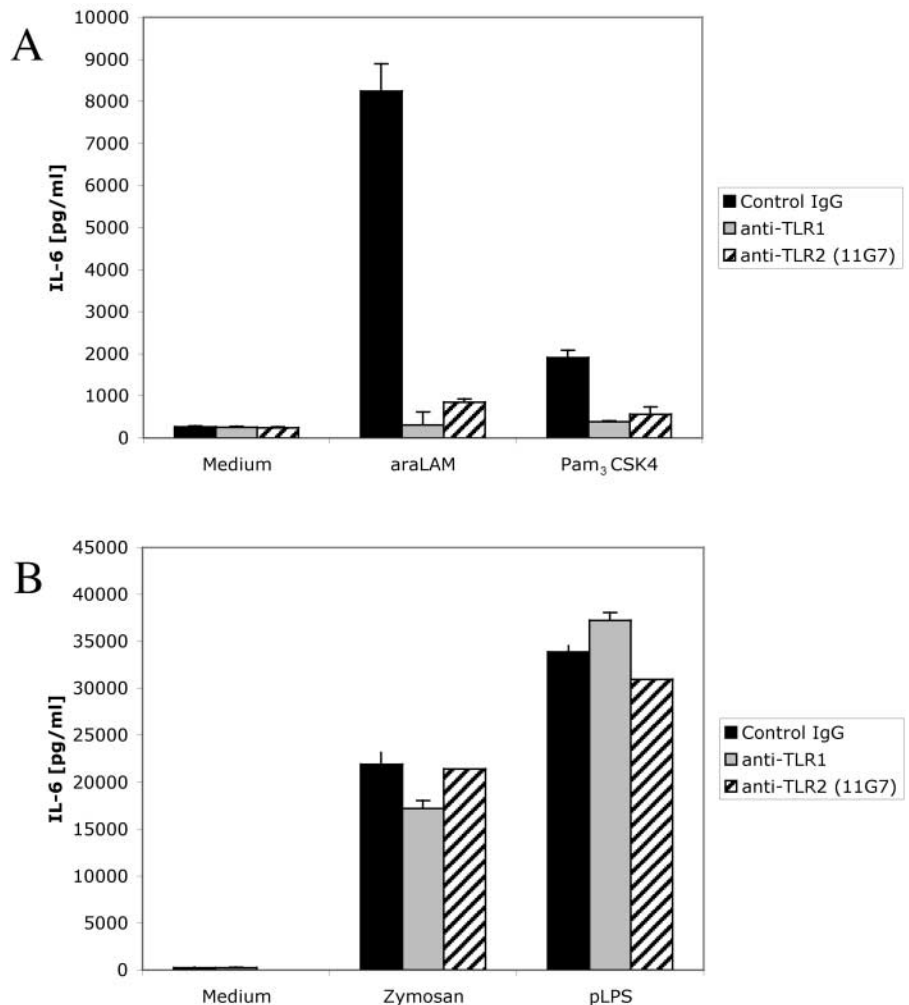
TLR2 in the response of normal human PBMCs to these ligands was analyzed in antibody-blocking experiments. Pretreatment of PBMCs with either anti-TLR1 or -TLR2 (11G7) antibodies blocked IL-6 cytokine production in response to araLAM and Pam_3CSK_4 (Fig. 1 A). In contrast, both antibodies failed to inhibit the IL-6 cytokine secretion after stimulation with zymosan. Moreover, as expected, addition of anti-TLR1 or -TLR2 antibodies to PBMCs did not exert any blocking effect to LPS stimulation (Fig. 1 B). These results suggest that TLR1 and TLR2 both participate in the response to araLAM and Pam_3CSK_4.

TLR1 cooperates with TLR2 on the cell surface to initiate signal activation: antibody cross-linking experiments

Both antibody-blocking experiments and TLR knockout animal analyses suggest that TLR2 signaling involves cooperation with other TLRs, particularly TLR1 and TLR6. Thus, a functional signal transduction complex seems to require elements of both receptors. We hypothesized that cross-linking TLR1 and TLR2 might mimic their engagement by a ligand and thus activate signal transduction and cytokine secretion. We analyzed the ability of plate-bound antibodies to TLRs to activate normal human cells. PBMCs were incubated on sterile tissue culture plates coated with mAbs to TLR1 (GD2.F4), TLR2 (2.1) alone, or in combination. We observed that a combination of anti-TLR1 and -TLR2 specific mAbs activated IL-8 secretion from PBMCs in a dose-dependent manner. It should be noted that the aggregation of TLRs by soluble antibodies did not activate these cells to secrete cytokines, whereas the same anti-TLR antibodies did activate cytokine secretion when they were prebound to tissue culture plates upon which the TLR-expressing cells were cultured. Individually, neither anti-TLR1, anti-TLR2, nor
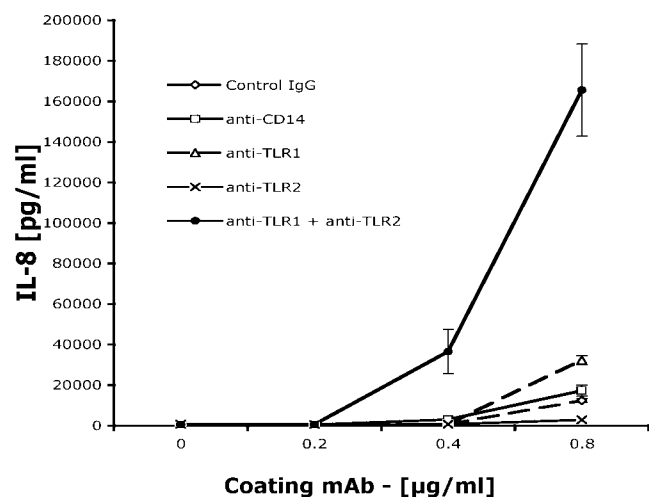

Figure 2.   TLR1 cooperates with TLR2 on the cell surface to initiate IL-8 secretion. Control antibody (OKT8), anti-CD14 (26ic), anti-TLR1 (GD2.F4), and anti-TLR2 (TL2.1) mAbs were immobilized on sterile high protein binding polystyrene 96-well plates from 0.2 to 0.8 µg/ml concentration. After blocking and washing, 7×10^5 human PBMCs were added to the antibody-coated wells in the presence of 5 µg/ml polymyxin B. After 18 h of incubation, supernatants were harvested and levels of IL-8 were measured by ELISA.

isotype control antibodies alone were sufficient to elicit IL-8 secretion (Fig. 2).

TLR1 colocalizes with TLR2 inside and on the surface of cells

The antibody blocking and cross-linking experiments suggested that TLR1 and TLR2 can associate in a functional signaling complex. Therefore, we investigated the possible interaction between TLR1 and TLR2 by confocal microscopy. HEK cells were stably transfected with TLR1 and TLR2 tagged at their COOH termini with YFP or CFP. This method of epitope tagging appears to have no effect on TLR function (unpublished data; Latz et al., 2002).

Cells transfected with $TLR2^{YFP}$ or $TLR4^{YFP}$ fusion proteins alone displayed a predominant membrane localization of the receptors. In contrast, $TLR1^{YFP}$-expressing cells displayed a diffuse pattern of TLR1 distribution (Fig. 3 A). Cotransfection of cells with $TLR1^{YFP}$ and $TLR2^{CFP}$ fusion proteins resulted in aggregation of TLR1 and TLR2 both on the surface and inside the cells (Fig. 3 B). Antibody-induced aggregation of TLR2 on the surface of the cells led to coaggregation of TLR1 (Fig. 3 C), whereas antibody-induced surface aggregation of MHC I did not coaggregate TLR1 or TLR2 (Fig. 3 D). As a further control, we surface aggregated TLR2 in $TLR4^{YFP}$-expressing cells. Capping of TLR2 (Fig. 3 E, red) did not lead to coaggregation of TLR4 (Fig. 3 E, green), indicating the specificity of the observed coaggregation of TLR2 and TLR1. These results suggest that TLR1 and TLR2 are preassembled into heteromultimeric complexes on the cell surface.

