7 Use of Toll-Like Receptor Chimeras to Dissect Mechanisms of Receptor Localization and Signaling

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7.1 BACKGROUND

Immune protection in mammals results from the combination of innate immune recognition molecules that provide genome-encoded sensing of conserved components of infectious organisms[1] with the somatic recombination-generated antigen recognition molecules of lymphocytes that recognize individualized features of particular infecting organisms. Among the molecules that provide hardwired recognition of infection by bacteria, fungi, parasites, and viruses, the Toll-like receptors (TLRs) are perhaps the most important for immune function. They are present in the three types of immune cells resident in tissues—the macrophage, the immature dendritic cell, and the mast cell—all of which act as sentinels that respond to recognition of infection by TLRs by inducing inflammation and/or taking antigen to the draining lymph node to initiate an adaptive immune response. TLRs are also present in a variety of other cell types, including B lymphocytes, endothelial cells, and epithelial cells, the latter two of which may also contribute to initial recognition of infection in some circumstances.

TLRs are composed of a ligand-binding N-terminal extracellular domain made up of leucine-rich repeats that pack together to form a horseshoe structure,[2] a single hydrophobic transmembrane domain, and a C-t erminal cytoplasmic domain made up of a short spacer followed by a globular TIR domain, named for its presence in *Drosophila melanogaster* Toll and in the mammalian TLRs, in the interleukin-1 receptor, and in many plant disease-resistance gene products. The TIR domain is also present in five intracellular adaptor molecules in mammals, and these adaptors interact with subsets of TLRs via TIR-TIR domain interactions to mediate their signaling.[3]

Whereas the TLRs that recognize bacterial cell wall components are present on the surface of macrophages and other cells, the TLRs that recognize nucleic acid ligands are localized to intracellular compartments.[4] Both types of TLRs are thought to recognize their ligands within phagosomes, but presumably those TLRs present on the cell surface can also recognize their ligands prior to phagocytosis. Therefore, it has been proposed that the intracellular localization of TLR3, TLR7, TLR8, and TLR9 is an adaptation to minimize responses to the nucleic acids of the host.[5] Interestingly, in this regard, there is accumulating evidence that TLR7 and TLR9 participate in the aberrant production of the anti-DNA, antichromatin, and antiribonucleoprotein autoantibodies seen in most patients with the autoimmune disease systemic lupus erythematosus.[6] Clearly there is a lot still to be learned with regard to how the nucleic acid–sensing TLRs distinguish self–nucleic acids, such as those present in an apoptotic cell that has been phagocytosed by a macrophage or a dendritic cell, from the viral or bacterial nucleic acids present in an apoptotic virus-infected cell or in phagocytosed virions or microbes, but the restriction of these TLRs to intracellular compartments is likely to be part of the answer.

The function of TLRs has been elucidated by three main approaches: (1) definition of the ligands for individual TLRs coupled with analysis of the responses of immune cells to those ligands; (2) genetic loss-of-function experiments, especially analysis of mice with natural or targeted ablation of particular TLRs; and (3) the use of chimeric TLRs to dissect the signaling properties and signaling mechanisms

of this family of receptors. With regard to the chimeric TLR approach, chimeras have been constructed that constitutively dimerize or trimerize TLR TIR domains, and such chimeras induce constitutive signaling,[7–10] suggesting that ligand-induced oligomerization may initiate TLR signaling. The constitutive nature of these chimeras, however, makes it difficult to study the mechanisms of receptor signaling, and, moreover, the high-level expression of these molecules in nonimmune cells may not fully reflect functional properties of TLRs in immune cells. Therefore, we set out to develop a TLR chimera system to dissect the functionally distinct properties of individual TLRs in the context of ligand-induced oligomerization and expression at roughly normal levels in immune cells. For this purpose, we made chimeras of TLRs in which the extracellular domain was derived from TLR4 and the transmembrane domain and intracellular domain were derived from another TLR. These chimeric molecules were expressed in bone marrow–derived macrophages (BMDMs) at roughly normal levels by a highly efficient viral transduction method. In addition, the macrophages used were derived from C57BL/10ScN mice, which carry a deletion of the TLR4 gene,[11–14] so when LPS was added to these cells, it would oligomerize the chimeric TLRs via binding to the extracellular domain of TLR4 complexed with MD-2, but would not bind any endogenous TLRs.

7.1.1 Intracellular Localization of Nucleic Acid–Recognizing TLRs

The TLR chimeras made using the extracellular domain of TLR4 exhibited a cellular localization that was very similar to that of the TLR that supplied the transmembrane domain and cytoplasmic domain. Chimeras with TLR1, TLR2, TLR5, and TLR6 were expressed on the cell surface at levels similar to that of full-length TLR4 expressed from the same retroviral vector.[15] In contrast, the chimera with TLR8 was expressed on the cell surface at a low but detectable level, and the chimeras with TLR3, TLR7, and TLR9 were expressed inside the cell but not on the cell surface. Subsequent studies demonstrated that these chimeras co-localized strongly with full-length TLR3 or TLR7 fused to green fluorescent protein (GFP) or one of its derivatives.[16] These TLRs were found to localize to abundant small vesicles within macrophages. This compartment was not definitively identified, although it was shown to be different from early endosomes. Moreover, TLR3 and TLR7 were seen to accumulate in or near phagosomes containing apoptotic T cells, suggesting that these TLRs are able to sense ligands within phagosomes. Other studies have suggested that TLR9 localizes to the endoplasmic reticulum.[17] In any case, this localization was specified by the transmembrane and/or cytoplasmic domains of these receptors.

The mechanism by which TLR3 and TLR7 are retained within intracellular compartments was explored by a random mutagenesis approach. The transmembrane domain– and cytoplasmic domain–encoding regions of the cDNA were subjected to random mutagenesis by error-prone PCR. The mutagenized sequences were reinserted into a vector containing the extracellular domain of TLR4 and enhanced GFP downstream from an internal ribosome entry site (IRES) sequence, and pools of approximately 40,000 mutagenized TLRs were transfected into HEK293T cells that stably expressed transfected CD14 and MD-2. Intact transfected cells were stained with a

fluorescent anti-TLR4/MD-2 antibody, and cells positive for both GFP and TLR4/MD-2 were isolated by cell sorting, which was repeated a second time to further enrich for mutated versions of the TLR chimera that had lost intracellular localization.[16]

Mutations of TLR3 causing loss of intracellular retention were mostly frameshift mutations that truncated the cytoplasmic domain of TLR3 within the spacer sequence between the transmembrane domain and the TIR domain. In contrast, deletion of just the TIR domain did not change the localization of the TLR3 chimera. These studies identified a 23-amino-acid sequence as being necessary for intracellular localization of the TLR4/TLR3 chimera. Grafting this membrane proximal spacer sequence onto the heterologous protein CD25 (IL-2 receptor α chain) caused CD25 to adopt a primarily intracellular localization, demonstrating that this sequence is also largely sufficient to mediate internal localization. This sequence is not similar to any of the well-characterized sequences known to target intracellular membrane proteins to their location, suggesting that TLR3 uses a novel mechanism to achieve its localization.

