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## Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the *Lps* Gene Product<sup>1</sup>

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Yoshifumi Takeda,<sup>†</sup> Kiyoshi Takeda,<sup>\*,§</sup> and  
Shizuo Akira<sup>2\*§</sup>

The human homologue of *Drosophila* Toll (hToll), also called Toll-like receptor 4 (TLR4), is a recently cloned receptor of the IL-1/Toll receptor family. Interestingly, the TLR4 gene has been localized to the same region to which the *Lps* locus (endotoxin unresponsive gene locus) is mapped. To examine the role of TLR4 in LPS responsiveness, we have generated mice lacking TLR4. Macrophages and B cells from TLR4-deficient mice did not respond to LPS. All these manifestations were quite similar to those of LPS-hyporesponsive C3H/HeJ mice. Furthermore, C3H/HeJ mice have, in the cytoplasmic portion of TLR4, a single point mutation of the amino acid that is highly conserved among the IL-1/Toll receptor family. Overexpression of wild-type TLR4 but not the mutant TLR4 from C3H/HeJ mice activated NF- $\kappa$ B. Taken together, the present study demonstrates that TLR4 is the gene product that regulates LPS response. *The Journal of Immunology*, 1999, 162: 3749–3752.

In adult *Drosophila*, the toll protein participates in host defense against fungal infection (1). The cytoplasmic region of *Drosophila* toll is homologous to that of the IL-1R family (2). Both *Drosophila* toll and IL-1R are known to signal through the NF- $\kappa$ B pathway (3, 4). Recently, human homologues of

*Drosophila* toll, termed Toll-like receptors (TLR)<sup>3</sup>, have been cloned, and it is implicated that they activate both innate and adaptive immune responses in vertebrates (5–8). TLR2 has been shown to be a signaling receptor that is activated by LPS (9, 10).

The C3H/HeJ mouse strain is characterized by hyporesponsiveness to LPS (11). Macrophages from C3H/HeJ mice fail to induce inflammatory cytokines, including TNF- $\alpha$ , IL-1, and IL-6. Their splenic B cells do not proliferate after exposure to LPS. The molecular basis of this hyporesponsiveness is unknown, but it may result from defective membrane signal transduction after LPS binding. The hyporesponsive phenotype of the C3H/HeJ mouse maps to the *Lps* locus (endotoxin unresponsive gene locus) on mouse chromosome 4 (12). The corresponding chromosomal location in the human genome is chromosome 9q32–33; that is the same region to which human TLR4 has been mapped (8). Recent genetic and physical mapping of the *Lps* locus identifies TLR4 as a candidate gene in the critical region (12).

In the present study, we have generated TLR4-deficient (TLR4<sup>-/-</sup>) mice and examined the LPS responsiveness. TLR4<sup>-/-</sup> mice showed hyporesponsive to LPS to an extent similar to that of C3H/HeJ mice. We also detected a single point mutation in the TLR4 gene of C3H/HeJ mice. These results demonstrate that TLR4 is the gene product of the *Lps* locus.

### Materials and Methods

#### Reagents

LPS from *Escherichia coli* serotype O55:B5 prepared by Westphal method and *Salmonella minnesota* Re-595 (R mutants) prepared by phenol-chloroform-petroleum ether extraction procedure were purchased from Sigma (St. Louis, MO). *E. coli*-type synthetic lipid A (compound 506) was described previously (13). Biotinylated anti-mouse I-A Ab were purchased from PharMingen (San Diego, CA).

#### Generation of murine TLR4<sup>-/-</sup> mice

The murine TLR4 genomic clone was screened from the 129/SvJ mouse genomic library (Stratagene, La Jolla, CA). A targeting vector was designed to replace a 2.54-kbp genomic fragment with neomycin resistance

