Toll-like receptors (TLRs) are sensors for the detection of invading infectious agents and can initiate innate immune responses. Because the innate immune system induces an appropriate defense against different pathogens, different TLR signaling domains may have unique properties that are responsible for eliciting distinct responses to different types of pathogens. To test this hypothesis, we created ligand-regulated TLR chimeric receptors composed of the extracellular region of TLR4 and the transmembrane and cytoplasmic regions of other TLRs and expressed these chimeras in macrophages lacking endogenous TLR4. Interestingly, the chimeras between TLR4 and either TLR3, TLR7, or TLR9 were localized completely intracellularly whereas other chimeras were expressed on the cell surface. Lipopolysaccharide (LPS), a ligand for these chimeras, induced the activation of nuclear factor \( \kappa B \) and mitogen-activated protein kinases and the subsequent production of pro-inflammatory cytokines in macrophages expressing TLR4, TLR4/TLR5, or TLR4/TLR8 chimeras but not in macrophages expressing TLR4/TLR1, TLR4/TLR2, or TLR4/TLR6 chimeras. Co-expression of unresponsive chimeras in some combinations (chimeras with TLR1+TLR2 or TLR2+TLR6 but not TLR1+TLR6) resulted in LPS responsiveness, indicating functional complementarity. Furthermore, the pair of TLR2+TLR6 chimera required approximately 10-fold less LPS to induce the same responses compared with the TLR1+TLR2 pair. Finally, LPS induced effective interferon-\( \beta \) production and subsequent Stat1 phosphorylation in macrophages expressing full-length TLR4 but not other cell surface TLR chimeras. These results suggest that the functions of TLRs are diversified not only in their extracellular regions for ligand recognition but also in their transmembrane and cytoplasmic regions for subcellular localization and signaling properties.

Vertebrates have developed innate and adaptive immune systems to combat infections by bacteria, fungi, and viruses (1). The innate immune system is a first line of the defense against invading agents. It contains elements conserved between vertebrates and invertebrates, and includes receptors recognizing molecular structures highly conserved among pathogens, referred to as pathogen-associated molecular patterns. In vertebrates, the innate immune system also has a critical role in triggering the activation of adaptive immune responses by promoting presentation of antigens and up-regulation of various cytokines and co-stimulatory molecules (2).

Toll-like receptors (TLRs), \(^1\) mammalian homologs of the Drosophila receptor Toll, are an integral part of the innate immune system (1, 3). To date, 10 different human and 9 different mouse TLRs have been identified. All TLRs are type I transmembrane proteins composed of an N-terminal extracellular domain with leucine-rich repeats involved in ligand recognition, a single transmembrane domain, and a cytoplasmic domain largely made up of a TIR (Toll/IL-1 receptor homology) domain required for downstream signaling. Previous experiments with TLR knockout mice and dominant negative mutants of TLRs have demonstrated that TLRs recognize a wide variety of molecules associated with pathogens, such as bacterial cell wall components and pathogen nucleic acids (1, 3).

Because important aspects of infection such as replication and dissemination are distinctive for different types of pathogens, it would seem that optimal innate immune responses to different pathogens would include elements especially appropriate for the type of pathogen. For example, the induction of pro-inflammatory cytokines, chemokines, and inducible nitric-oxide synthase (iNOS) are important for the elimination of bacterial infections. In contrast, type I IFNs are more critical for the elimination of many viral infections. Because these immune responses are mediated by TLRs in innate immune cells, such as macrophages and dendritic cells, we hypothesized that different TLRs may have different properties which allow innate immune cells to tailor their responses to individual pathogens by producing specific patterns of cytokines and inflammatory mediators.