TLR3 was found to co-localize strongly with TLR7,[16] but surprisingly, there was no clear homology between the membrane proximal spacer region of TLR3 and the corresponding region in TLR7. Moreover, random mutagenesis of the TLR4/TLR7 chimera did not yield any mutants that relocalized the TLR4/TLR7 chimera to the cell surface. Subsequent site-directed mutagenesis of the TLR4/TLR7 chimera demonstrated that the membrane proximal spacer region and the TIR domain were both dispensable for intracellular location and that the transmembrane domain was responsible.[16] Remarkably, TLR3 and TLR7 use different regions of their protein sequence to target themselves to the identical intracellular localization in macrophages.

7.1.2 Distinctive Signaling Properties of TLRs

The TLR chimeras that were expressed exclusively in intracellular vesicles failed to respond to LPS,[16] suggesting that LPS does not easily gain access to this compartment, in agreement with direct demonstration that these chimeras did not localize to early endosomes.

The TLR chimeras that are expressed on the cell surface mediated responses to LPS, including production of TNF and activation of signaling responses, such as NF-κB activation and activation of the Erk, JNK, and p38 MAP kinases.[15] These responses were seen in cells expressing full-length TLR4 and in cells expressing the TLR4/TLR5 chimera, indicating that the cytoplasmic domains of TLR5 were able to signal efficiently in the context of the TLR4/TLR5 chimera. Whereas the TLR4/TLR5 chimera was reasonably efficient at inducing TNF production and signaling events downstream of MyD88,[15] it was only weakly capable of inducing type 1 interferon production and subsequent STAT1 tyrosine phosphorylation. The latter responses were strongly induced by LPS stimulation of cells expressing full-length TLR4, presumably because the cytoplasmic domain of TLR4 associates with the adaptor TRIF, which can induce interferon-β production, whereas the cytoplasmic domain of TLR5 does not. The TLR4/TLR8 chimera was able to reduce responses similar to those induced by the TLR4/TLR5 chimera, except at a lower level, presumably reflecting the low level of cell surface expression of this chimera.

In contrast, chimeras containing the transmembrane and cytoplasmic domains of TLR1, TLR2, or TLR6 were expressed on the cell surface at levels similar to those of full-length TLR4 or the TLR4/TLR5 chimera, but failed to induce TNF or signaling responses to LPS when expressed alone in bone marrow–derived macrophages. In contrast, in macrophages expressing TLR4/TLR2 together with either TLR4/TLR1 or TLR4/TLR6, LPS induced robust production of TNF, substantial activation of NF-κB, and activation of MAP kinases.[16] In none of these cases was the combination of TLR chimeras able to activate a substantial amount of interferon-β production. Thus, the cytoplasmic domains of TLR2, TLR1, and TLR6, all of which have TIR domains, exhibit functional complementarity in certain combinations (TLR2 with TLR1 or TLR6), but not in other combinations (TLR1 with TLR6), and activate the MyD88 signaling pathway but not the TRIF signaling pathway.

Previous work had indicated that TLR2 functions together with TLR1 for responses to triacylated lipoproteins and that TLR2 functions together with TLR6 for responses to diacylated lipoproteins.[1] These distinct lipoproteins are found in bacterial cell walls. Previously, it had been thought that the complementarity of TLR2 with TLR1 or TLR6 reflects binding specificity of the extracellular domains of TLR1 and TLR6, and this is likely true, but our findings indicate that in addition TLR2 requires a complementary TLR TIR domain to promote efficient signaling. Interesting in this regard is that additional ligands for TLR2 have been identified, including peptidoglycan, porins from *Neisseria*, lipoarabinomannan from mycobacteria, and phospholipomannan from certain fungal pathogens. For these ligands, a second TLR in addition to TLR2 is likely to be required for signaling to be generated, but this complementary TLR has not yet been identified.

7.2 CONSTRUCTION OF TLR CHIMERAS

All TLRs are type I transmembrane proteins composed of an NH_2-terminal signal peptide, an extracellular domain involved in ligand recognition, a single transmembrane domain, and a cytoplasmic domain largely made up of the TIR domain. All TLRs have a cysteine-rich region proximal to the transmembrane domain in their extracellular region. Four cysteine residues are located in this region. Mutation analysis of *Drosophila* Toll protein has shown that these cysteine residues are important for its function.[18] For this reason, the highly conserved last cysteine in the cysteine-rich region of TLR4 and other TLRs was used to align the sequences and to provide the junctions of the chimeras (Figure 7.1). We used two distinct methods to construct TLR chimeras: a triple ligation method and the PCR sewing method, as described in Figure 7.2.

7.2.1 Triple Ligation Method

This method is simple. As shown in Figure 7.2A, cDNAs encoding the extracellular region of TLR4 (4EX) and the transmembrane and cytoplasmic regions of other TLRs (TM-CP) were amplified by PCR using primers containing desired restriction enzyme sites and 5′ phosphate modification: the 5′ primer for 4EX and the 3′ primer for TM-CP contained a restriction enzyme site, and the 3′ primer for 4EX and the 5′

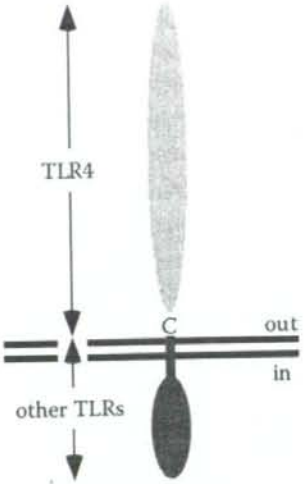


FIGURE 7.1 The TLR Chimeras

Note: TLR chimeras are composed of the extracellular region of TLR4 and the transmembrane and cytoplasmic regions of other TLRs. The highly conserved last cysteine (c) in the cysteine-rich regions of TLR4 and other TLRs is used to align the sequences and to provide the junctions of the chimera.

primer for TM-CP had a 5′ phosphate modification, which is indispensable for the blunt-end ligation between 4EX and TM-CP. The PCR products were digested with restriction enzymes and then ligated with the vector.

