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J Immunol 2013; 190:195-204; Prepublished online 30 November 2012;
doi: 10.4049/jimmunol.1201047
http://www.jimmunol.org/content/190/1/195

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/11/30/jimmunol.1201047.DC1

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Reduced Surface Expression of TLR4 by a V254I Point Mutation Accounts for the Low Lipopolysaccharide Responder Phenotype of BALB/c B Cells

Hiroki Tsukamoto,*† Kenji Fukudome,* Shoko Takao,* Naoko Tsuneyoshi,* Shoichiro Ohta,‡ Yoshinori Nagai,§ Hideyuki Ihara,‡ Kensuke Miyake,¶ Yoshitaka Ikeda,‡ and Masao Kimoto*

LPS is recognized by TLR4 and radioprotective 105 kDa in B cells. Susceptibility to LPS in murine B cells is most closely linked to the locus containing the TLR4 gene. However, the molecular mechanism underlying genetic control of LPS sensitivity by this locus has not been fully elucidated. In this study, we revealed that C57BL/6 (B6) B cells respond to mAb-induced, TLR4-specific signals stronger than BALB/c (BALB) B cells, as assessed by proliferation and upregulation of CD69 and CD86. In contrast, BALB B cells were not hyporesponsive to agonistic anti–radioprotective 105 kDa mAb or the TLR9 agonist CpG. Although the level of TLR4 mRNA in BALB B cells was comparable with that in B6 B cells, surface TLR4 expression in BALB B cells was lower than that in B6 B cells. This lower surface expression of BALB TLR4 was also observed when HEK293 and Ba/F3 cells were transfected with a BALB TLR4 expression construct. We identified a V254I mutation as the responsible single nucleotide polymorphism for lower surface expression of BALB TLR4. Furthermore, cotransfection of myeloid differentiation factor-2 increased BALB TLR4 expression, although it was still lower than B6 TLR4 expression. In concordance with reduced expression, Ba/F3 cells transfected with BALB TLR4 and myeloid differentiation factor-2 were hyporesponsive compared with those with B6 TLR4, as assessed by LPS-induced NF-κB activation. In conclusion, we revealed that LPS sensitivity is genetically controlled by the level of surface TLR4 expression on B cells. A V254I mutation accounts for the LPS hyporesponsive phenotype of BALB B cells. The Journal of Immunology, 2013, 190: 195–204.

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Received for publication April 9, 2012. Accepted for publication November 1, 2012.

This work was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (Grant 24790112 to H.T., Grant 22591064 to K.F., Grant 21590538 to M.K.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; BALB, BALB/c; Bio, biotinylated; BMDC, bone marrow-derived dendritic cell; BMM, bone marrow–derived macrophage; CM, conditioned medium; LRR, leucine-rich repeat; MD-2, myeloid differentiation factor-2; MFI, mean fluorescence intensity; PRAT4A, protein associated with TLR4; RP105, radioprotective 105 kDa; SNP, single nucleotide polymorphism; stv, streptavidin; TLR4C, C-terminal half of TLR4; TLR4N, N-terminal half of TLR4; wt, wild-type.

In addition, the TLR4 homologous receptor, radioprotective 105 kDa (RP105), has been reported to be involved in LPS recognition (5).

TLR4 is an indispensable innate immune receptor for recognition of LPS (4). With the help of its associate molecule MD-2 (6), TLR4 transmits activation signals caused by LPS via an MyD88-dependent and Toll/IL-1R domain–containing adapter–inducing IFN-β–dependent pathway (7). MD-2 directly binds to LPS via its hydrophobic binding pocket in concert with TLR4 (8). TLR4-deficient mice, as well as MD-2–deficient mice, are unresponsive to LPS and succumb to Gram-negative bacterial infection (4, 6). The surface localization of TLR4 is regulated by a complex mechanism, in which chaperone-like molecules are involved, that varies among TLR4-expressing cell types (6, 9, 10). MD-2 plays a role in transporting TLR4 to the cell surface (6, 11, 12), although it has been reported to be dispensable in dendritic cells and macrophages (11, 13). Coexpression of MD-2 enhances surface TLR4 levels in HEK293 cells (11, 12).