Dominant-negative TLR2 and TLR1 inhibit response to araLAM and zymosan: loss of function analyses

HEK293 cells constitutively express a cytoplasmic pool of TLR1 that can be detected by fluorescent staining analysis of permeabilized cells and TLR1 mRNA-specific RT-PCR (unpublished data). Transfection of these cells with wild-type TLR2 is sufficient to confer responsiveness to both araLAM and zymosan (Kurt-Jones et al., 2002). To investigate the role of TLR2 and TLR1 in response to araLAM and zymosan, various mutants of TLR2 and TLR1 proteins were generated. Based on a dominant-negative mutation of the TIR domain of TLR4 found in the C3H/HeJ mice (Poltorak et al., 1998; Hoshino et al., 1999), the corresponding homologous conserved proline within the TIR domain of TLR2 protein was mutated to histidine TLR2 P681H. A second TLR2 mutant was generated in which a stop codon was introduced at aa 643, resulting in the deletion of the conserved intracellular TIR domain TLR2ΔTIR. Co-transfection experiments were performed using increasing amounts of TLR2 mutant proteins with a constant amount of TLR2 wild-type protein. The response of transfected cells to araLAM and zymosan was determined. Transient transfection of HEK293 cells stably expressing CD14 on the surface (HEK293-CD14) with increasing amounts of TLR2 mutants resulted in dose-dependent impairment of NFκB activation in response to both araLAM and zymosan (Fig. 4, A and B).

To analyze the role of TLR1, a cytoplasmic deletion mutant (TLR1Δcyt) was tested (Wyllie et al., 2000). In this

A Single expression of TLR1, TLR2, and TLR4

B Co-expression of TLR2 and TLR1

C TLR2 + TLR1: Surface antibody-patching of TLR2

D TLR2 + TLR1: Surface antibody-patching of MHC I

E TLR2 + TLR4: Surface antibody-patching of TLR2

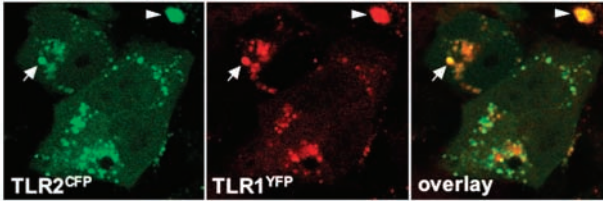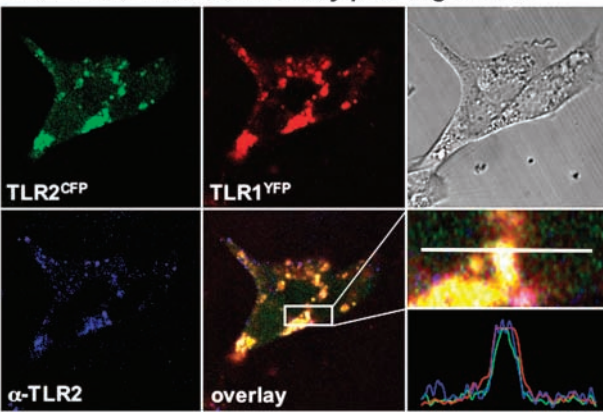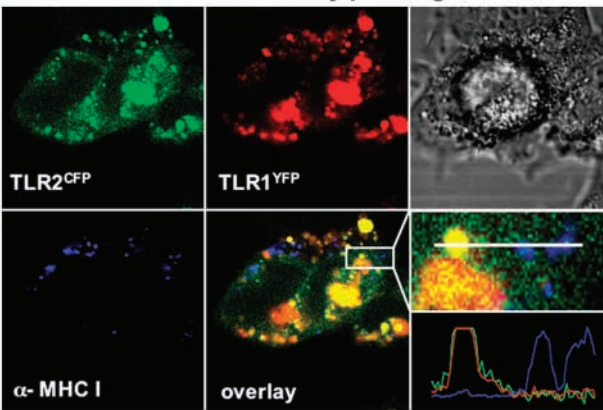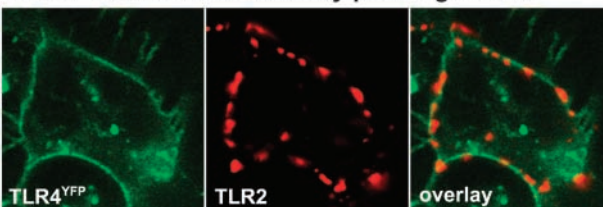Figure 3. **Confocal imaging of live HEK cells expressing fluorescent protein–tagged TLR1, TLR2, and TLR4.** (A) Confocal microscopy of HEK cells stably expressing TLR1^{YFP}, TLR2^{YFP}, or TLR4^{YFP}. HEK cells stably expressing either TLR1^{YFP}, TLR2^{YFP}, or TLR4^{YFP} were grown on glass-bottom tissue culture dishes, and living cells were analyzed by confocal microscopy at 37°C without any manipulation. Shown are confocal sections in representative cells. (B) TLR1 colocalizes with

construct, the intracellular protein sequence was deleted immediately after the transmembrane region, thus the TLR1 protein lacked the entire cytoplasmic domain (including the TIR domain). This TLR1Δcyt truncation construct, when transfected together with TLR2 wild-type protein into HeLa cells, has been shown to abrogate the response to *Salmonella minnesota* LPS preparation (Wyllie et al., 2000). Transfection of HEK293-CD14 cells with increasing amounts of TLR1Δcyt truncation construct resulted in a dose-dependent decrease in NFκB activation in response to both araLAM and zymosan (Fig. 4 C). Thus, dominant-negative forms of both TLR1 and TLR2 inhibit the NFκB response to araLAM and zymosan.

Neither the extracellular domain nor the intracellular domain of TLR2 alone is sufficient to activate an NFκB-dependent response to araLAM, Pam₃CSK₄, or zymosan

To further understand the role of TLR1 and TLR2 in ligand recognition, various types of TLR chimeric (fusion) proteins were generated by domain swapping of the extracellular and intracellular domains of TLR1, TLR2, and TLR4. Chimeric TLR proteins were transfected into HEK293-CD14 cells, and the response of the cells to TLR ligands was determined. The expression level of chimeric TLR protein constructs on the cell surface was comparable to the wild-type TLR protein expression (unpublished data). Cells transfected with a