Protocol 7.1: Construction of TLR Chimeras by a Triple Ligation Method

1. Amplify cDNA encoding 4EX and TM-CP of other TLRs by PCR using PfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA). The composition of the reaction is below.

   distilled H_2O: 78 µl
   10× buffer: 10µl
   DMSO: 5µl
   0.1 µg/µl template: 1µl
   200 ng/µl upper primer: 1.25 µl
   200 ng/µl lower primer: 1.25 µl
   10 mM dNTP: 2 µl
   PfuUltra: 1.5 µl
   Total volume: 100 µl (50 µl × 2 tubes)

   The PCR is performed for 18 cycles at 95 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 3 minutes and 30 seconds. After the last cycle, the reaction is incubated at 72 °C for 10 minutes to complete extension of PCR products. Eighteen cycles is enough to amplify PCR products; additional cycles would increase the chance of PCR errors.
2. Purify PCR products by using the Gel Purification Kit (Qiagen, Valencia, CA). Gel purification is used to confirm that PCR products are successfully amplified.
3. Digest PCR products with restriction enzymes overnight.
4. Purify PCR products by using the PCR Purification Kit (Qiagen).

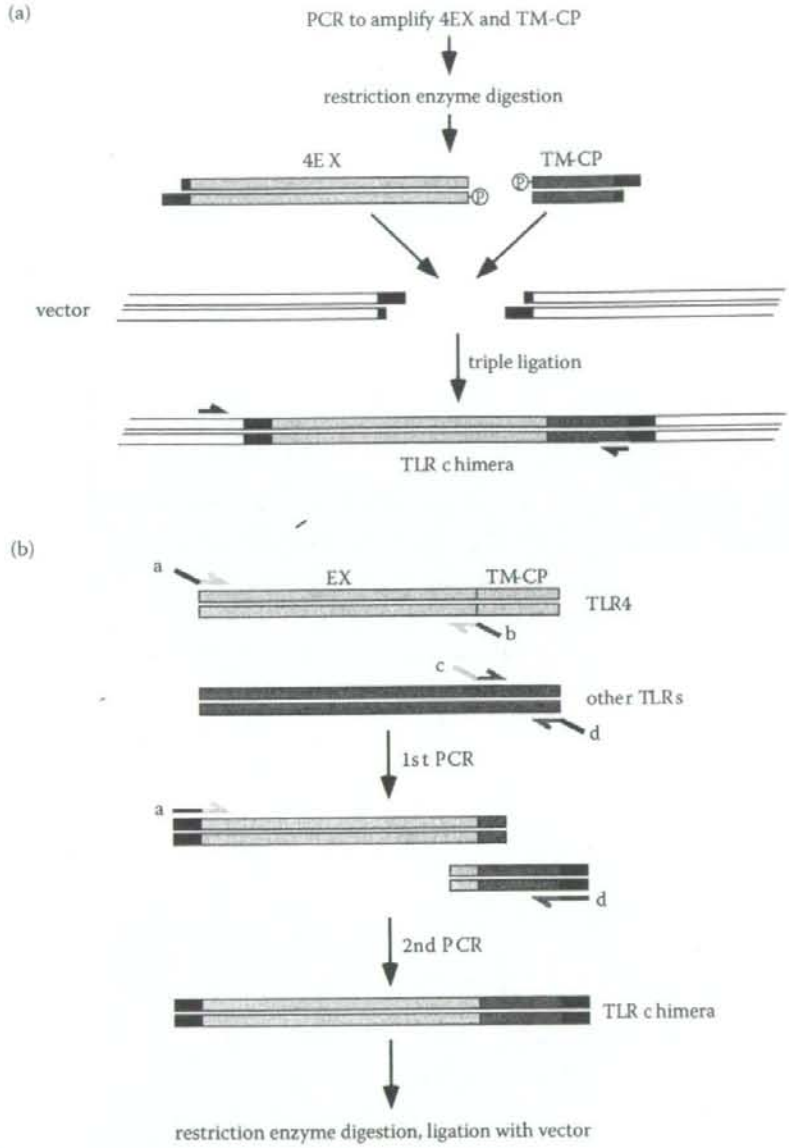


FIGURE 7.2 Two Methods Used for Construction of TLR Chimeras

Note: (A) Triple ligation method. cDNAs encoding the extracellular region of TLR4 (4EX) and the transmembrane and cytoplasmic regions of other TLRs (TM-CP) were amplified by PCR. After restriction enzyme digestion, the triple ligation was performed. The primers shown for the final product were used to screen transfected bacterial colonies. (B) PCR sewing method. The cDNAs encoding 4EX and TM-CP were amplified in the first PCR reaction. The second PCR was performed by using primers a and d and the first PCR products as templates. The resulting PCR products encode TLR chimeras, which were then digested with restriction enzymes and ligated with the vector. The gray bar shows 4EX, the dark-gray bar shows TM-CP, the open bar shows vector sequence, and black bars show restriction enzyme sites. Primers a and d contain a restriction enzyme site. Primers b and c contain the other TLR sequence and the TLR4 sequence, respectively, so that an overlapping sequence can be created between the two PCR products.

5. Ligate 4EX, TM-CP, and vector. The molecular ratio of 4EX, TM-CP, and vector should be 10:1:1. This ratio was chosen to optimize the efficiency of the triple ligation because TM-CP (about 600 bp) is much smaller than 4EX (about 1,900 bp). In general, the ligation efficiency varies inversely in proportion to the size of the fragment.
6. Transform *E. coli* competent cells with ligation mixture.
7. Perform colony PCR to identify bacterial colonies carrying vectors incorporating the TLR chimeras. A vector sequence upstream of the insert was used for the upper primer, and the lower primer used to amplify TM-CP was used for the lower primer of colony PCR (Figure 7.2A). The composition of this reaction is as follows:

   Distilled H_2O: 91.7 µl
   DMSO: 10.9 µl
   200 ng/µl upper primer: 2.2 µl
   200 ng/µl lower primer: 2.2 µl
   GoTaq Green Master Mix (Promega, Madison, WI): 109 µl
   Total volume: 218 µl (for 10 colonies; 20 µl × 10 tubes)

   This PCR is performed for 24 cycles at 95 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 3 minutes.
8. DNA sequencing to confirm that the chimera's sequence is correct.

The triple ligation method is simple, but the ligation efficiency is not high; usually, 60% of the bacterial colonies carried vectors containing TM-CP alone, about 20% of the bacterial colonies carried vectors containing TLR chimeras, and the rest of the colonies carried self-ligated vectors. The ligation of vector with TM-CP alone and the self-ligation of vector were not prevented, although use of different restriction enzyme sites for 4EX and TM-CP to the vector helped to minimize unwanted products. Therefore, to find bacterial colonies carrying plasmids containing TLR chimeras, at least 16 different colonies were screened by colony PCR.