Comparing the signaling capabilities of endogenous TLRs is not entirely straightforward as these receptors are not all expressed on the cell surface (4, 5), their expression levels in cells may differ (4, 5), not all have known ligands, and some TLRs probably function in combinations rather than alone (6–10). For these reasons, the functional properties of altered forms of TLRs, such as chimeric molecules with constitutively active signaling properties, have been examined. Although those chimeras systems such as CD4-TLR (9, 11), Fas-TLR (7), or inte-
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grin-TLR chimeras (12) have been informative, the noninducible nature of these chimeric TLRs makes a number of issues, such as the kinetic aspects of signaling, hard to address. In addition, most TLR chimera experiments have been carried out in nonimmune cells, such as 293T or Chinese hamster ovary cells, rather than innate immune cells and have involved high levels of expression. Thus we wanted to develop a ligand-regulated chimeric system that improves on these aspects of previous TLR chimera systems, and examined the diversity of TLRs originating from the transmembrane and cytoplasmic domains of TLRs. Here we describe studies with a system in which ligand-regulated TLR chimeras were expressed at near physiological levels in primary macrophages. These studies demonstrated the functional diversity of TLR transmembrane and cytoplasmic domains for intracellular localization and signaling properties.

EXPERIMENTAL PROCEDURES

Reagents, Mice, and Cell Culture—Highly purified LPS from Salmonella minnesota R595 (Re) and peptidoglycan from Staphylococcus aureus were purchased from List Biological Laboratories (Campbell, CA) and Sigma, respectively. Recombinant mouse interferon-γ (IFN-γ) was purchased from Peprotech (Rocky Hill, NJ). Antibodies to unmodified forms of LPS, ERK1/2, JNK1, p38, and Stat1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phosphorylated IκBα, ERK1/2, JNK1/2, p38, and Stat1 were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse TNF-α monoclonal antibody (clone 7F.12, a rat IgG1) was purchased from Seikagaku America (Falmouth, MA). GolgiPlug reagent was purchased from BD Pharmingen (San Diego, CA).

C57BL/10ScN and C57BL/10ScSn mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our colony. Bone marrow-derived macrophages were prepared from 8–10-week-old mice, plated onto non-tissue culture-treated plates (Becton Dickinson Lab Ware, Franklin Lakes, NJ), and were grown in RPMI 1640 medium containing 25 mM Hepes and supplemented with 10% fetal bovine serum, 2 mM sodium pyruvate, and 15% CMG 120 cell culture medium as a source of CSF-1 (13). Macrophages at day 4 or 5 after isolation from the bone marrow were used for retroviral infection.

cDNAs and Plasmids—cDNAs for murine TLR4, TLR6, and TLR9 were kindly provided by Drs. B. Beutler (Scripps Research Institute, La Jolla, CA), A. Aderem (Institute for Systems Biology, Seattle, WA), and S. Akira (Osaka University, Osaka, Japan), respectively. Murine TLR1 and TLR2 were isolated by screening of a mouse spleen cDNA library —(amino acids 586–795), TLR2 (amino acids 631–820), TLR6 (amino acids 594–806), TLR7 (amino acids 830–1050), TLR8 (amino acids 814–1032), and TLR9 (amino acids 811–1032). In all cases, the junctions were made at the highly conserved last cysteine of the extracellular cysteine-rich domain. All TLR chimeras had the preprotryptic signal sequence followed by a FLAG-tag sequence followed by the amino terminus of the extracellular domain of TLR4. A TLR4 chimera modified in this way has been shown to respond normally to LPS (14). The TLR chimeras were cloned into the pMX-pie bicistronic retroviral vector, which encodes the gene of interest followed by an internal ribosomal entry site element and the enhanced green fluorescent protein gene (15). GFP fluorescence is therefore an indication of infection efficiency and expression level of the gene of interest. TLR4 transmembrane and intracellular domain mutant (TLR4TIRDL or 4DL, amino acids 22–664) was amplified by PCR and was cloned into pMX-pie.

Viral Production and Infection—The TLR chimeras were transduced into bone marrow-derived macrophages by retroviral gene transfer. Briefly, TLR chimeras-encoding retroviruses were produced by triple transfection of HEK293T cells with retroviral constructs along with gag-pol and amphotropic mouse ecotropic virus G glycoprotein expression constructs (16). Viral supernatants were collected 48 h after transfection and added to macrophages. Cells and virions were then centrifuged at 2,400 rpm for 1 h at room temperature followed by incubation for 6 h at 37°C in 5% CO2, 95% air. Then, viral supernatants were replaced with culture media. After 48 h, the infection efficiency was assessed by measuring GFP fluorescence with a FACSscan, and the cells were used for experiments.