TLR2 in resting HEK cells stably expressing both TLR1^{YFP} and TLR2^{CFP}. HEK cells stably expressing both TLR1^{YFP} and TLR2^{CFP} were grown on glass-bottom tissue culture dishes, and living cells were analyzed as described in A. Left, Localization of TLR2; center, distribution of TLR1; right, overlay of the left and middle images. In these cells without any manipulation and without ligand stimulation, TLR1 colocalizes with TLR2 (arrows and arrowheads). Representative confocal sections of cells are shown. (C) Surface cross-linking of TLR2^{CFP} by antibody leads to coaggregation of TLR1^{YFP} on the cell surface. Double transfectants of TLR2^{CFP} and TLR1^{YFP} were surface stained with anti-TLR2 antibody (clone TL2.1), and further cross-linked with Alexa® 647–conjugated polyclonal rabbit anti–mouse antibody (2° Ab^{Alexa674}, shown in blue) on ice. After incubation for 10 min at 37°C to induce capping, living cells were analyzed by confocal microscopy, and representative cells are shown. Left, distribution (green) and surface labeling of TLR2^{CFP} with anti-TLR2 + 2° Ab^{Alexa674} (blue); middle, TLR1^{YFP} distribution (red) and the overlay image of all three colors. The transmitted light image and the fluorescence intensities of a representative section of the overlay image is shown in the right panels. Surface cross-linking of TLR2^{CFP} by antibody (blue) induced coaggregation of TLR2^{CFP} and TLR1^{YFP}. (D) Antibody-induced capping of MHC I does not induce coaggregation with TLR2^{CFP} or TLR1^{YFP}. HEK cells stably expressing TLR2^{CFP} and TLR1^{YFP} were stained for MHC I using a mouse monoclonal anti-HLA I antibody, counterstained and processed as described in B. Distribution of TLR2^{CFP} (green) and surface labeling of MHC class I with anti-MHC I + 2° Ab^{Alexa674} (blue) are shown in the left panels; the middle panels demonstrate TLR1^{YFP} localization (red) and the overlay of all three colors. The transmitted light image and fluorescence intensities of a representative section of the overlay image are displayed in the right panels. MHC I cross-linking (blue) does not co-patch either TLR2^{CFP} or TLR1^{YFP}. (E) Antibody-induced capping of TLR2 does not coaggregate TLR4. Stably transfected HEK-TLR4^{YFP} cells (green) were transiently transfected with TLR2. Antibody-induced capping of TLR2 was induced by sequentially incubating the cells with anti-TLR2 (clone TL2.1) and anti-mouse IgG (Alexa® 647 conjugated; red). Aggregation of TLR2 did not influence the expression pattern of TLR4.
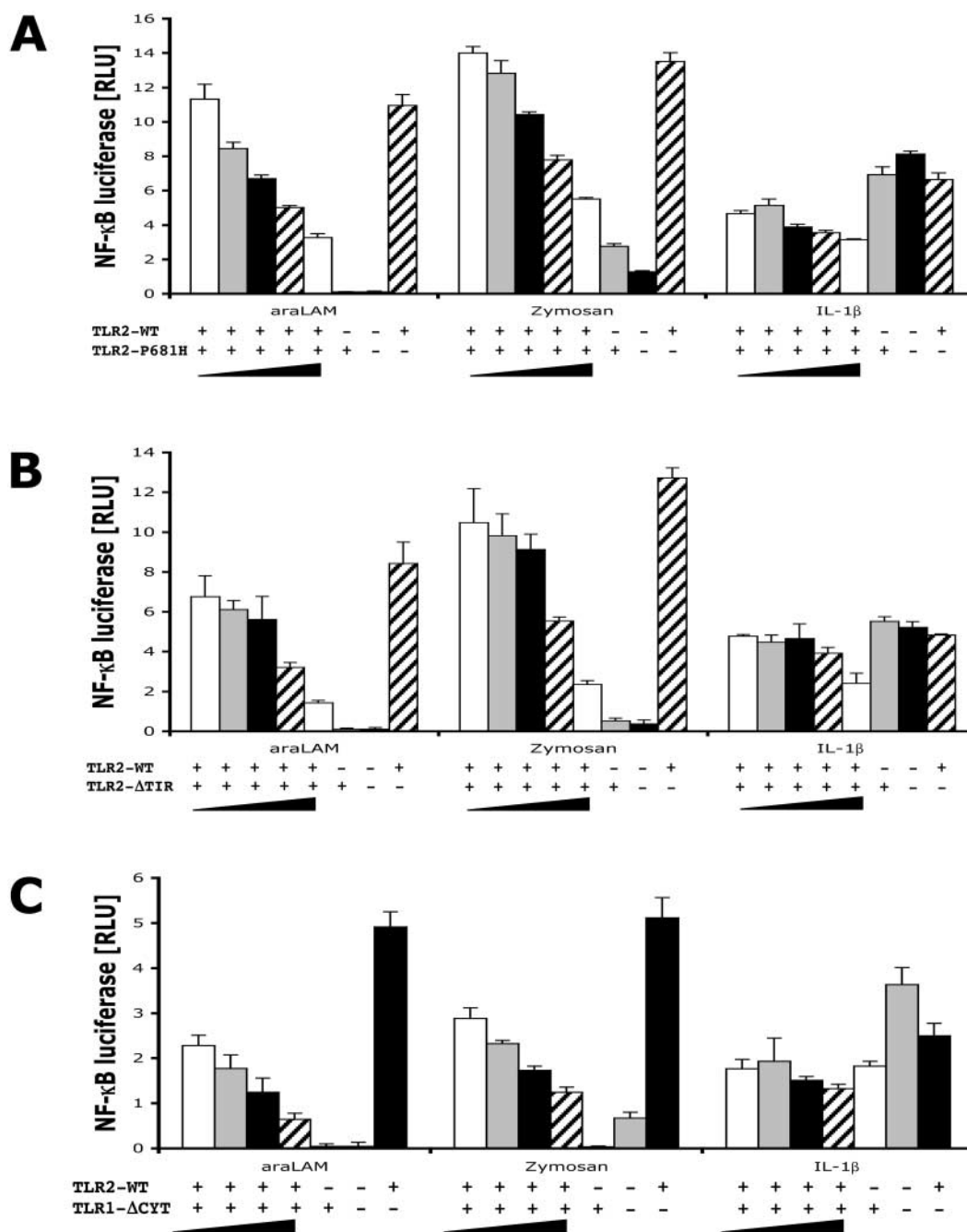
Figure 4.   **TLR2 and TLR1 mutants inhibit NFκB activation in HEK 293 cells in stimulation with araLAM and zymosan.** (A) The response of HEK293-CD14 cells to araLAM and zymosan is inhibited by a single point mutated TLR2 construct, TLR2-P681H. HEK293-CD14 cells were cotransfected with 5 ng TLR2-WT DNA and increasing amounts of TLR2-P681H (from 5 to 200 ng DNA). After 6 h of stimulation with 1 μg/ml araLAM and 10 μg/ml zymosan, cells were lysed and NFκB luciferase reporter gene activity was measured. IL-1β was used as a positive control. Luciferase activity is expressed in normalized RLU as the ratio of NFκB-dependent firefly luciferase activity to NFκB-independent renilla luciferase activity. Data shown are the mean ± SD of triplicate wells. (B) The response of HEK293-CD14 cells to araLAM and zymosan is inhibited by expression of a TLR2 mutant missing the TIR domain TLR2-ΔTIR. HEK293-CD14 cells were cotransfected with 5 ng TLR2-WT DNA and increasing amounts of TLR2-ΔTIR (from 5 to 200 ng DNA). After 6 h of stimulation with 1 μg/ml araLAM and 10 μg/ml zymosan, cells were lysed and NFκB luciferase reporter gene activity was measured. IL-1β was used as a positive control. (C) The response of HEK293-CD14 cells to araLAM and zymosan is inhibited by expression of a cytoplasmic deletion mutant of TLR1, TLR1-Δcyt. HEK293-CD14 cells were cotransfected with 5 ng TLR2-WT DNA and increasing amounts of TLR1-Δcyt (from 5 to 200 ng DNA). After 6 h of stimulation with 1 μg/ml araLAM and 10 μg/ml zymosan, cells were lysed and NFκB luciferase reporter gene activity was measured. IL-1β was used as a positive control.