7.2.2 PCR Sewing Method

This method is composed of two steps of PCR. As described in Figure 7.2B, in the first step of PCR, cDNA encoding 4EX containing a part of TM-CP was amplified using primers a and b. Similarly, cDNA encoding TM-CP of other TLRs containing a part of 4EX was amplified using primers c and d. Primers a and d also contained restriction enzyme sites, whereas b and c contained the sequence encoding a part of 4EX or TM-CP of other TLRs. In the second step, the cDNA encoding the TLR chimera was amplified by using primers a and d and PCR products obtained from the first-step PCR as templates. The second PCR products encode TLR chimeras, which are then digested with restriction enzymes to generate sticky ends for ligation into the vector.

Protocol 7.2: Construction of TLR Chimeras by a PCR Sewing Method

1. Perform first PCR to amplify cDNAs encoding 4EX and TM-CP by using PfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA), as described in Protocol 2.1.
2. Perform second PCR to obtain cDNA encoding TLR chimeras. The composition of this reaction is as follows:
   10× buffer: 10 μl
   DMSO: 5μl
   4EX: 30 ng
   TM-CP: 10 ng
   200 ng/μl upper primer: 1.25 μl
   200 ng/μl lower primer: 1.25 μl
   10 mM dNTP: 2 μl
   PfuUltra: 1.5 μl
   Add distilled H_2O to 100 μl.
   This PCR is performed for 18 cycles at 95 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 3 minutes and 30 seconds. After the last cycle, the reaction is incubated at 72 °C for 10 minutes to complete extension of PCR products. The molecular ratio of 4EX to TM-CP is 1:1.
3. Purify PCR products by using the Gel Purification Kit (Qiagen, Valencia, CA).
4. Digest PCR products with restriction enzymes overnight.
5. Purify PCR products by using the PCR Purification Kit (Qiagen).
6. Ligate PCR products with vector. The molecular ratio of PCR product to vector is 10:1.
7. Transform *E. coli* competent cells with ligation mixture.
8. Perform colony PCR to identify colonies carrying TLR chimeras, as described in Protocol 7.1.
9. Sequence DNA to confirm correct sequence.

The ligation efficiency of the PCR sewing method is much higher than that of the triple ligation method. Although this method has more steps than the triple ligation method, most of the bacterial colonies carried the vector with the sequences encoding the TLR chimeras.

7.3 INTRODUCTION OF TLR CHIMERAS INTO BONE MARROW–DERIVED MACROPHAGES BY RETROVIRAL GENE TRANSFER

Because the major functions of TLRs are to induce innate immune responses in immune cells, such as macrophages and dendritic cells, assessing the function of the TLR chimeras in those cells was desired. However, innate immune cells are difficult to transfect efficiently by using typical transfection methods, such as liposome-based methods, DEAE-dextran, or calcium-phosphate. In contrast, VSV-G pseudotype retroviruses efficiently infect primary macrophages. In this protocol, we describe a

retroviral gene transfer to introduce TLR chimeras into bone marrow–derived macrophages with excellent efficiency.

7.3.1 Preparation of Bone Marrow–Derived Macrophages from Mice

Typically, we obtained 2–3 × 10^7 macrophages from one mouse (two sets of femora and tibias).

Protocol 7.3: Preparation of Bone Marrow–Derived Macrophages from Mice

1. Euthanize the mouse with carbon dioxide gas followed by cervical dislocation.
2. Sterilize the mouse fur with 70% ethanol.
3. Remove the skin, fur, and muscles. Next, cut the bone that joins the spine and patella.
4. Clean the bones with Kimwipes (Kimberly-Clark, Dallas, TX) as much as possible. Then, wash the exterior of the bones with 70% ethanol, and store in tissue culture medium until the cleanup of the last bone is finished. *Note*: Subsequent steps are performed in a laminar flow biosafety cabinet.
5. Holding the bones with sterile forceps, cut off each end using sterile scissors.
6. Flush the bones with approximately 2.5 ml of medium per femur or tibia, using a 26 gauge needle or syringe at both ends until the bones turn white.
7. Centrifuge the collected marrow at 500 × g for 5 minutes at 4 °C. The pellets should be red because of red blood cells.
8. Resuspend the pellets with 5 ml of red blood cell lysis buffer, and incubate for 5 minutes at room temperature.
9. Add 5 ml of medium to the cells, and centrifuge. After centrifugation, the cell pellet should be white or light pink.
10. Resuspend the cells in 20 ml of macrophage medium. We use CMG12-14 cell culture supernatant for CSF-1 source.[19] Alternatively, L929 cell supernatant can be used at 30% of final concentration.
11. Spread the bone marrow cells onto a 15 cm plastic Petri dish (not treated for tissue culture). *Note*: The compositions of the red blood cell lysis buffer and the primary macrophage media are described below.

Red Blood Cell Lysis Buffer (500 ml)

Hepes: 1.19 g (10 mM)
NH_4Cl: 4.1 g (150 mM)
$KHCO_3$: 0.5 g (10 mM)
0.5 mM EDTA: 100 µl (0.1 mM)

Add water to 500 ml. Adjust pH to 7.3 with 1 M KOH. Sterilize through 0.2 μm filter, and store at room temperature.

Culture Media for Bone Marrow–Derived Macrophages (500 ml)

RPMI 1640 containing 25 mM Hepes: 400 ml
FBS: 50 ml
100 mM sodium pyruvate: 5 ml
CMG12-14 cell condition medium: 45 ml

7.3.2 Preparation of Retroviruses

VSV-G retroviruses producing TLR chimeras are prepared by triple transfection of HEK293T cells with retroviral constructs along with gag-pol and VSV-G expression constructs.[20]

Protocol 7.4: Preparation of Retroviruses

1. Split HEK 293T cells at a density of 1.8×10^6 cells per well of a 6-well plate.
2. The next day, transform the cells with 3.6 μg of retroviral vector, 1.4 μg of gag-pol expression vector, and 2.1 μg of VSV-G expression vector by using 10 μl of LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA).
3. Twenty-four hours after transfection, change medium with fresh media (DMEM containing 10% FBS), and further incubate the cells for 24 hours.
4. Collect the media containing viruses (virus supernatants), and use these media for infection or store them at –70 °C until use.

VSV-G retroviruses are stable for at least 1 year at –70 °C, and the titer is not significantly changed by a couple of freeze-thaw steps. Virus supernatants can be collected three times; repeat steps 3 and 4 (above) three times.

7.3.3 Infection of Bone Marrow–Derived Macrophages with Retroviruses

Approximately 30–50% of bone marrow–derived macrophages can be successfully infected with VSV-G retroviruses by this protocol.

Protocol 7.5: Infection of Bone Marrow–Derived Macrophages with Retroviruses

1. Plate BMDMs at day 4 or 5 after the preparation from the bone marrow at a density of 7.5×10^5 cells per well in a nontissue culture–treated 6-well plate.