Flow Cytometric Analysis—Cells were released from adherence by chelation of divalent cations with phosphate-buffered saline containing 5 mM EDTA, incubated with Fc Block (anti-mouse CD16/CD32 antibody, BD Pharmingen), and then stained with phycoerythrin (PE)-conjugated monoclonal anti-mouse TLR4/MD2 antibody (clone 45-7S, eBioscience, San Diego, CA) or isotype control (PE-conjugated anti-rat IgG2a, eBioscience). After washes, stained cells were analyzed by flow cytometry using a FACSscan and CellQuest software (Becton Dickinson, San Jose, CA). For intracellular staining, the cells were preincubated with Fc Block, fixed, and then permeabilized with the Cytofix/Cytoperm reagent (BD Pharmingen) before staining with antibodies.

Quantitation of Cytokines and Nitrite in Culture Medium—One day before stimulation, 2 × 105 cells/well in 0.5 ml of culture medium were plated in 24-well plates. After stimulation, the concentration of TNF, IL-6, and IL-12 p40 in cell culture medium was measured by enzyme-linked immunosorbent assay according to the instructions from the manufacturer (BD Pharmingen). Production of nitrite was measured by using the Griess reagent as described previously (17). For the intracellular TNF staining, cells were stained with PE-conjugated anti-mouse TNF-α monoclonal antibody (BD Pharmingen) after cells were treated with Fc Block, fixed, and permeabilized as described above.

Western Blot Analysis—One day before stimulation, cells were pelleted at 8,750 × g for 5 min at room temperature followed by incubation for 6 h at 4°C. Two μg of total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) in a total volume of 20 μl. Two μl of this reaction was used as a template for PCR amplification with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) for 24, 27, and 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (25 μg/ml of each primer). PCR products were resolved by electrophoresis. Gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA), and blotted with indicated antibodies.

PCR with Reverse Transcription (RT-PCR)—Cells were seeded, and given a 3-h rest in low growth factor medium as described above. After stimulation, total RNA was isolated with TRIzol reagent (Invitrogen) according to the instructions from the manufacturer. Two μg of total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) in a total volume of 20 μl. Two μl of this reaction was used as a template for PCR amplification with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) for 24, 27, and 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (25 μg/ml of each primer). PCR products were resolved by electrophoresis. Gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA), and blotted with indicated antibodies.

RESULTS

Ligand-regulated Chimeric Toll-like Receptors—To investigate the signaling properties and biological functions of different TLRs in a ligand-dependent manner, we established a new system in which chimeric receptors were composed of the extracellular region of TLR4 fused with the transmembrane and cytoplasmic region of other TLRs and expressed in primary macrophages lacking endogenous TLR4 (Fig. 1A). In this way, the activity of chimeric receptors could be regulated by a TLR4 ligand, LPS. Retroviral vectors were used to introduce these chimeras into bone marrow-derived macrophages derived from C57BL/10ScN mice, which carry a null mutation in the TLR4 gene (19–21). These macrophages are unresponsive to optimal concentrations of a highly purified preparation of rough LPS, and introduction of full-length TLR4 cDNA into these macrophages restored LPS responsiveness (Fig. 1B). The cell surface level of TLR4 produced by retrovirus-mediated expression was estimated to be approximately 2–3-fold elevated compared with endogenous TLR4 in bone marrow macrophages derived from wild-type mice (C57BL/10ScN) (Fig. 1C). In addition, TNF production levels in response to LPS were almost the same between wild-type macrophages and TLR4-reconstituted macrophages.
C57BL/10ScN macrophages (Fig. 1D), suggesting that this experimental system is highly physiological. Approximately 35% of these primary macrophages expressed the chimeric receptors in each case, as judged by expression of GFP, which was also encoded in the retrovirus (data not shown).