TLR [2–1] chimeric protein (consisting of the extracellular domain of the TLR2 fused to the intracellular domain of TLR1) did not respond to stimulation with araLAM, Pam₃CSK₄, and zymosan. Similarly, the reciprocal construct

TLR [1–2] (with the extracellular domain of TLR1 fused to the intracellular domain of TLR2) was not able to elicit a response to any of these stimulants (Fig. 5, A and B). These results suggested that neither the extracellular domain of
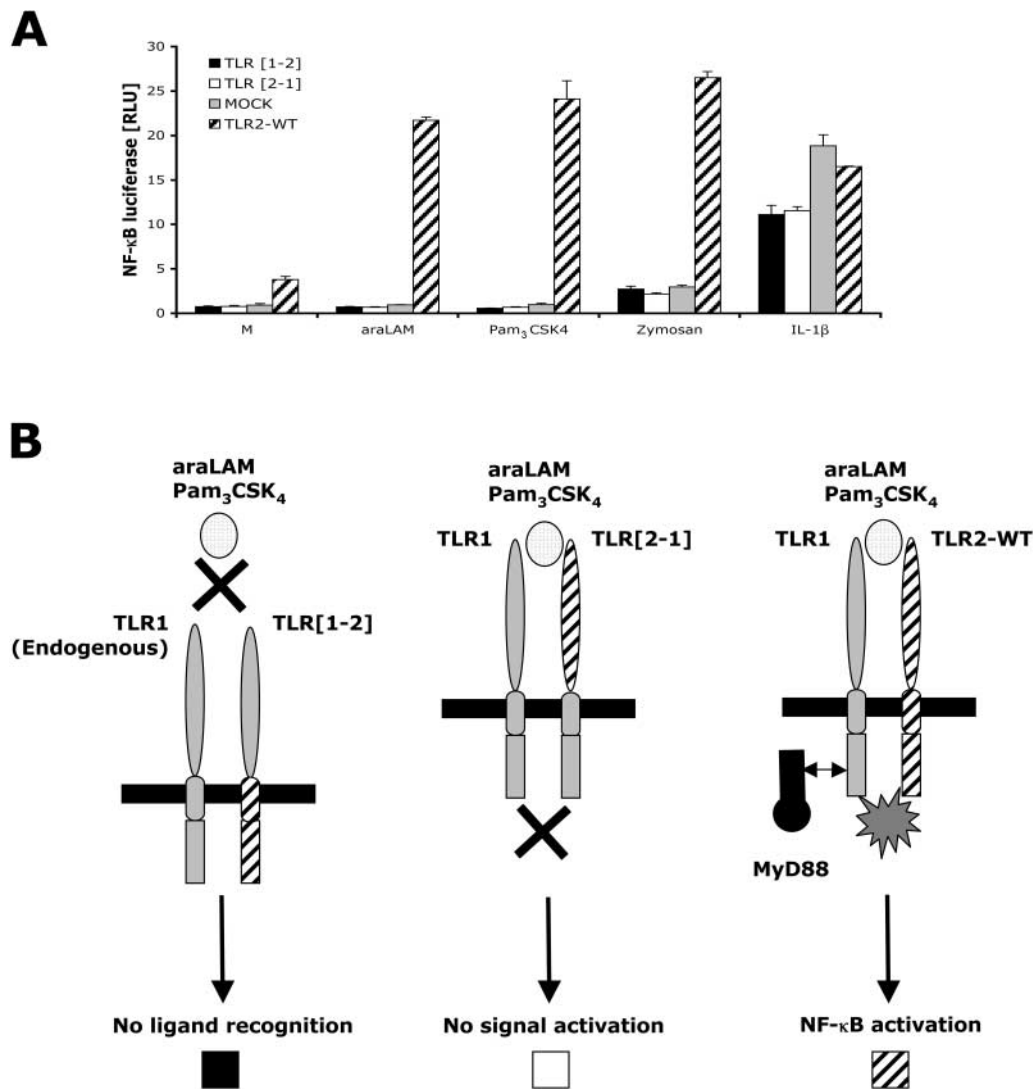
Figure 5. **Neither the intracellular nor the extracellular domain of TLR2 is sufficient to confer NFκB signal activation.** (A) HEK293-CD14 cells were transfected with either TLR [1–2] or TLR [2–1] DNA encoding chimeric protein and wild-type TLR2 DNA. After 6 h of stimulation with 1 μg/ml araLAM, 100 ng/ml Pam₃CSK₄, or 10 μg/ml zymosan, cells were lysed and NFκB luciferase reporter gene activity was measured. IL-1β was used as a positive control. (B) A schematic model of separate TLR1 and TLR2 chimeric protein transfection. With TLR1 endogenously expressed, transfection only of the intracellular portion of TLR2 (TLR [1–2]) does not confer responsiveness as a result of missing TLR2 extracellular domain causing ligand recognition failure. Transfection with only the extracellular portion of TLR2 (TLR [2–1]) is not sufficient to confer responsiveness because the TLR1 intracellular domain lacks the TLR2 intracellular domain for effective initiation of signaling pathways. With TLR1 endogenously present, transfection of TLR2-WT protein confers responsiveness by providing both extra- and intracellular domains needed for ligand recognition and signal activation respectively.

TLR2 nor the intracellular domain of TLR2 alone was sufficient to confer signaling in response to tested ligands. Similar results were obtained using TLR [1–4], TLR [2–3], TLR [4–1], and TLR [2–5] chimeric fusion proteins including their combinations (Table I).

Both extracellular and intracellular domains of TLR1 and TLR2 are required for signaling in response to araLAM or Pam₃CSK₄

Neither the TLR [1–2] nor the TLR [2–1] chimeric proteins alone were sufficient to confer responsiveness to TLR2 ligands. Therefore, we examined the ability of combinations of these chimeric fusion proteins to signal. HEK293-CD14 cells were cotransfected with TLR [1–2] and TLR [2–1]

fusion proteins, and the response of cells to araLAM, Pam₃CSK₄, and zymosan was tested. This combination of chimeric receptors was sufficient to confer responsiveness to araLAM and Pam₃CSK₄. Interestingly, the combination of TLR [1–2] and TLR [2–1] did not confer responsiveness to zymosan (Fig. 6, A and B). These results suggest that both intracellular and extracellular domains of TLR1 and TLR2 are required in recognition of araLAM and Pam₃CSK₄. We hypothesize that within the intracellular domains of both receptors, dimerization of TIR domains is essential for subsequent signal activation.

To analyze the role of the intracellular domain in signaling, another TLR fusion protein was generated (TLR [2–1 TIR]) in which the TIR domain of TLR2 was replaced with

Table I. **Transfection of HEK-CD14 cells with various types of TLR chimeric constructs**

Transfected vectors	araLAM	Zymosan	IL-1β
TLR [1-2]	-	-	+
TLR [2-1]	-	-	+
TLR [2-1 TIR]	-	-	+
TLR [1-4]	-	-	+
TLR [4-1]	-	-	+
TLR [2-3]	-	-	+
TLR [2-4]	-	-	+
TLR [2-5]	-	-	+
TLR [1-2] + TLR [2-1]	+	-	+
TLR [1-2] + TLR [2-1 TIR]	+	-	+
TLR [1-2] + TLR [2-3]	-	-	+
TLR [1-2] + TLR [2-4]	-	-	+
TLR [1-2] + TLR [2-5]	-	-	+

the TIR domain of TLR1. Transfection with the TLR [2–1 TIR] construct alone did not confer responsiveness to araLAM and zymosan (Table I). HEK293-CD14 cells cotransfected with TLR [1–2] fusion protein and TLR [2–1 TIR] fusion protein were activated in response to araLAM and Pam$_3$CSK$_4$, but not to zymosan (Fig. 7, A and B). These

data suggest that the heterodimerization of the TIR domains of TLR1 and TLR2 is essential for the signaling in response to some (but not all) TLR2 ligands (i.e., araLAM and Pam$_3$CSK$_4$, but not zymosan).