2. The next day, mix viral supernatants with polybrene at a final concentration of 10 μg/ml, and then add to the cells.
3. Centrifuge cells and viruses at 1,300 × g for 1 hour at room temperature. This is a critical step to obtain maximum infection efficiency.
4. Incubate the cells for 6 hours at 37 °C in 5% CO_2 and 95% air. In general, 6 hours of incubation is enough to complete the integration of virus into the genome of cells.
5. Replace viral supernatants with culture media. After 48 hours, assess the infection efficiency by flow cytometric analysis as described in the next protocol.

It is critical to use BMDMs at day 4 to 6 after the preparation from the bone marrow for retroviral infection, because the infection efficiency is significantly decreased at day 7 or later. The efficiency of retroviral infection is totally dependent on the proliferation rate of cells. BMDMs proliferate very fast until day 6, and suddenly become slow after day 7.

Before mixing with polybrene, the virus supernatants should be centrifuged at 500 × g for 10 minutes at 4 °C to remove contaminating HEK293T cells. The filtration of viral supernatants with a 0.45 μm filter to remove HEK293T cells from viral supernatants is not recommended because the titer of retroviruses is significantly decreased by this operation.

It has been suggested that the concentration of retroviruses by polyethylene glycol (PEG) precipitation increases virus titer. However, we have observed no significant increment of virus titer by PEG precipitation.

7.3.4 Determination of Infection Efficiency by Flow Cytometry

Retroviral vectors carrying an IRES element and the enhanced green fluorescent protein (eGFP) gene are useful to easily assess the infection efficiency for cells. We have used the bicistronic retroviral vector pMX-pie[21] for the introduction of TLR chimeras into BMDMs. By using this vector, the cells expressing the gene of interest also express eGFP proteins. Therefore, eGFP fluorescence is an indication of infection efficiency and expression level of the gene of interest. eGFP fluorescence is simply detected by the use of a flow cytometer. In this protocol, we describe the preparation of cells for flow cytometry.

Protocol 7.6: Determination of Infection Efficiency by Flow Cytometry

1. Perform retroviral infection of BMDMs as described in Protocol 7.5.
2. Forty-eight hours after infection, incubate the cells in phosphate buffered saline (PBS) containing 5 mM EDTA for 5–10 minutes at 37 °C.
3. Detach the cells from the plate by gentle pipetting. Cells should be easily detached if plastic plates that have not been treated for tissue culture are used.
4. Centrifuge the cells at 900 × g for 1 minute at 4 °C.

TABLE 7.1
The Efficiency of Infection in Bone Marrow–Derived Macrophages

TLR Chimeras	% Green Fluorescent Protein + Cells
TLR4/TLR1	36.8 ± 6.4
TLR4/TLR2	37.5 ± 5.6
TLR4/TLR3	36.3 ± 4.1
TLR4	37.0 ± 6.4
TLR4/TLR5	38.7 ± 5.6
TLR4/TLR6	38.0 ± 4.6
TLR4/TLR7	34.5 ± 1.3
TLR4/TLR8	36.7 ± 4.7
TLR4/TLR9	35.0 ± 3.2
Vector	52.3 ± 4.3

Note: Infection efficiency of TLR chimeras was assessed as described in Protocol 7.6, and was shown as average ± *SD* of three independent infections.

5. Resuspend the cells with FACS-staining buffer consisting of 1% FBS and 0.09% sodium azide in PBS.
6. Measure GFP fluorescence by flow cytometer. As shown in Table 7.1, approximately 35% of BMDMs express GFP in our experiments.

7.4 CHARACTERIZATION OF TLRS BY USING TLR CHIMERAS

TLR chimeras are useful to examine both subcellular localization and signaling properties of TLRs. In this protocol, we describe protocols for studies of subcellular localization and signaling of TLRs by using TLR chimeras.

7.4.1 Examination of the Subcellular Localization of TLRs by Flow Cytometry

Flow cytometry is a useful method to determine whether the receptor is expressed on the cell surface or resides in intracellular compartments. To detect receptors on the cell surface, intact cells are stained with antibodies specific to the extracellular portion of the receptors of interest. To detect receptors inside cells, fixation and permeabilization of the cells are needed before staining with antibodies. Miyake's group has generated an anti-mouse TLR4/MD-2 antibody,[22] which recognizes the extracellular region of TLR4 associated with MD-2, an accessory molecule required for cell surface expression and LPS responsiveness of TLR4.[23] Because the TLR chimeras constructed by the method described above possess the extracellular region of

TLR4 (Figure 7.1), the anti-mouse TLR4/MD-2 antibody is a useful tool to examine the subcellular distribution of the TLR chimeras.

To specifically detect TLR chimeras with the anti-mouse TLR4/MD-2 antibody, TLR chimeras must be expressed in macrophages prepared from TLR4-deficient mice. We have prepared bone marrow–derived macrophages from C57BL/10ScN mice, which carry a null mutation in the TLR4 gene.[11,12,14] In this protocol, we describe the sample preparation for flow cytometry using an anti-mouse TLR4/MD-2 antibody.

Protocol 7.7: Measuring the Expression Levels of TLR Chimeras on the Cell Surface and inside the Cells by Flow Cytometry