**Different Subcellular Distributions of the TLR Chimeras in Macrophages**—We first examined whether TLR chimeras were expressed on the plasma membrane by flow cytometry using an anti-TLR4/MD2 monoclonal antibody, which recognizes the extracellular region of TLR4 associated with MD2, an accessory molecule required for cell surface expression and LPS responsiveness of TLR4 (22). Interestingly, only some TLR chimeras were expressed on the cell surface. In intact cells, TLR4/TLR1, TLR4/TLR2, TLR4/TLR5, TLR4/TLR6, and, to a lesser extent, TLR4/TLR8 chimeras were detected on the cell surface (Fig. 2). In contrast, the TLR4/TLR3, TLR4/TLR7, and TLR4/TLR9 chimeras were not detected on the cell surface. These chimeras, however, were readily detected when cells were fixed and permeabilized before staining (Fig. 2). Identical results were obtained in 3T3 fibroblasts (data not shown). These results were confirmed by fluorescence microscopy (data not shown). We also observed that the TLR4/TLR9 chimera was strongly co-localized with hemagglutinin-tagged wild type TLR9 in intracellular compartments, suggesting that TLR chimeras were localized in macrophages similarly to wild type TLRs.

**Cytoplasmic Domains of Individual TLRs Exhibit Distinct Signaling Properties**—This ligand-regulated chimeric receptor system allowed us to compare the ability of the different chimeras to trigger signaling cascades involved in the production of pro-inflammatory cytokines. We examined the ability of TLR chimeras to activate NF-κB, ERK1/2, JNK1/2, and p38 MAPK, because all these molecules are activated by TLR ligands, and are also involved in the induction of inflammatory responses (23). As shown in Fig. 3A, the phosphorylation and degradation

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2 T. Nishiya and A. L. DeFranco, unpublished data.
of IkBα were detected in macrophages expressing TLR4, TLR4/TLR5, and, with slower kinetics, TLR4/TLR8 chimeras, whereas these events were not detected in macrophages expressing TLR4/TLR1, TLR4/TLR2, or TLR4/TLR6 chimeras. Similarly, phosphorylated ERK1/2, JNK1/2, and p38 were detected in macrophages expressing TLR4 and the TLR4/TLR5 chimera. Phosphorylated ERK1/2 and p38, but not JNK1/2, were also detected in macrophages expressing the TLR4/TLR8 chimera, with slower but prolonged activation. In contrast, activation of these signaling events was undetectable in macrophages expressing TLR4/TLR1, TLR4/TLR2, or TLR4/TLR6 chimeras, with the exception of a weakly phosphorylated

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**Fig. 2. Expression and cellular distribution of the TLR chimeras.** Expression of the TLR chimeras in macrophages was determined by flow cytometric analysis using anti-TLR4/MD2 antibody as described under "Experimental Procedures." 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; **solid line**, anti-TLR4/MD2 antibody. The fraction of cells that was GFP-positive was essentially identical for all cells expressing TLR chimeras, ranging from 34.5 ± 1.0% to 38.7 ± 2.5% of the macrophages.

**Fig. 3. Differential activation of signaling cascades by individual TLR chimeras.** A and B, cells were stimulated with 10 ng/ml (A) or 100 ng/ml (B) LPS for the indicated periods. Cell lysates were prepared, and the activity of NF-κB, ERK1/2, JNK1/2, and p38 was determined by Western blotting using antibodies to unmodified and phosphorylated proteins. Equal loading of lanes was verified by immunoblotting of unphosphorylated ERK, JNK, and p38. Note that IkBα becomes phosphorylated and degraded to activate NF-κB, and that IkBα synthesis is rapidly increased by NF-κB.