Discussion

Together, our data support the hypothesis that optimal activation of cytokine secretion by TLRs involves TLR1 and TLR2 clustering on the cell surface. Work by several authors has suggested that an association between TLR1 and TLR2 was necessary for activation of cytokine secretion by certain ligands. Experiments with knockout mice have confirmed a requirement for both TLRs in the response to certain TLR2 ligands. By mAb blocking, we have demonstrated that both TLR1 and TLR2 are required for extracellular recognition of araLAM and Pam$_3$CSK$_4$. This dual requirement (for both TLR1 and TLR2) is confirmed by transfection of cells with dominant-negative mutants of either TLR1 or TLR2. Antibody cross-linking experiments demonstrated that aggregation of TLR1 and TLR2 into the same domain was sufficient to induce signal transduction events independently of ligand recognition. Interestingly, transfection of cells with labeled TLRs demonstrated the surprising finding that
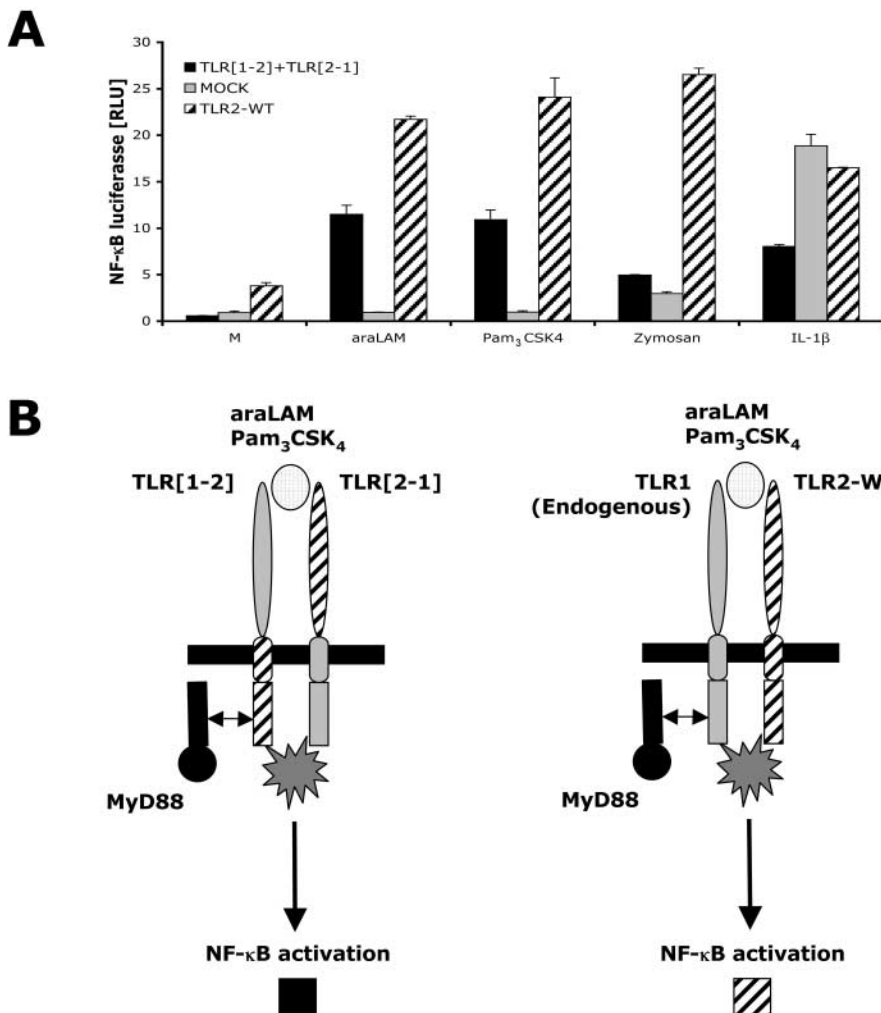

Figure 6. **NFκB signal activation requires both extracellular and intracellular domains of TLR1 and TLR2.** (A) HEK293-CD14 cells were cotransfected with TLR [1–2] and TLR [2–1] DNA encoding chimeric proteins or wild-type TLR2 DNA. After 6 h of stimulation with 1 μg/ml araLAM, 100 ng/ml Pam$_3$CSK$_4$, or 10 μg/ml zymosan, cells were lysed and NFκB luciferase reporter gene activity was measured. IL-1β was used as a positive control. (B) A schematic model of cotransfection of TLR [1–2] and TLR [2–1] chimeric proteins. Co-transfection of both chimeric proteins confers responsiveness as a result of concomitant expression of both intracellular and extracellular domains of TLR1 and TLR2. With TLR1 endogenously present, transfection with TLR2-WT alone is sufficient to confer responsiveness.

Figure 7. **Heterologous expression of the extracellular domains of TLR1 and TLR2 together with heterologous expression of the TIR domains of TLR1 and TLR2 is sufficient for NFκB signal activation.** (A) HEK293-CD14 cells were cotransfected with TLR [1–2] and TLR [2–1 TIR] DNA encoding chimeric proteins or wild-type TLR2 DNA. After 6 h of stimulation with 1 μg/ml araLAM, 100 ng/ml Pam$_3$CSK$_4$, or 10 μg/ml zymosan, cells were lysed and NFκB luciferase reporter gene activity was measured. IL-1β was used as a positive control. (B) A schematic model of cotransfection of TLR [1–2] and TLR [2–1 TIR] chimeric proteins. Co-transfection of TLR [1–2] chimeric protein containing the entire intracellular domain of TLR2 (including the TIR domain) with the TLR [2–1 TIR] chimeric protein containing the TIR domain of TLR1, confers responsiveness. The transfected cells express the extracellular domains of TLR1 and TLR2 (ligand recognition) as well as both the TIR domains of TLR1 and TLR2 (signal transduction). With TLR1 endogenously present, transfection with TLR2-WT alone is sufficient to confer responsiveness.
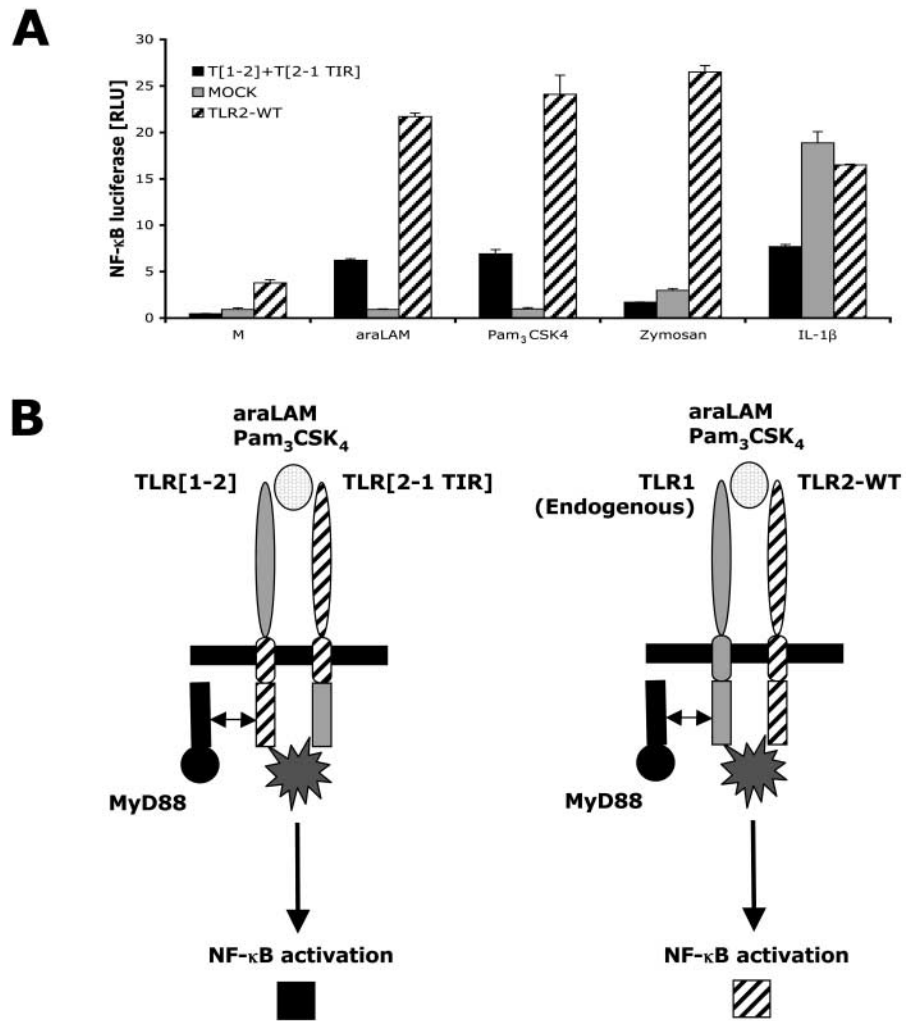
TLR1 and TLR2 are associated before ligand stimulation or cross-linking. Using confocal microscopy and expressing TLR1 in one color and TLR2 in another, we found both receptors are expressed on the surface of dual receptor-transfected cells and are in close association without ligand or antibody cross-linking.