RP105 is homologous to TLR4 in that it is composed of leucine-rich repeat (LRR) motifs (14) and associates with MD-1, an MD-2 homolog (15). We previously demonstrated that RP105+/− (5) and MD-1−/− mice (15) are hyporesponsive to LPS challenge. Agonistic anti-RP105 mAbs induce robust activation of B cells (14, 16). These data suggest the involvement of RP105 in LPS-induced B cell activation. Recent crystallography experiments demonstrated that the MD-1 binding pocket does not accommodate LPS (17), although it was able to bind lipid IVa, a tetra-acylated LPS analog (18). The role of RP105 has been considered to extend beyond LPS recognition, because RP105 deficiency also limits the signaling events driven by TLR2 ligands that are structurally unrelated to LPS (19, 20).
Susceptibility to LPS challenge is associated with genetic variations in mice (21–24) and humans (25). Allelic mutations of the \textit{ips} locus in the C57BL/10ScCr and C3H/HeJ mutant strains of mice are known to completely abrogate the immune response to LPS (24). The \textit{TLR4} gene was identified as the responsible \textit{ips} gene by positional cloning experiments (24), which was confirmed by generation of \textit{TLR4}\textsuperscript{-/-} mice (4). In addition to these LPS unresponsive strains, C57Bl/6 (B6) B cells have been known to be good responders to LPS, whereas BALB/c (BALB) B cells are known to be poor responders (21, 22). A genome-wide search implicated the locus containing the \textit{TLR4} and \textit{MHC class II} genes as major genetic factors controlling B cell responsiveness to LPS (22). Genetic studies using congenic strains of mice revealed that MHC\textsuperscript{a} haplotype is responsible for higher responsiveness to LPS, compared with MHC\textsuperscript{b} haplotype (22). However, the molecular mechanism by which the \textit{TLR4} gene determines high or low responsiveness to LPS has not yet been addressed.

In this study, we dissected B cell activation using agonistic mAbs against TLR4 (26) and RP105 (16), in addition to LPS. Use of these agonistic mAbs provides a distinct advantage, because cross talk with TLR family members other than TLR4 and RP105 because of contaminated pathogen-derived stimulants is negligible. Furthermore, these Abs enable specific analysis of activation via TLR4 and RP105, respectively. In this article, we demonstrate that the low LPS responsiveness of BALB B cells is attributed to MHC\textsuperscript{a} haplotype by generation of TLR4 to the proper subcellular compartment.

Materials and Methods

Animals and cells

B6 and BALB mice and Wistar rats were obtained from Charles River Japan (Yokohama, Japan). MD-2\textsuperscript{2/-} (6) and RP105\textsuperscript{-/-} (5) mice were previously established in our laboratory. TLR4\textsuperscript{-/-} mice (4) were a gift from Dr. S. Akira (Osaka University, Osaka, Japan). The animals were maintained and bred at the Animal Facility, Saga Medical School, under a 12 h/12 h light/dark photoperiod, and given food and water ad libitum. All animal experiments were done in accordance with the Saga Medical School guidelines for the care and treatment of animals used in experimentation.

Human embryonic kidney HEK293 (CRL-1573), mouse myeloma SP2/O (CRL-1581), and rat NRK-52E (CRL-1571) cell lines were purchased from the American Type Culture Collection (Rockville, MD). HEK293, NRK-52E, and derivative transfected cells were maintained in DMEM supplemented with 10% FCS, SP2/O cells and derivative hybridoma clones were maintained in RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-ME. Ba/F3 and derived transfected cells were maintained as previously described (5).