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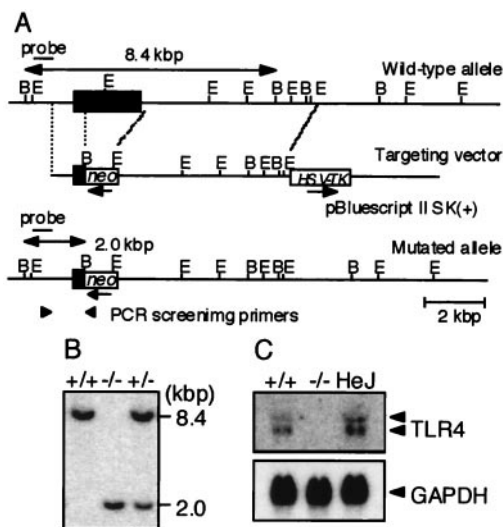
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<sup>3</sup> Abbreviations used in this paper: TLR, Toll-like receptor; TLR4<sup>-/-</sup> mice, TLR4-deficient mice; *neo*, neomycin resistance gene; *HSV-TK*, herpes simplex virus-thymidine kinase gene; ES cell, embryonic stem cell; NO<sub>2</sub><sup>-</sup>, nitric oxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



**FIGURE 1.** Targeted disruption of the mouse *TLR4* gene. **A**, Targeting vector and restriction map of the *Tlr4* locus. The restriction map of the wild-type allele, targeting vector and the mutated allele are shown. Filled boxes denote the coding exons. The orientation of *neo* and *HSV-TK* are indicated by the arrow. E, *EcoRI*; B, *BamHI*. **B**, Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with *BamHI*, electrophoresed, and hybridized with the probe as shown in Fig. 1A. The approximate size of the wild-type band is 8.4 kbp, and the mutated band is 2.0 kbp. +/+, Wild-type; +/-, heterozygous mutant; -/-, homozygous mutant. **C**, Northern blot analysis of mouse *TLR4* mRNA. Total RNA was isolated from the splenocytes of wild-type, *TLR4*<sup>-/-</sup>, and C3H/HeJ mice, electrophoresed and transferred to a nylon membrane. A mouse *TLR4* cDNA probe was used for hybridization. The same membrane was rehybridized with a GAPDH probe.

gene (*neo*) from pMC1-neo-poly(A) (Stratagene). A herpes simplex virus-thymidine kinase cassette (*HSV-TK*) was inserted into the 3' end of the vector. The resultant targeting vector was electroporated into E14.1 ES cells. Generation of chimeric mice and mutant mice was essentially as described previously (14).

#### B cell assay

Proliferative response of B cells and I-A expression on B cells were analyzed as described (14).

#### Cytokine production

Peritoneal macrophages were isolated 3 days after i.p. thioglycolate injection, and then  $5 \times 10^4$  cells were cultured with various reagents for 24 h. Production of TNF- $\alpha$  was measured by ELISA (Genzyme, Boston, MA), and production of NO<sub>2</sub><sup>-</sup> was measured by NO<sub>2</sub>/NO<sub>3</sub> assay kit-C (Dojindo, Kumamoto, Japan).

#### Sequence analysis of mouse *TLR4* cDNA

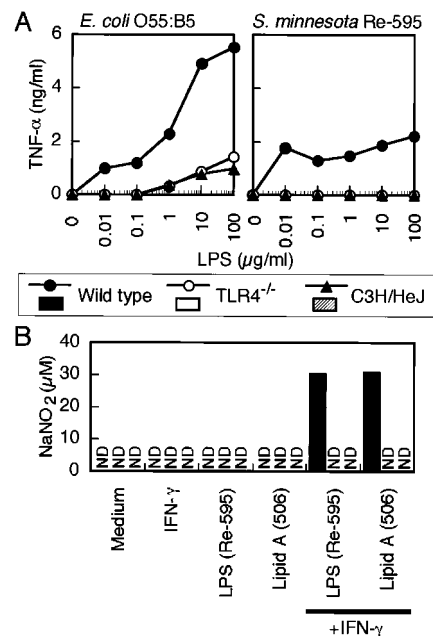
Total RNA was extracted from splenocytes of C3H/HeJ and C3H/HeJ mice, reverse-transcribed, and amplified by PCR using a set of primers. The resulting DNA fragments were sequenced. The primer sequences were available upon request.