Overexpression of TLR1 in HEK cells produced a diffuse pattern of distribution, TLR1 being mainly localized inside the cells with a minimal cell membrane involvement. On the other hand, expression of TLR2 resulted in a more prominent cell membrane localization (Fig. 3 A). Overexpressing TLR1 in a TLR2-positive cell line produced aggregation of TLR1 and TLR2 inside the cells as well as in the cell membrane (Fig. 3 B). A similar colocalization pattern was observed in several cell clones expressing fluorescent protein–labeled TLR1 and TLR2. TLR1 and TLR2 were present in a preassembled complex, and as such, are transported to the cell membrane. This interaction was independent of ligand, and the data suggest that these receptors heterodimerize before expression on the cell surface, perhaps by a cotranslational association of the two receptors. The morphologic redistribution of fluorescent TLR1 and TLR2 molecules in the double-labeled cell line (HEK-TLR1^{YFP} + TLR2^{CFP}) was also functionally reflected in 2–3-fold higher basal IL-8 cytokine level secretion when compared with cells transfected with TLR1^{YFP} or TLR2^{CFP} alone. Similarly, an overexpression of TLR4 has been observed to cause higher basal NFκB activation and cytokine production (Medzhitov et al., 1997; Kurt-Jones et al., 2002). However, the levels of basal cytokine secretion of TLR4 overexpressing HEK cells are ~5–10 times higher than the double-labeled cell lines used in our experiments. Our confocal data are consistent with the work of Ozinsky et al. (2000), where TLR2 and TLR1 were found to be expressed on the cell surface, and the work of Takeuchi et al. (2002), where association of TLR1 and TLR2 was demonstrated by coimmunoprecipitation experiments, supporting the idea of ligand-independent association of TLR1 and TLR2.

Experiments with chimeric proteins indicated that neither expression of TLR1 nor expression of TLR2 on the surface by itself was sufficient for cytokine induction by araLAM or Pam$_3$CSK$_4$. The easiest explanation for this result is to postulate that araLAM and Pam$_3$CSK$_4$ must bind to the NH$_2$-terminal portions of both TLR1 and TLR2. Each TLR may contribute to a combined ligand-binding site for araLAM and Pam$_3$CSK$_4$. Interestingly, use of COOH-terminal chimeric proteins revealed that the COOH termini of both TLR1 and TLR2 are necessary for ligand-mediated induction of cytokines. This result suggests that either TLR1 is associated with different signaling or adaptor proteins than

TLR2, or that both proteins interact with one another, and this interaction is required for the association of adaptor molecules. Further experiments will be necessary to define the different associated molecules, but these experiments underscore the fact that (1) TLR1–TLR2 interactions occur naturally without ligand binding; (2) certain ligands appear to be able to bind to both TLR1 and TLR2 extracellularly; and (3) intracellular interaction between TLR1 and TLR2 is necessary, suggesting that different adaptor or signaling proteins are associated with different TLRs.

Several recent analyses performed in knockout mice confirmed the cooperation of TLR2 with other TLRs, namely TLR1 and TLR6 (Hajjar et al., 2001; Takeuchi et al., 2001, 2002; Alexopoulou et al., 2002). These analyses suggest that both TLR1 and TLR2 are involved in recognition of a native mycobacterial peptide as well as several triacylated lipopeptides, whereas coexpression of TLR2 and TLR6 is required for recognition of diacylated lipopeptides. In the phylogenetic tree of human TLR2, TLR1, and TLR6 proteins belong to so-called "TLR2 subfamily." TLR1 and TLR6 proteins share 69.3% overall homology, but the TIR domains of these receptors share >90% identity. Moreover, these proteins have similar genomic structures consisting of one exon and are located in tandem in the same chromosome, 4p14 (Rock et al., 1998; Takeuchi et al., 1999). It is hypothesized that they may be a product of an evolutionary duplication (Takeda et al., 2003). This gives the rationale of TLR1 and TLR6 having the capability to distinguish subtle differences in lipopeptide recognition; TLR1 being responsible for recognition of triacylated peptides, and TLR6 being responsible for recognition of diacylated peptides.

TLR2, on the other hand, has been shown to recognize a large array of microbial ligands. We hypothesize that TLR2 may have multiple ligand-binding sites itself or when heteromerized to either TLR1 or TLR6. This hypothesis is supported by our blocking data with the 11G7 mAb and by our analyses of chimeric receptor combinations. Anti-TLR2 (11G7) antibody blocked the cytokine production in stimulation with araLAM and Pam_3CSK_4 (Fig. 1 A); however, this antibody did not inhibit cytokine secretion induced by zymosan (Fig. 1 B), despite the fact that several analyses clearly indicate that zymosan signals in TLR2- and TLR6-dependent fashion (Ozinsky et al., 2000). Thus, the 11G7 anti-TLR2 may fail to block zymosan signaling because the epitope recognized by 11G7 is involved in araLAM, but not zymosan or to an epitope involved in TLR1–TLR2 heterodimer activation. Our blocking data parallel previously published reports in which another type of anti-TLR2 (2393) mAb, but not an isotype control antibody, was able to block synthetic bacterial lipopeptide (Pam_3CSK_4)–mediated reactive oxygen species generation in peripheral blood leukocytes (Aliprantis et al., 1999).