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**TABLE**

| TLR4/TLR1 | TLR4/TLR2 | TLR4/TLR3 | TLR4/TLR4 | TLR4/TLR5 | TLR4/TLR6 | TLR4/TLR8 | TLR4/TLR9 | TLR4/MD2 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|----------|
| Intact    | Intact    | Intact    | Intact    | Intact    | Intact    | Intact    | Intact    | Intact    |
| Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized | Fixed/Permeabilized |

**Graphs**

- **A**
  - phospho-IκBα
  - IkBα
  - phospho-ERK1/2
  - ERK1/2
  - phospho-JNK1/2
  - JNK1
  - phospho-p38
  - p38

- **B**
  - phospho-p38
  - p38
ERK1/2 in macrophages expressing the TLR4/TLR2 chimera, and of a phosphorylated p38 with slow kinetics in macrophages expressing the TLR4/TLR6 chimera when cells were stimulated with a high dose of LPS (100 ng/ml) (Fig. 3B).

LPS-induced Homo-multimerization of the Cytoplasmic Domains of TLR4, TLR5, and TLR8, but Not TLR1, TLR2, and TLR6, Can Mediate Inflammatory Responses—One of the most important functions of TLRs is to induce early inflammatory responses by the production of pro-inflammatory cytokines. Therefore, we next examined whether the cytoplasmic domains of individual TLRs can mediate inflammatory responses. Nitrite accumulation, which reflects the expression of the iNOS gene, was detected in a ligand-dependent manner in macrophages expressing TLR4 or the TLR4/TLR5 chimera (Fig. 4).

Nitrite production in response to TLR4 or TLR4/TLR5 stimulation was significantly enhanced in the presence of IFN-γ as expected, because iNOS induction is known to be regulated by both TLR and IFN signaling (24). Macrophages expressing the TLR4/TLR8 chimera made less nitrite, but a small amount was detected in response to LPS plus IFN-γ. Similar results were seen for production of TNF, IL-6, and IL-12, except no significant enhancement was seen in the presence of IFN-γ. Inflammatory responses mediated by TLR4/TLR5 and TLR4/TLR8 chimeras required approximately 10 and 100 times more LPS compared with the amount of LPS needed to induce a comparable response in macrophages expressing TLR4. These differences could be because of the cell surface levels of each chimera as the TLR4/TLR8 chimera was expressed at 10–20 times lower levels than TLR4 and the TLR4/TLR5 chimera was expressed at approximately 4 times lower levels (Table I).

In contrast, macrophages expressing TLR4/TLR1, TLR4/TLR2, or TLR4/TLR6 chimeras did not induce any inflammatory responses tested. TNF was normally induced in the transduced macrophages expressing different chimeras by the TLR2 ligand, peptidoglycan and the TLR9 ligand, CpG-DNA (data not shown), suggesting that signaling components involved in the induction of inflammatory responses were intact in all macrophages. Production or lack of production of inflammatory mediators correlated with the ability of the chimeras to activate signaling molecules. The macrophages expressing TLR chimeras that were seen only intracellularly failed to respond to LPS even at high concentration (100 ng/ml) (data not shown).

Complementary Signaling Functions of Some TLR Chimeras—Aderem’s group has suggested that TLR2 may function as a hetero-multimer with TLR1 or TLR6 (9), and the response to some bacterial lipoproteins has been shown to be lost in macrophages lacking either TLR2 or TLR6 (7). Therefore, we examined whether the combination of two of the unresponsive TLR chimeras, TLR4/TLR1, TLR4/TLR2, and TLR4/TLR6, could trigger activation of intracellular signaling events. In these experiments, macrophages were infected with two different retroviruses to express two different TLR chimeras. Efficiency of dual infections and cell surface levels of TLR chimeras were almost the same between the different combinations tested (Table II). In agreement with the evidence that TLR2 and
It has recently been suggested that restricted to TLR4 Signaling.