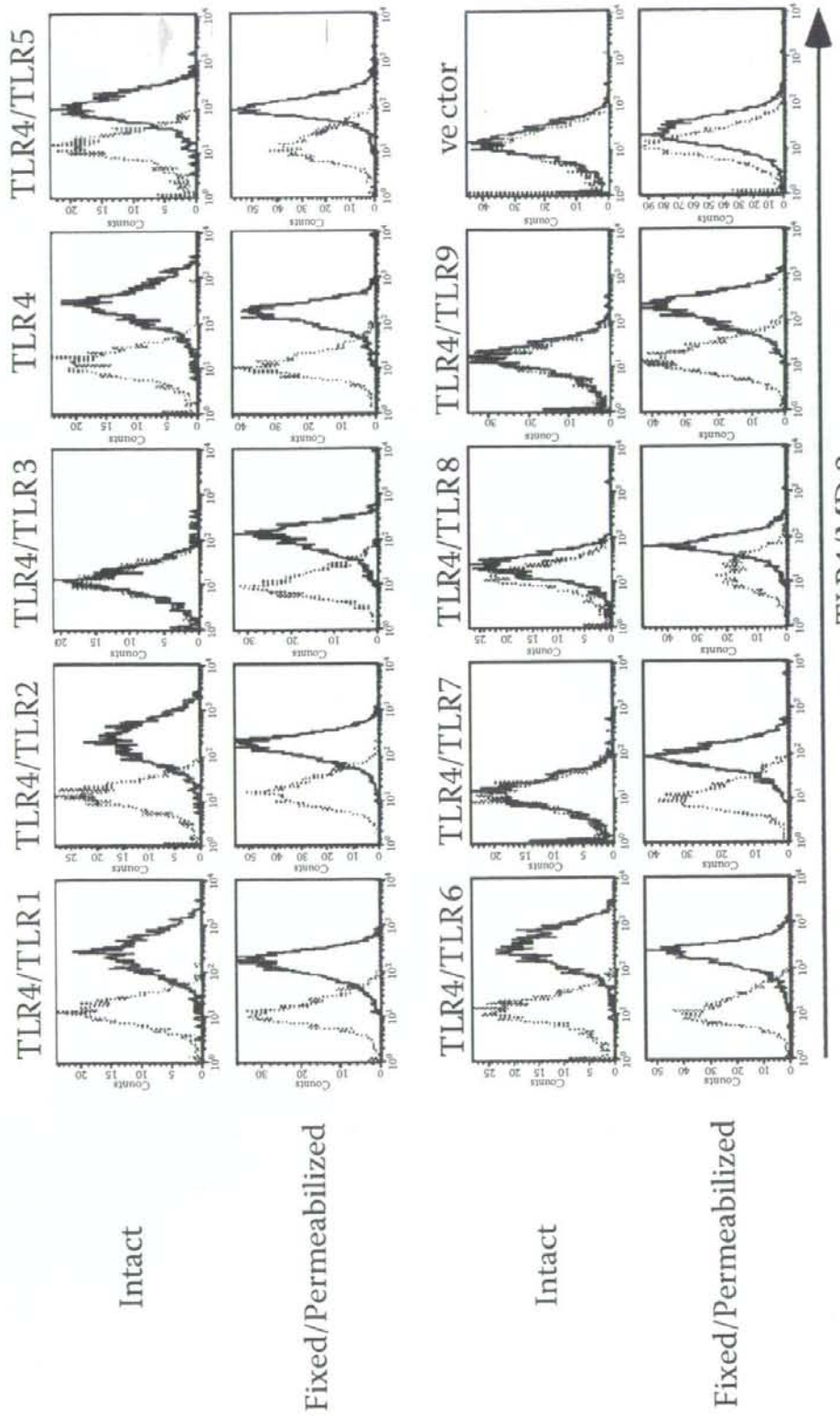
1. Prepare BMDMs from TLR4-deficient mice as described in Protocol 7.3.
2. Infect the cells at day 4 or 5 with retroviruses producing TLR chimeras as described in Protocol 7.5.
3. Forty-eight hours after infection, detach the cells by incubating in PBS containing 5 mM EDTA, followed by gentle pipetting.
4. Treat the cells with an anti-CD16/CD32 antibody (clone 2.4G2, BD Bioscience, San Jose, CA) to block Fcγ receptors II and III.
5. After washes, stain the cells with Phycoerythrin (PE) conjugated anti-mouse TLR4/MD-2 antibody (eBioscience, San Diego, CA) for 30 minutes at 4 °C. For intracellular staining, the cells are fixed and permeabilized with Cytofix/Cytoperm solution (BD Bioscience) before staining with PE–anti-mouse TLR4/MD-2 antibody.
6. After washes, resuspend the cells in FACS-staining buffer.
7. Measure the expression level of TLR chimeras on the cell surface by flow cytometry.

Figure 7.3 shows the subcellular distribution of TLR chimeras examined by flow cytometry. TLR4/TLR1, TLR4/TLR2, TLR4, TLR4/TLR5, and TLR4/TLR6 are expressed on the cell surface, whereas TLR4/TLR3, TLR4/TLR7, TLR4/TLR8, and TLR4/TLR9 are localized intracellularly. These data are consistent with data obtained by available anti-TLR antibodies or fluorescently labeled TLRs. The subcellular distribution of TLRs may be regulated by their transmembrane and/or cytoplasmic regions. It should be noted that if the extracellular region contains a targeting element, these TLR chimeras may not reflect the correct cellular localization of TLRs.

FIGURE 7.3 Subcellular Distribution of the TLR Chimeras Determined by Flow Cytometry

Note: Macrophages infected with retroviruses producing TLR chimeras were stained with anti-mouse TLR4/MD-2 antibody. GFP-positive cells were gated and displayed for TLR chimera staining. Intact: cells without fixation/permeabilization; fixed/permeabilized: cells with fixation/permeabilization: dotted line: isotype control; and solid line: anti-TLR4/MD-2 antibody.

Source: From Nishiya, T., and DeFranco, A. L. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. *J. Biol. Chem.* 279, 19008, 2004. With permission.


TLR4/TLR1
TLR4/TLR2
TLR4/TLR3
TLR4
TLR4/TLR5
TLR4/TLR6
TLR4/TLR7
TLR4/TLR8
TLR4/TLR9
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Intact
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Intact
Fixed/Permeabilized
TLR4/MD-2

7.4.2 Examination of the Subcellular Localization of TLRs by Microscopy

Fluorescent microscopy is useful to characterize the intracellular organelle, where intracellular receptors are localized. Anti-mouse TLR4/MD-2 antibody is suitable for microscopy as well as flow cytometry. Because antibodies against some TLRs have not been established yet, the microscopy of TLR chimeras using anti-mouse TLR4/MD-2 antibody has given us important information about TLR localization in cells.

Protocol 7.8: Determination of the Subcellular Localization of TLR Chimeras by Microscopy

1. Prepare BMDMs from TLR4-deficient mice as described in Protocol 7.3.
2. Infect the cells at day 4 or 5 with retroviruses producing TLR chimeras as described in Protocol 7.5.
3. Plate the cells at a density of 9×10^5 cells per dish in a 35 mm glass-bottom dish.
4. Next day, treat the cells with anti-CD16/CD32 antibody to block Fcγ receptors II and III.
5. Wash the cells with 2 ml of FACS-staining buffer three times.
6. Fix and permeabilize the cells with 100 μl of Cytofix/Cytoperm solution for 20 minutes at 4 °C.
7. Wash the cells with 2 ml of Perm Wash buffer (BD Bioscience, San Jose, CA) three times.
8. Treat the cells with appropriate antibodies against various subcellular marker proteins.
9. Wash the cells as in step 7.
10. Treat the cells with PE–anti-mouse TLR4/MD-2 antibody for 40 minutes at 4 °C.
11. Wash the cells as in step 7.
12. Treat the cells with 300 nM DAPI for 5 minutes at room temperature to visualize nucleus.
13. After washes with FACS-staining buffer, add 1 ml of FACS-staining buffer to the dish and perform microscopy. PE signals bleach very rapidly. You can stain the cells with unlabeled anti-mouse TLR4/MD-2 antibody and fluorescently-labeled anti-rat IgG antibody instead of PE-labeled anti-mouse TLR4/MD-2 antibody.