Our transfection experiments with chimeric fusion proteins suggest that dimerization of both the extracellular and the intracellular domains of TLR1 and TLR2 is essential in response to either araLAM or Pam_3CSK_4. HEK293-CD14 cells express endogenous TLR1 (but not TLR2; unpublished data). Therefore, transfection with wild-type TLR2 protein alone confers responsiveness to araLAM, zymosan, or bacterial lipopeptides (Kurt-Jones et al., 2002). Separate transfec-

tion with TLR [1–2] or TLR [2–1] chimeric fusion proteins demonstrated that neither the extracellular domain of TLR2 nor the intracellular domain of TLR2 alone is sufficient to confer responsiveness to araLAM and Pam_3CSK_4 (Fig. 5, A and B). These data are consistent with previous experiments using CD4-TLR1 or CD4-TLR2 fusion proteins, which suggested that neither TLR2 homodimers nor TLR1 homodimers were sufficient to initiate signaling (Ozinsky et al., 2000). Our data are consistent with the hypothesis that heteromers of both extracellular and intracellular domains of TLR1 and TLR2 may be necessary for ligand-dependent signal activation. Thus, cells cotransfected with TLR [1–2] plus TLR [2–1] chimeric constructs responded to araLAM and Pam_3CSK_4 (Fig. 6 A). These cotransfectants simultaneously expressed the extracellular domains of TLR1 and TLR2, as well as the intracellular domains of both TLR1 and TLR2 (Fig. 6 B). The data are consistent with recent reports in knockout animals demonstrating that TLR1 pairs with TLR2 in recognition of Pam_3CSK_4 and 19-kD mycobacterial lipoprotein (Takeuchi et al., 2002). Similarly, cooperative interactions of TLR2 with another TLR (TLR6) have been demonstrated. These reports suggest that a functional interaction between TLR2 and TLR6 is required for responses to group B streptococci (Henneke et al., 2002), diacylated lipopeptide MALP-2 isolated from *Mycoplasma fermentans* (Takeuchi et al., 2001), and phenol-soluble modulin (Hajjar et al., 2001). Our results are further consistent with experiments using dominant-negative constructs that demonstrated signaling in a TLR1- and TLR2-dependent manner by bacterial products (Wyllie et al., 2000). Other recently performed experiments suggest that TLR1 and TLR2 are required for *B. burgdorferi* outer-surface lipoprotein recognition (Alexopoulou et al., 2002). Together, our complementation experiments with TLR1 and TLR2 chimeric proteins further support the hypothesis that multimerization of various TLRs is required for recognition of distinct ligands.

In our loss of function experiments using various mutants of TLR1 and TLR2, we noted that TLR2 constructs with mutation or deletion of the TIR domain (TLR2 P681H and TLR2ΔTIR) exhibited a dose-dependent impairment of NFκB activation in cells stimulated with either araLAM or zymosan. Recent analyses found a similar effect of TLR2 dominant-negative mutant constructs on murine macrophages stimulated with peptidoglycan, zymosan, or triacylated bacterial lipopeptide (Ozinsky et al., 2000). Because all the mutations in our TLR2 mutant proteins involved the intracellular TIR domain and an inhibition in response to all tested stimulants was observed, we conclude that the TIR domain of TLR2 is essential for signal transduction in response to araLAM and zymosan. Thus, signaling by TLR2, like TLR4, requires an intact TIR domain; however, unlike TLR4, TLR2 cannot function as a homodimer, but may rely on sequences within the TIR domain of TLR1 for signal activation (Ozinsky et al., 2000).

Interestingly, inhibition of zymosan responses was observed using a TLR1Δcyt construct, although chimeric TLR1 and TLR2 constructs did not cooperate in the response to zymosan (compare Fig. 4 C with Fig. 6 A). Further, knockout experiments suggest that zymosan responses are dependent on TLR2–TLR6 interaction. This apparent

contradiction between the dominant-negative, chimeric receptor, and knockout experiments may be due to sequestering of TLR2 by the TLR1Δcyt construct. The TLR1Δcyt may form nonfunctional heterodimers with the TLR2 wild-type receptor, thus preventing TLR2 from associating with other TLRs such as TLR6, which is known to be involved in zymosan signaling (Takeuchi et al., 2001).

Sequestration may also be involved in the impairment of NFκB activation noted at high levels of TLR mutant gene expression. Thus, at high levels of TLR2 dominant-negative expression, the response to IL-1β is impaired. Because TLR2 signaling and IL-1β signaling are both dependent on TIR domains, this global inhibition may reflect sequestration of common intracellular signaling molecules (i.e., MyD 88, IRAK-1, TRAF 6) activated in the TLR and IL-1β signaling pathways. Experiments done by others have demonstrated that using dominant-negative MyD88, IRAK-1, IRAK-2, and TRAF6 inhibited IL-1β, and LPS induced NFκB activation (Burns et al., 1998; Zhang et al., 1999; Equils et al., 2001).

We have identified araLAM as a specific ligand for TLR1–TLR2. Our analyses indicate that both extracellular and intracellular domains of TLR1 and TLR2 are required for ligand recognition and subsequent ligand-induced signal activation (Ozinsky et al., 2000; Takeuchi et al., 2000, 2001, 2002; Wyllie et al., 2000; Akira et al., 2001; Alexopoulou et al., 2002). TLR1 and TLR2 are present on the cell surface as a preassembled heterodimer. Together, our experiments indicate that TLR1 and TLR2 colocalize and associate functionally in a signaling complex, explaining the requirement for both proteins for ligand recognition.

Materials and methods

DNA expression vectors
Human TLR2 and TLR4 cDNAs were obtained from Tularik. The TLR cDNAs encoded Flag-epitope–tagged proteins and had been cloned into the pFlag-CMV-1 vector. Chimeric TLR2–TLR1 (TLR [2–1]) and TLR1–TLR2 (TLR [1–2]) constructs were generated by PCR as follows: An XhoI restriction site was added just upstream of Cys 577 of TLR1 and Cys 585 of TLR2 and used for the domain swapping. Intracellular and extracellular domains were PCR amplified using Pfu Turbo DNA polymerase (Stratagene) and cross-assembled into pBlueScript® II KS(+) (Stratagene).

The transmembrane and cytoplasmic regions of TLR1 and TLR2 were PCR amplified using primers as follows: T2cyto-5, 5′-GCGCCTCGAGTGT-CACAGGACAGCACTGGTGTCTG-3′; TLR2–3, 5′-CGCGGGTACCCTAG-GACTTTATCGCAGCTCTCAG-3′; T1cyto-5, 5′-GCGCCTCGAGTGCAA-CATAACTCTGCTGATCGTCACC-3′; and TLR1-3, 5′-CGCGGGTAC-CCTATTTCTTTGCTTGCTCTGTCAGC-3′. The PCR products were digested and cloned into the KpnI and XhoI sites of pBlueScript® II KS (+) (Stratagene).

A portion of the extracellular domain of TLR1 was PCR amplified using primers as follows: TL1-Bam, 5′-CTTTCATTAGGATCCTCCAGCTG-GTTTG-3′; and T1ex-3, 5′-GCGCCTCGAGTTCAGACATGTGAAAGTC-CTTTAGTAGG-3′. The PCR product was digested with BamHI and XhoI and cloned into the BamHI and XhoI sites of the pBlueScript® vector already containing the cytoplasmic region of TLR2. This vector was then digested with BamHI and KpnI (KpnI site was blunted with Klenow enzyme), and the fragment containing the regions of TLR1 and TLR2 was inserted into the BamHI and SmaI sites of pFlag-CMV-1 TLR1.II KS(+) (Stratagene).