Reagents and Abs

LPS from \textit{E. coli} ATCC 25922 was prepared as described previously (27). Phosphorothioate-stabilized CpG oligonucleotide (5’-TCCATGACGTTC-CTGTAGTC-3’) was purchased from Hokkaido System Science (Sapporo, Japan). Anti-TLR4 (UT12, UT49) (26) and anti-RP105 (RP16) (16) mAbs were prepared as described previously. Rat anti-human CD14 mAb (1B12) was established in our laboratory, as described previously (28, 29). In brief, two Wistar rats were immunized in the foot pads with human CD14, and single clones were isolated by limiting dilution. Purified mAb was obtained from ascitic fluids of SCID mice by caprylic acid precipitation followed by DEAE ion exchange chromatography. Other Abs were purchased from the following companies: FITC-labeled anti-B220 and PE-conjugated anti-CD69 mAbs from eBiosciences (San Diego, CA); FITC-labeled anti-CD11b, -CD11c, and -CD19 and PE-conjugated anti-CD86 and -CD138 mAbs and PE-conjugated streptavidin (svt) from BD Biosciences (San Jose, CA); and PE-conjugated goat anti-mouse IgG Ab from Southern Biotechnology Associates (Birmingham, AL). Biotinylated (Bio)-mAbs were prepared using EZ-Link NHS-LS-Biotin (Pierce, Rockford, IL), according to the manufacturer’s instructions.

Cell staining and flow cytometric analysis

Cells were stained at 4°C with primary mAbs or Bio-mAbs in staining buffer (HBSS containing 2% FCS, and 0.1% sodium azide for splenocytes and BA/F3-derived cells; PBS containing 3% FCS, 10 mM EDTA, and 0.1% sodium azide for HEK293-derived cells, resident macrophages, bone marrow–derived macrophages (BMMs), and bone marrow–derived dendritic cells (BMDCs)). After washing three times, cells were incubated with PE-conjugated svt or goat anti-mouse IgG and subjected to flow cytometric analysis using a FACScan or FACS caliber (Beckton Dickinson, Franklin Lakes, NJ). Collected data were analyzed by the WinMDI program (J. Trotter, The Scripps Research Institute, La Jolla, CA).

MTT assay

Murine splenocytes (2–5 × 10^6 cells) were stimulated in 100 μl on 96-well plates. After a 3-d stimulation, 25 μl MTT (5 mg/ml; Sigma, St. Louis, MO) was added to the cells for an additional 5 h, and the resultant precipitates were dissolved in acidified isopropanol containing 10% Triton X-100. OD measurements were made at 540 and 650 nm using a SpectraMax microplate reader (Molecular Devices LLC, Sunnyvale, CA). Cell growth was calculated by subtracting OD540 values from OD510 values.

Real-time PCR for quantification of TLR4 and MD-2 mRNA

B220\textsuperscript{+} B cells were isolated from splenocytes using Pan-B Dynabeads (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Total RNA was isolated using the RNaseasy mini kit (Qiagen, Valencia, CA) with on-column DNase treatment and reverse transcribed to cDNA using Superscript-II (Invitrogen). TLR4, MD-2, and \textit{β}-actin cDNA were detected using Bio-Rad Premix Ex Taq and a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). The mAbs were determined using standard curves prepared by plotting defined amounts of plasmids containing the target gene against its respective threshold cycle. Levels of TLR4 and MD-2 mRNA were normalized to that of \textit{β}-actin mRNA. Specific amplification was confirmed by running the products on agarose gels by electrophoresis.