7.4.3 Determination of the Intracellular Targeting Elements of TLRs by Random Mutagenesis Approach

Error-prone PCR-based random mutagenesis can be applied to the identification of the targeting elements for intracellular TLRs: TLR3, TLR7, TLR8, and TLR9. The

subcellular distribution of TLRs appears to be regulated by their transmembrane and/or cytoplasmic regions. The introduction of mutation(s) into the targeting elements may disrupt the conformational characteristics involved in the intracellular localization of TLRs, and therefore the mutants will be expressed on the cell surface.

There are some important considerations for performing this experiment. First, a retroviral vector should be used to express the mutant TLRs, because it is critical to stably express distinct mutants in as many cells as possible. Second, mutants should be introduced to HEK293T cells stably expressing mouse CD14 and MD-2 (designated as 293TCM cells), because (1) the infection efficiency of HEK293T cells (> 90%) is much higher than that of primary macrophages (~ 35%), (2) the HEK293T cells do not express endogenous human TLR4, and (3) HEK293T cells are easily cloned because they proliferate well. Finally, supercompetent *E. coli* cells, such as cDNA library grade, should be used for the transformation with vectors carrying TLR4/TLR chimeras with point mutations (TLR4/TLR^{Mu} chimeras).

Protocol 7.9: Determination of the Intracellular Targeting Elements of TLRs by Random Mutagenesis Approach

1. The entire transmembrane and cytoplasmic regions of TLR chimeras are amplified by error-prone PCR using the Genemorph II Random Mutagenesis Kit (Stratagene, Santa Clara, CA) (Figure 7.4). The composition of reaction is given below.

   Distilled H_2O: 38.7 µl
   10× buffer: 5 µl
   DMSO: 2.5 µl
   10 ng/ml template: 0.5 µl
   200 ng/ml upper primer: 0.63 µl
   200 ng/ml lower primer: 0.63 µl
   10 mM dNTP: 1 µl
   Mutazyme II: 1 µl
   Total volume: 50 µl

   PCR is performed for 30 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute. After the last cycle, the reaction mixture is incubated at 72 °C for 10 minutes to complete extension of PCR products. The error rate can be controlled by both amount of template and cycle number of PCR reaction. Following this protocol, the mutation rates of the TLR4/TLR3 and TLR4/TLR7 chimeras were 1.7 ± 0.3 and 2.9 ± 0.4 per clone at the nucleotide level and 1.1 ± 0.4 and 1.6 ± 0.3 per clone at the amino acid level, respectively.
2. Purify PCR products by using the Gel Purification Kit (Qiagen, Valencia, CA).
3. Digest PCR products with *SalI* and *NotI* restriction enzymes overnight.
4. Purify PCR products by using the PCR Purification Kit (Qiagen).

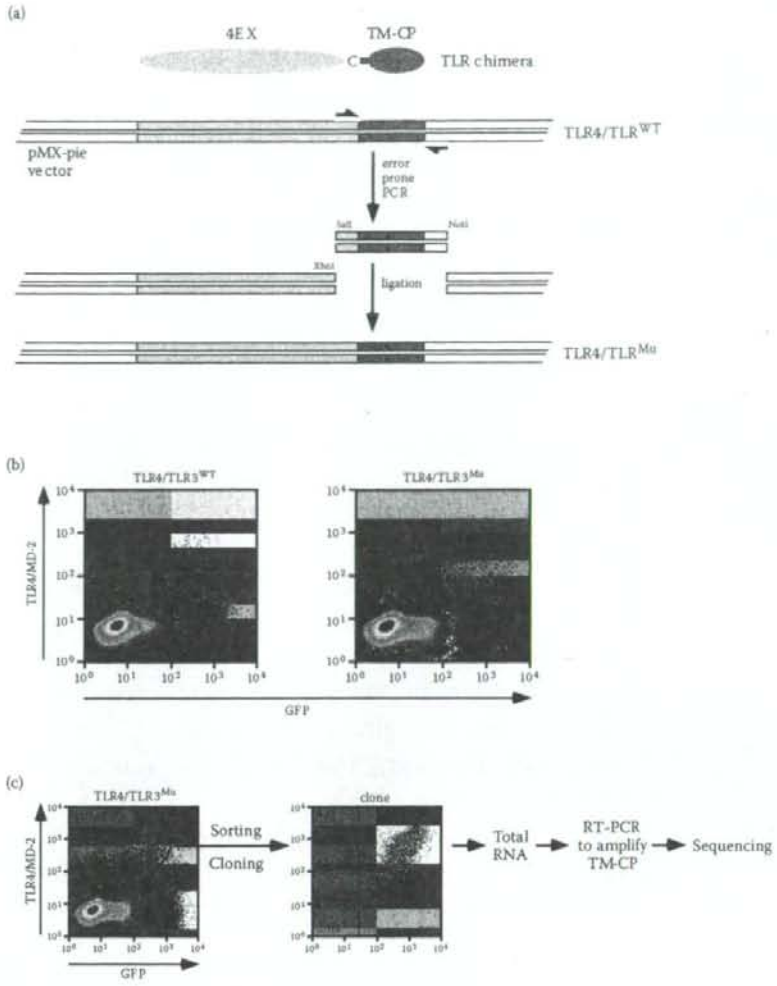

FIGURE 7.4 Random Mutagenesis of the Transmembrane and Cytoplasmic Regions of TLR Chimeras

Note: (A) Schematic of random mutagenesis. Random mutagenesis of the transmembrane and cytoplasmic regions of TLR chimeras was performed by error-prone PCR using primers indicated by the arrows. PCR products carrying mutation(s) were cloned into the pMX-pie-TLR4(EX) vector. (B) Flow cytometric analysis of cell surface expression of wild-type and mutated TLR4/TLR3 chimeras. HEK293TCM cells were infected with retroviruses encoding the TLR4/TLR3 chimera (TLR4/TLR3^{WT}) or encoding pooled chimeras containing random mutations in the transmembrane and cytoplasmic domains of TLR3 (TLR4/TLR3^{Mu}). Forty-eight hours after infection, the cell surface level of TLR4/TLR3 chimeras was determined by flow cytometric analysis using anti-mouse TLR4/MD-2 antibody. (C) Isolation of mutations in TLR4/TLR3^{Mu} chimeras that express on the cell surface. The GFP+ TLR4/MD-2+ population gated by the oval shown was sorted twice, and then individual cells were cloned by limiting dilution. About 200 clones were isolated, and the cell surface level of the TLR4/TLR3^{Mu} chimera in each clone was determined by flow cytometry using anti-mouse TLR4/MD-2 antibody.

Source: Adapted from Nishiya, T., Kajita, E., Miwa, S., and DeFranco, A. L. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J. Biol. Chem.* 280, 37107, 2005. With permission.