A portion of the extracellular domain of TLR2 was PCR amplified using primers as follows: TL2-RV, 5′-CTAACATTGATATCAGTAAGAAT-AGTTTTC-3′; and T2ex-3, 5′-GCGCCTCGAGCACCGAGAGGCGGA-CATCCTGAACC-3′. The PCR product was digested with EcoRV and XhoI and cloned into the EcoRV and XhoI sites of the pBlueScript® vector already containing the cytoplasmic region of TLR1. This vector was then digested with EcoRV and KpnI (KpnI site was blunted with Klenow enzyme),

and the fragment containing the cytoplasmic region of TLR1 was inserted into the EcoRV and SmaI sites of pFlag-CMV-1 TLR2.

To construct the vectors TLR [1–2 TIR] and TLR [2–1 TIR], the domain swapping was done using a NsiI restriction site that is conserved in TLR1 and TLR2. pCMV-Flag TLR1 was cut with NotI and NsiI (NsiI partial digestion), and the fragment of 1,917 bp was cloned into the NotI and NsiI sites of the pBlueScript® containing the cytoplasmic region of TLR2. The resulting plasmid was then cut with NotI and KpnI, and the fragment containing TLR [1–2 TIR] was inserted into the NotI and KpnI sites of pFlag-CMV-1. Similar strategy was used to construct the TLR [2–1 TIR] vector.

Stimulants
Yeast zymosan was purchased from Sigma-Aldrich. Mycobacterial araLAM, purified from rapidly growing avirulent mycobacteria, was provided by Dr. John Belisle (Colorado State University, Fort Collins, CO) under the National Institutes of Health, National Institute of Allergy and Infectious Diseases contract N01-AI-75320 entitled "Tuberculosis Research Materials and Vaccine Testing." Pam₃CSK₄ was obtained from EMC Microcollections. Rough LPS (Sigma-Aldrich) was phenol extracted and used at 10 ng/ml for stimulation assays. Recombinant human IL-1β was purchased from R&D Systems, and used as a positive control for NFκB activation.

In vitro stimulation of human PBMCs: antibody-blocking experiments
Human PBMCs were isolated from peripheral blood using Lymphocyte Separation Medium (Mediatech). PBMCs were cultured in RPMI 1640 medium supplemented with 10% FCS (Atlanta Biologicals) in 24-well plates at 10^6 cells/well. For blocking experiments, PBMCs were preincubated for 30 min at 37°C in 5% CO_2 with anti-TLR1 (clone GD2.F4; eBiosciences), or anti-TLR2 (clone 11G7) mAbs or isotype control antibody (eBiosciences) at 10 μg/ml concentration/well before the addition of stimulants. Culture supernatants were collected after an overnight incubation at 37°C in 5% CO_2. Secreted IL-6 levels were determined by ELISA according to manufacturer's instructions (OptEIA; BD Biosciences). Data are representative of at least three independent experiments.

Antibody cross-linking experiments
Anti-TLR1 (clone GD2.F4; eBiosciences), anti-TLR2 (clone TL2.1; a gift of Dr. Egil Lien, University of Massachusetts Medical School, Worcester, MA), or isotype control OKT8 (CRL-8014; American Type Culture Collection) mAbs were added to sterile high protein binding capacity 96-well plates (Costar) at varying concentrations in PBS and incubated overnight at 4°C. The plates were washed three times with PBS and blocked with 10% FCS (Atlanta Biologicals) in PBS for 2 h. 7×10^5 PBMCs in RPMI 1640 with 10% FCS were added to each well and incubated for 18 h at 37°C in a 5% CO_2 humidified incubator. Low endotoxin mAb preparations were used in all experiments. As an additional control, polymyxin B (cat.# P4932; Sigma-Aldrich) at a 5-μg/ml concentration was added to the culture medium to neutralize potential endotoxin contamination. Supernatants were harvested and IL-8 levels were determined by ELISA according to manufacturer's instructions (BD Biosciences). The results shown are representative of three independent experiments.

Confocal microscopy imaging experiments
Stable cell lines of HEK293 cells expressing the fluorescent protein TLR constructs were engineered as described previously (Latz et al., 2002). Confocal microscopy was performed with living cells that were seeded on 35-mm glass-bottom tissue culture dishes (MatTek Corp.) 24–48 h before examination. Images were taken with a confocal microscope (TCS SP2 AOBS; Leica) equipped with an acousto-optical beamsplitter using version 2 of the Leica Confocal Software, and the images were further processed with Adobe Photoshop® software, version 6 and 7. The cells were kept at 37°C during imaging using a warm stage apparatus. CFP-tagged proteins were visualized using the 458-nm argon laser line; for YFP, the 514-nm line of a 100-mW argon laser was used. Alexa® 647 was excited with a 2.5-mW helium/neon laser emitting at 633 nm. Cells expressing CFP and YFP proteins were sequentially scanned using only one laser line active per scan.

Antibody-patching experiments
For antibody-patching experiments, either stably transfected HEK cells (TLR1-YFP/TLR2-CFP) or TLR4-YFP cells transiently transfected with pcDNA3-TLR2 were grown on glass-bottom 35-mm tissue culture dishes and washed twice with ice-cold HBSS/1% FBS. The cells were then incubated with 5 μg/ml anti-TLR2 (clone TLR 2.1) or anti–human HLA I (clone W6/32HL, cat.# RDI-CBL139–1XP; Research Diagnostics, Inc.) antibody in HBSS/1% FBS as primary antibodies on ice for 30 min. After two washes

with cold HBSS, the cells were counterstained with Alexa® 647–conjugated goat anti–mouse secondary antibody (Molecular Probes, Inc.). After washing, the cells were incubated in prewarmed complete growth medium for 10 min at 37°C and immediately analyzed by confocal microscopy. Representative results of three independent experiments are shown.

Transfection and reporter gene assays in HEK 293-CD14 cells

HEK293 cells (CRL-1573; American Type Culture Collection) stably expressing human CD14 (HEK293-CD14) were cloned as described previously (Kurt-Jones et al., 2002). Transfections of HEK293-CD14 cells were performed using GeneJuice® Transfection Reagent (Novagen) according to manufacturer's instructions. The cells were plated to 96-well plates at 2.5×10^4/well and transfected 24 h later with a total of 0.3 µg DNA per well. The transfected DNA included 80 ng NFκB-driven firefly luciferase plasmid (pGL-3-Basic Vector, cat.# E1751; Promega) and 20 ng HSV-TK promoter-driven renilla luciferase plasmid (phRL-TK Vector, cat.# E6241; Promega) along with wild-type or chimeric TLR constructs cloned into in pFlag-CMV-1 vector (cat.# E7273; Sigma-Aldrich). TLR plasmids were transfected at concentrations ranging from 5 to 200 ng/well. Empty pFlag-CMV-1 vector was used to bring up the total amount of transfected DNA to 0.3 µg per well.

The transfected cells were incubated overnight at 37°C in 5% CO_2 humidified incubator and then stimulated for 6 h with 10 µg/ml zymosan, 1 µg/ml araLAM, 100 ng/ml Pam$_3$CSK$_4$, or 100 ng/ml IL-1β. Cells were lysed using 50 µl Passive Lysis Buffer (cat.# E1941; Promega), and firefly luciferase activity was measured using Dual-Glo™ Luciferase Assay System (cat.# E2940; Promega) according to the manufacturer's instructions. Luciferase activity was calculated in RLU as a ratio of NFκB-dependent firefly luciferase activity to NFκB-independent renilla luciferase activity. The results are shown as the mean ± SD of triplicate wells, and are representative of three independent experiments.

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