**Table I**

| Viruses       | Cell surface levels of TLR4/MD2 |
|---------------|---------------------------------|
|               | MFI                             |
| TLR4/TLR1     | 376 ± 44                        |
| TLR4/TLR2     | 366 ± 28                        |
| TLR4          | 484 ± 56                        |
| TLR4/TLR5     | 124 ± 8                         |
| TLR4/TLR6     | 473 ± 54                        |
| TLR4/TLR8     | 26 ± 2                          |
| TLR4/TLR1 + TLR4/TLR6 | 548 ± 32 |
| Vector        | 18 ± 1                          |

**Table II**

| Viruses       | GFP + cells | MFI |
|---------------|-------------|-----|
| TLR4/TLR1 + TLR4/TLR2 | 35.4 ± 3.5 | 430 ± 30 |
| TLR4/TLR2 + TLR4/TLR6 | 38.4 ± 2.5 | 486 ± 16 |
| TLR4/TLR1 + TLR4/TLR6 | 35.7 ± 2.6 | 485 ± 33 |

TLR6 can function together to promote responses to bacterial lipopeptides, the combination of TLR4/TLR2 plus TLR4/TLR6 induced activation of all signaling molecules tested (Fig. 5). Similar but smaller activity was detected in the combination of TLR4/TLR1 plus TLR4/TLR2, whereas TLR4/TLR1 plus TLR4/TLR6 showed a minimal ability to activate those signaling molecules.

Consistent with their relative abilities to promote activation of signaling pathways, the combination of TLR4/TLR2 plus TLR4/TLR6 showed much higher production of pro-inflammatory cytokines than the combination of TLR4/TLR1 plus TLR4/TLR2, and no cytokine production was seen upon addition of LPS to cells expressing TLR4/TLR1 plus TLR4/TLR6 (Fig. 6).

Effective Antiviral Response by IFN-β Production Is Restricted to TLR4 Signaling—It has recently been suggested that TLR signaling also mediates antiviral innate immune responses by directing production of type I IFNs, especially IFN-β (25–27). To investigate which TLR cytoplasmic domains can mediate type I IFN production, we conducted RT-PCR to detect IFN-β mRNA in macrophages expressing cell surface TLR chimeras. As shown in Fig. 7A, IFN-β mRNA was strongly induced by LPS in macrophages expressing TLR4, but not in cells expressing TLR4/TLR1, TLR4/TLR2, or TLR4/TLR6 chimeras. Low levels of IFN-β mRNA were detected in LPS-stimulated macrophages expressing TLR4/TLR5 and TLR4/TLR8 chimeras. These inductions were detected after 24 cycles of PCR reaction and did not saturate even at 30 cycles (data not shown). The phosphorylation of tyrosine 701 on Stat1, a critical event for the induction of antiviral responses by IFN-β and its receptor, was next examined. Macrophages expressing TLR4 showed robust tyrosine phosphorylation of Stat1 at 2 h after LPS stimulation (Fig. 7B). This response was inhibited by anti-IFN-β antibody, indicating that it was caused by IFN-β production. In contrast, no significant Stat1 phosphorylation was observed in macrophages expressing other TLR chimeras even at 4 h after stimulation.

We then investigated whether combinations of unresponsive TLR chimeras could induce IFN-β. As shown in Fig. 7C, IFN-β mRNA was detected at low levels in macrophages expressing TLR4/TLR1 plus TLR4/TLR2 or TLR4/TLR2 plus TLR4/TLR6 at 30 cycles of PCR reaction, but was not detected at 24 or 27 cycles (data not shown). Even this low level of IFN-β mRNA was not detected in macrophages expressing TLR4/TLR1 plus TLR4/TLR6. In addition, no significant phosphorylation of Stat1 was observed in macrophages expressing these combinations of TLR chimeras (Fig. 7D). The failure of TLR4/TLR1 + TLR4/TLR2 or TLR4/TLR2 + TLR4/TLR6 to make nitrites (Fig. 6) supports the previous observation that LPS-induced iNOS is IFN-β-dependent (25). These data together demonstrate that, among cell surface TLRs, only TLR4 is able to produce the amount of IFN-β required for signaling for the antiviral response.