5. Ligate PCR products with the *XhoI/NotI* site of pMX-pie-TLR4(EX) vector, which contains the cDNA encoding amino acids 1–598 of TLR4.
6. Transform 200 µl of OmniMAX supercompetent *E. coli* cells (Invitrogen, Carlsbad, CA) with 20 µl of ligation mixture, and plate onto 5 × 15 cm LB agar plates. In the case of TLR4/TLR3^{Mu} and TLR4/TLR7^{Mu} chimera experiments, we obtained about 40,000 bacterial colonies from the plates.
7. Count and collect all colonies on the plates, and expand them in 200 ml of LB media.
8. Purify the plasmid DNAs from the pooled bacteria.
9. Generate a pool of retroviruses encoding TLR4/TLR^{Mu} chimeras.
10. Infect 293TCM cells with a pool of retroviruses encoding TLR4/TLR^{Mu} chimeras.
11. Twenty-four hours after infection, start puromycin selection.
12. Three days later, detect the cell surface TLR4/TLR3^{Mu} in puromycin-resistant cells by flow cytometry using PE-anti-mouse TLR4/MD-2 antibody.
13. Enrich the anti-mouse TLR4/MD-2 positive cells by using a cell sorter. Cells with TLR4/MD-2 expressed on the surface are obtained by two rounds of sorting to enrich strongly for real positive cells.
14. Clone the anti-mouse TLR4/MD-2 positive cells by limiting dilution; about 40 cells are resuspended with 20 ml media, distributed in the wells of 96-well plates (200 µl/well), and cultured for 2 weeks.
15. Test individual clones expressing mutated TLR4 chimeras for cell surface expression by flow cytometry.
16. Prepare total RNAs from each clone, and reverse-transcribe.
17. Amplify the resulting cDNA by PCR, and perform DNA sequencing to determine the nature of the mutation causing relocalization to the cell surface.

7.4.4 Examination of the Signaling Properties of TLRs

TLR chimeras are expressed in BMDMs from TLR4-deficient mice, and stimulated with a TLR4 ligand (Figure 7.5). Highly purified LPS is necessary for this experiment, because crude LPS preparation may contain ligands for other TLRs, such as peptidoglycan or lipopeptides, which may induce a response in untransfected CS7BL/10SCN macrophages. Highly purified LPS is now commercially available from several companies.

Protocol 7.10: Examination of the Signaling Properties of TLRs

1. Prepare BMDMs from TLR4-deficient mice as in Protocol 7.3.
2. Infect the cells on day 4 or 5 with retroviruses producing TLR chimeras as described in Protocol 7.5.
3. Plate the cells at a density of 4×10^5 cells per well in wells of a 24-well plate, or at a density of 9×10^5 cells per well of a 6-well plate. We usually use

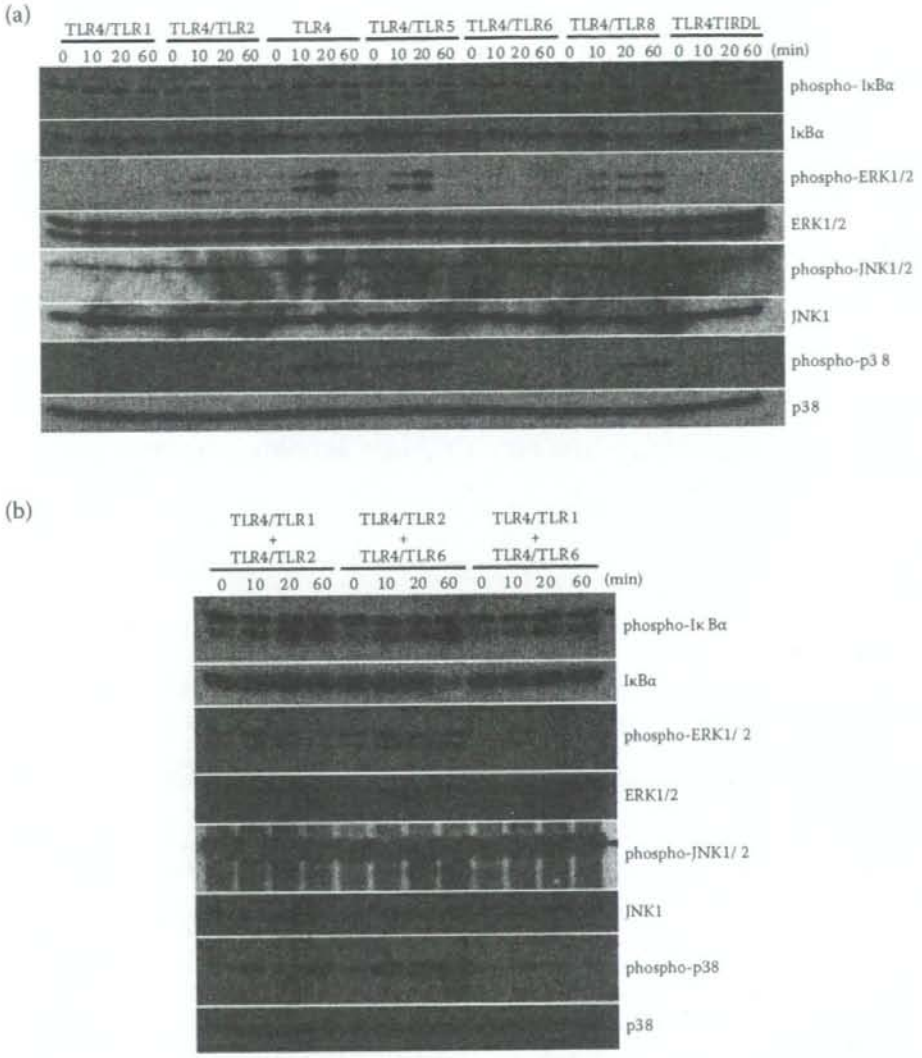

FIGURE 7.5 Analysis of the Activation of Signaling Molecules in Ligand-Regulated TLR Chimera System

Note: Ligand-induced activation of signaling molecules derived from the cytoplasmic domain of individual TLR (A) and combinations of two distinct TLRs (B). TLR4-deficient bone marrow–derived macrophages expressing an individual TLR chimera (A) or two distinct TLR chimeras (B) were stimulated with 10 ng/ml LPS for the indicated periods. Cell lysates were prepared, and the activity of NF-κB, ERK1/2, JNK1/2, and p38 was determined by immunoblotting using antibodies to unmodified and phosphorylated ERK, JNK, and p38. Note that IκBα becomes phosphorylated and degraded to activate NF-κB, and that IκBα synthesis is rapidly increased by NF-κB. TLR4TIRDL: truncated TLR4 in which the TIR domain was deleted.

Source: Modified from Nishiya, T., and DeFranco, A. L. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. *J. Biol. Chem.* 279, 19008, 2004. With permission.