#### Reporter assay

The transmembrane and the cytoplasmic domain of murine *TLR4* (amino acid residue 623 to 835) were fused to the extracellular domain of murine CD4 (amino acid residue 1 to 384). The chimeras were ligated into a mammalian expression vector pEF-BOS (15). Two hundred ninety-three cells ( $1 \times 10^5$ ) seeded on 6-well plates were transiently cotransfected with 2  $\mu$ g of indicated expression plasmids together with NF- $\kappa$ B reporter plasmid. After 24 h, the reporter gene activity was measured and normalized as described previously (15).

## Results and Discussion

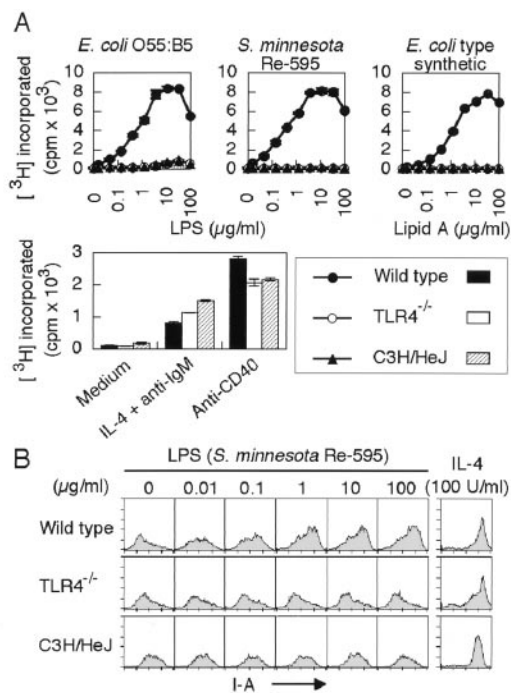
We generated the mice deficient in *TLR4* and examined the LPS responsiveness. The mouse *TLR4* gene was disrupted by homol-



**FIGURE 2.** Impaired LPS responsiveness in *TLR4*<sup>-/-</sup> macrophages. **A**, Peritoneal macrophages from wild-type, *TLR4*<sup>-/-</sup>, and C3H/HeJ mice were isolated 3 days after i.p. thioglycolate injection. The cells were cultured with the indicated concentrations of *E. coli* O55:B5 LPS or *S. minnesota* Re-595 LPS for 24 h. Concentrations of TNF- $\alpha$  in the culture supernatants were measured by ELISA. **B**, Peritoneal macrophages were isolated and cultured with 1.0  $\mu$ g/ml *S. minnesota* Re-595 LPS (Re-595) or 1.0  $\mu$ g/ml *E. coli*-type synthetic lipid A (506) in the presence or absence of 30 U/ml IFN- $\gamma$  for 24 h. Concentrations of NO<sub>2</sub><sup>-</sup> in the culture supernatants were measured. Indicated values are means of duplicates. ND, Not detected.

ogous recombination in E14.1 embryonic stem (ES) cells. A targeting vector was designed to replace both the transmembrane and cytoplasmic regions of *TLR4* (amino acid residue 86–835) with *neo* (Fig. 1A). The targeted ES clones successfully transmitted the disrupted *TLR4* gene through the germline (Fig. 1B). *TLR4*<sup>-/-</sup> mice were born normally, grew healthy, and showed no obvious abnormalities until 10 wk. Northern blot analysis using total RNA from splenocytes confirmed the absence of expression of *TLR4* mRNA in *TLR4*<sup>-/-</sup> mice (Fig. 1C). FACS analysis of the expression of CD3, B220, CD4, CD8, IgM, I-Ab, and Fc $\gamma$ R on thymocytes, splenocytes, and peritoneal exclude cells showed normal composition in 6-wk-old *TLR4*<sup>-/-</sup> mice (data not shown).