**DISCUSSION**

The current understanding of TLR signaling comes from three types of experiments: 1) signaling and cytokine responses to TLR ligands of macrophages or other cell types, 2) effects of TLR knockouts, and 3) signaling and cytokine responses of TLR chimeras in which the cytoplasmic domain of TLRs is used. The first approach has the caveat that, in most cases, it is not completely clear which TLRs are responsible for mediating the response to a particular ligand preparation; in addition, reagents are not available in most cases to measure TLR expression levels, and therefore it is unclear whether the differences seen are because of different signaling abilities or are influenced by different expression levels. The knockout approach is extremely powerful, and addresses what is necessary but not what is sufficient, which is what the chimera approach provides. In addition, knockout studies do not distinguish between the roles of TLR for ligand binding versus signaling. Although TLR chimera experiments have been informative, the TLR chimera systems used previously have had the problem that they were constitutive not ligand-activated. The constitutive nature of these chimeric receptors limits the analysis of early signaling events and other cellular responses that are dependent on the timing of activation. In addition, many of the TLR chimera experiments have involved overexpression in HEK293 or Chinese hamster ovary cells, systems that have been prone to artifacts in studying other receptor systems.

In this study, we have succeeded in creating a novel TLR
chimera system that is an improvement over previous studies in that the chimera was expressed in primary macrophages at physiological levels and exhibited ligand-regulated activity. This highly physiological system demonstrated that the chimeras between TLR4 and either TLR3, TLR7, and TLR9 were localized to intracellular organelles without cell surface expression (Fig. 2). Other chimeras were expressed at the cell surface, but exhibited distinctive signaling properties. TLR4, TLR4/TLR5, and TLR4/TLR8 chimeras were able to activate signaling cascades for the production of pro-inflammatory cytokines when expressed individually (Figs. 3 and 4). On the other hand, chimeras containing the transmembrane and cytoplasmic domains of TLR1, TLR2, and TLR6 were only functional as heterodimers in certain combinations (TLR1+TLR2 or TLR2+TLR6 but not TLR1+TLR6) and could not function individually (Figs. 5 and 6). Moreover, the pair of TLR2+TLR6 chimeras required a much lower concentration of ligand to induce the same responses compared with the TLR1+TLR2 pair. In addition, only TLR4 and none of the cell surface TLR chimeras were able to induce significant IFN-β production and subsequent Stat1 phosphorylation (Fig. 7). These observations demonstrate that the transmembrane and cytoplasmic domains of TLRs define both different subcellular targeting and signaling properties of TLRs.

The TLR chimeras newly developed here utilized the extracellular domain of TLR4 to regulate the activity of chimeras by the TLR4 ligand, LPS. The structural similarity to other TLRs may explain the success of achieving ligand-regulated activity of these chimeric receptors. There is considerable evidence that the TLR4-MD2 complex functions as a homo-multimer to mediate responses to LPS (28). For example, the LPS response is absent in TLR4-deficient cells but intact in mice lacking TLR1 (8), TLR2 (29), TLR3 (18), TLR6 (7), TLR7 (30), and TLR9 (31). Moreover, introduction of TLR4 and MD2 into heterologous cell types such as 293T cells will confer LPS responsiveness (32) reflecting the fine specificity of the species source of TLR4 and MD2 (33, 34). Therefore, the TLR chimeras used here expressed in TLR4-deficient macrophages likely exhibit localization and signaling responses emanating from their transmembrane and cytoplasmic domains without contributions of endogenous TLRs.

The results described here are for the most part in agreement with earlier results obtained with constitutively active chimeric receptors, such as CD4-TLR (9, 11, 35), Fas-TLR (7), or integrin-TLR chimeras (12). Those studies have suggested that constitutive homo-multimerization of TLR4 and TLR5 cytoplasmic domains can induce pro-inflammatory cytokine production, whereas TLR1, TLR2, and TLR6 cannot induce responses alone, but can induce cytokine production if present in complementary combinations (TLR2 with TLR1 or TLR6 but not TLR1 with TLR6) (9, 12, 35). Our results with ligand-responsive TLR chimeras gave similar results with regard to cytokine induction, and extended these studies to demonstrate signaling properties of the chimeric receptors that paralleled cytokine production. Taken together, our work and previous work indicate that the functional properties of particular TLRs are intrinsic to the TLR cytoplasmic domains, and are triggered similarly by TLR ligands and by artificial multimerization.