We first examined the LPS response in macrophages from *TLR4*<sup>-/-</sup> mice. LPS contains polysaccharide and lipid A portion, and much of LPS responses are mediated by the lipid A portion (11). LPS (*S. minnesota* Re-595 or *E. coli* O55:B5), and synthetic *E. coli*-type lipid A (compound 506) were used in this study. It has been demonstrated that C3H/HeJ mice lack the responsiveness to LPS (Re-595) and synthetic lipid A (506) (13). Peritoneal macrophages were isolated from wild-type, *TLR4*<sup>-/-</sup>, and C3H/HeJ mice, cultured in the presence of various concentration of LPS from Re-595 or O55:B5, and production of TNF- $\alpha$  was measured (Fig. 2A). Wild-type macrophages produced an increased level of TNF- $\alpha$  in response to each LPS in a dose-dependent manner. In contrast, *TLR4*<sup>-/-</sup> and C3H/HeJ macrophages did not produce any detectable level of TNF- $\alpha$  in response to Re-595 LPS but did produce low levels of TNF- $\alpha$  in response to a high concentration of O55:B5 LPS. Next, the macrophages were cultured with LPS or lipid A in the presence or absence of IFN- $\gamma$  for 24 h, and production of nitric oxide (NO<sub>2</sub><sup>-</sup>) was measured (Fig. 2B). Wild-type

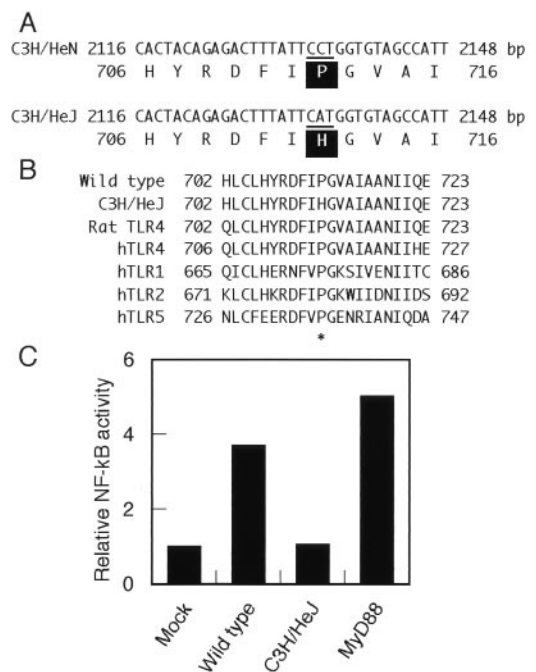


**FIGURE 3.** Impaired LPS responsiveness in TLR4<sup>-/-</sup> B cells. *A*, Splenocytes ( $1 \times 10^5$ ) from wild-type, TLR4 and C3H/HeJ mice were cultured with the indicated concentrations of O55:B5 LPS, Re-595 LPS, or *E. coli*-type synthetic lipid A. Splenocytes were also cultured with 100 U/ml IL-4 plus 5 μg/ml anti-IgM Ab, or 500 ng/ml anti-CD40 Ab for 48 h. [<sup>3</sup>H]thymidine (37 kBq) was pulsed for the last 6 h. [<sup>3</sup>H] incorporation was measured with a scintillation counter. Indicated values are means ± SD of triplicates. *B*, Splenocytes ( $1 \times 10^6$ ) were cultured with the indicated concentrations of Re-595 LPS or 100 U/ml IL-4. At 48 h culture period, the cells were harvested and stained with biotin-conjugated anti-I-A Ab and phycoerythrin-conjugated anti-B220 Ab followed by streptavidin-FITC. Stained cells were analyzed on FACScalibur using Cell Quest software (Becton Dickinson, San Jose, CA).

macrophages produced NO<sub>2</sub><sup>-</sup> in response to all LPS and lipid A as tested when cocultured with IFN-γ. In contrast, macrophages from TLR4<sup>-/-</sup> and C3H/HeJ did not produce any detectable level of NO<sub>2</sub><sup>-</sup> in response to both Re-595 LPS and *E. coli*-type lipid A 506.

We next analyzed LPS responsiveness of B cells. Splenic B cells were cultured in the presence of various concentrations of LPS (O55:B5, Re-595) or lipid A (506). Wild-type B cells showed an increased proliferative response to LPS and lipid A in a dose-dependent manner (Fig. 3*A*). In contrast, both TLR4<sup>-/-</sup> and C3H/HeJ B cells did not proliferate in response to O55:B5 LPS, Re-595, or lipid A 506. In addition, TLR4<sup>-/-</sup> B cells normally proliferated in response to IL-4 plus anti-IgM Ab or anti-CD40 Ab, indicating that the defective proliferative response is LPS specific (Fig. 3*A*). We further analyzed an augmentation of MHC class II expression on B cells in response to LPS (Re-595). Wild-type B cells showed the increased expression of MHC class II in response to LPS. However, LPS-induced augmentation of MHC class II expression on TLR4<sup>-/-</sup> and C3H/HeJ B cells was severely impaired (Fig. 3*B*). Both wild-type, TLR4<sup>-/-</sup>, and C3H/HeJ B cells expressed almost the same level of MHC class II in response to IL-4. Thus, B cells of TLR4<sup>-/-</sup> mice are specifically hyporesponsive to LPS. Taken together, all these findings demonstrate that TLR4<sup>-/-</sup> mice present almost the same phenotype with C3H/HeJ mice.

We therefore examined whether there is any abnormality in the TLR4 gene of C3H/HeJ mice. As shown in Fig. 1*C*, expression of TLR4 mRNA was detected in splenocytes of C3H/HeJ mice at the



**FIGURE 4.** A point mutation of the TLR4 gene in C3H/HeJ mice. *A*, Sequence comparison of mouse TLR4 cDNA obtained from C3H/HeJ and C3H/HeN mice. C3H/HeJ mice had one amino acid replacement (aa residue 712) in intracellular domain compared with wild-type mice. This amino acid residue is thought to be a critical for TLR signaling. *B*, The amino acid sequences of mouse TLR4 cytoplasmic domain obtained from wild-type and C3H/HeJ mice are aligned to those of other TLR family members (8). *C*, Two hundred ninety-three cells were transiently cotransfected with the expression plasmid for CD4-TLR4 from C3H/HeN or C3H/HeJ together with NF-κB-dependent reporter gene plasmid. The expression plasmid for MyD88 was used as a positive control (4). A similar result was obtained from another independent experiment.

similar level relative to the wild-type mice. We next searched for a mutation in the TLR4 gene of C3H/HeJ mice. Using a set of primers, we amplified the coding region of TLR4 gene from the spleen of LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice after reverse transcription of mRNA, and the nucleotide sequences of the resultant PCR products were compared. TLR4 cDNA from C3H/HeN mice contained an open reading frame of 2508 bp, and predicted protein sequence produces an 835-aa residue. Murine TLR4 showed 86.0% and 64.5% overall amino acid similarity to rat and human TLR4, respectively (data not shown). The sequence analysis revealed one nucleotide difference in the cytoplasmic region of the TLR4 gene between C3H/HeN and C3H/HeJ mice (Fig. 4*A*). Transversion from C to A in C3H/HeJ mice resulted in an amino acid change from proline to histidine. As shown in Fig. 4*B*, this proline residue is highly conserved in the Toll receptor family. We next examined whether this mutation is the cause of defective LPS signaling in C3H/HeJ mice. We constructed two versions of expression vectors for TLR4 from C3H/HeN and C3H/HeJ mice. The extracellular portion of both constructs was replaced to that of CD4 because CD4-mediated aggregation may induce the activation of the downstream events, as in the case of the human TLR4 (5). As shown in Fig. 4*C*, CD4-TLR4 from C3H/HeN (wild-type) significantly induced the activation of NF-κB-dependent reporter gene expression. In contrast, TLR4 from C3H/HeJ failed to activate NF-κB, suggesting that this portion is critical for its signaling leading to the activation of NF-κB.

In the present study we have generated mice deficient in TLR4 and examined LPS responsiveness, comparing with C3H/HeJ mice. TLR4-deficient mice showed hyporesponsiveness to LPS to an extent similar to that of C3H/HeJ mice. We also detected a missense mutation in the cytoplasmic portion of TLR4 in C3H/HeJ mice. Although there was a slight difference in the LPS response between TLR4<sup>-/-</sup> and C3H/HeJ mice, this may be due to a difference in the mutation (null mutation in TLR4<sup>-/-</sup> but a point mutation in C3H/HeJ mice), as well as in the strain. Taken together, these results demonstrate that TLR4 is the gene product of *Lps* locus, the defect of which results in hyporesponsiveness to LPS in C3H/HeJ mice. During preparation of our manuscript, a similar finding was published through the analyses of genetic and physical mapping of the *Lps* locus (16).

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