Interestingly, we found that the activity of the TLR2+TLR6 chimera pair was much higher than that of the TLR1+TLR2 pair (Figs. 5 and 6). This difference has not been observed in studies using constitutively active chimeras. There were no significant differences in infection efficiency of each chimera-producing retrovirus and cell surface levels of those chimeras (Table I, II), suggesting that this difference was not the result of differential expression of the TLR4/TLR1 versus the TLR4/TLR6 chimera. It has been shown that TLR2 and TLR6 associate with each other in the absence of ligand in a manner that...
requires the TLR2 extracellular domain but not the TLR2 cytoplasmic domain (9) suggesting that the TLR4/TLR2 chimera does not associate with endogenous TLR6 and providing an explanation for why the TLR4/TLR2 chimera fails to signal unless expressed together with the TLR4/TLR6 chimera. In addition to the TLR4/TLR6 chimera's ability to induce stronger signaling as a hetero-multimer with the TLR4/TLR2 chimera, we observed partial signaling ability by this chimera on its own. At a relatively high dose of LPS (100 ng/ml), TLR4/TLR6 but not TLR4/TLR1 stimulated p38 activation (Fig. 3B), suggesting that the higher activity of TLR2+TLR6 chimera pair may result from the better function of the signaling domain of TLR6. Although TLR1 and TLR6 have significant similarity in terms of chromosomal location (human chromosome 4p14), gene structure (a single exon), and the protein sequences of their transmembrane and cytoplasmic domains (>90% identity), the results presented here demonstrate that TLR1 and TLR6 have distinctive signaling properties. It should be noted that the amino acid sequences of the last 10 amino acids at the COOH termini of TLR1 and TLR6 are significantly different (36). Whether this region confers the observed quantitative difference in signaling capacity is unclear at present.

In addition to the distinction between TLRs that can signal by themselves versus those that can only signal in a complementary fashion, there were also some differences in what signaling reactions are activated by different TLRs. Indeed, it had previously been recognized that some TLR ligands, such as CpG-DNA, poly(I:C), and LPS, induce the antiviral innate immune response of producing IFN-β (25–27). Similarly, we found that, among TLR chimeras expressed at the cell surface, only TLR4 produced enough IFN-β to induce robust phosphorylation of Stat1, which is essential for the induction of the antiviral response. Two adaptor molecules, TRIF/TICAM-1 (37, 38) and TRAM/TICAM2 (39–41), have recently been identified as the likely mediator of this response. TRAM is likely to be specific to TLR4, because it seems to link TLR4, but not TLR3, with TRIF (39–41). Both poly(I:C)- and LPS-induced IFN-β production are dependent on TRIF, as demonstrated by analysis of mice genetically inactivated for TRIF (42, 43). A slight up-regulation of IFN-β and IFN-β-inducible genes was detected in embryonic fibroblasts from TRIF-deficient mice, but was undetectable in embryonic fibroblasts from TRIF/MyD88 dou-

![Fig. 7. Effective antiviral response by IFN-β production is restricted to TLR4. A and C, RT-PCR analysis of IFN-β production. Cells expressing the indicated TLR chimeras were stimulated with 100 ng/ml LPS for 4 h. Then, total RNA was isolated, and the expression of IFN-β and HPRT were determined by RT-PCR. Data after 30 and 24 cycles are shown for IFN-β and HPRT, respectively. B and D, phosphorylation of Stat1 induced by IFN-β production. Cells were stimulated with 100 ng/ml LPS for the indicated periods, and lysates were prepared and subjected to Western blotting using antibodies to unmodified or phosphorylated Stat1. To neutralize IFN-β, anti-IFN-β antibody, or anti-FcγRII/III antibody as a negative control, was added to the cells at the indicated dilutions, 5 min before stimulation with LPS. 4FL, full-length TLR4; 4DL, TIR domain-deleted TLR4.](http://www.jbc.org/Downloaded from)
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