Construction of expression vectors

A panel of TLR4 expression vectors was constructed as follows: cDNA fragments coding for the N-terminal (aa 1–438, TLR4N) and C-terminal (aa 439–835, TLR4C) halves of TLR4 were amplified from cDNA purified from B6 or BALB B cells by PCR using the primers 5’-caatctgatcgcaagaatctggagcatgtgc-3’ and 5’-GAA-CGCTGAAATTCCTGTTCAACC-3’ for TLR4; 5’-GTGTTTGCCTGCAAC-CTCTCCAGTGC-3’ and 5’-CAATGTTCCAGGCAACTGCTC-3’ for MD-2; 5’-GTGTTCTGACATCAAAGAAG-3’ and 5’-CGAGTGTCACG-GTCCACT-3’ for \textit{β}-actin. Real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). The mRNA levels were determined using standard curves prepared by plotting defined amounts of plasmids containing the target gene against its respective threshold cycle. Levels of TLR4 and MD-2 mRNA were normalized to that of \textit{β}-actin mRNA. Specific amplification was confirmed by running the products on agarose gels by electrophoresis.

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3′ and 5′-gagatcatGGACTTTATTTGAGCTTTCTC-3′. The PCR product was digested with Xhol and BglII, and subcloned into a pEFBOS vector, which was modified by introducing a FLAG tag at the C terminus, and at Xhol and compatible BamHI sites. A resultant vector expresses TLR2 tagged with FLAG at the C terminus. Full-length human CD14 was subcloned from an EST clone (accession no. BC010507; Open Biosystems) into pBluescript II-KS(+) at EcoRI and NotI sites. This insert was further subcloned into a pEFBOS vector at Xhol and NotI sites. Full-length mouse MD-2 was subcloned into a pCAGGS1 vector from a MD-2/pEFBOS vector (30) at Xhol and NotI sites.

**Transfection study**

In transient transfection experiments, TLR4/pEFBOS constructs were co-transfected into HEK293 cells with a pEFBOS vector containing human CD14 (as a transfection control) in the presence or absence of a pEFBOS vector containing mouse MD-2 (30) using Lipofectamine 2000 (Invitrogen). After 24–48 h cultivation, surface expression of TLR4 and CD14 was determined by flow cytometry. To establish stably transfected cells, TLR4/pEFBOS constructs were transfected into HEK293 cells with pBabePuro selection vectors using Lipofectamine 2000. After puromycin selection, stably transfected clones were screened by flow cytometry using UT12 or another TLR4 mAb, as described previously (26). Ba/F3 reporter cells carrying NF-κB-responsive luciferase gene (5) were transfected with a TLR4/pEFBOS construct with a MD-2/pCAOGS1 construct by the electroporation with GenePulser (Bio-Rad, Hercules, CA). After G418 selection, stably transfected clones were obtained as described earlier.

**NF-κB reporter assay**

Ten thousand Ba/F3-transfected cells carrying TLR4/MD-2/NF-κB reporter gene were stimulated with LPS for 5 h, and luciferase activity was measured as previously described (29).

**Preparation of resident macrophage**

Resident macrophages were collected by peritoneal lavage with ice-cold PBS, washed twice, and then suspended in staining buffer.

**Generation of BMMs and BMDCs**

BMMs and BMDCs were generated from bone marrow cells that were prepared from femurs and tibias, and subjected to RBC-lysis in a hypotonic solution (0.15 M NH4Cl, 1.0 mM KHCO3, and 0.1 mM Na2EDTA) as previously described (6) with slight modification. For BMM, bone marrow cells (1 × 10^6) were cultured in 10 ml DMEM containing 10% FCS, 50 kU/ml penicillin, 50 μg/ml streptomycin, and 1% sodium. After 24–48 h cultivation, surface expression of TLR4 and CD14 was determined by flow cytometry. To establish stably transfected cells, TLR4/pEFBOS constructs were transfected into HEK293 cells with pBabePuro selection vectors using Lipofectamine 2000. After puromycin selection, stably transfected clones were screened by flow cytometry using UT12 or another TLR4 mAb, as described previously (26). Ba/F3 reporter cells carrying NF-κB-responsive luciferase gene (5) were transfected with a TLR4/pEFBOS construct with a MD-2/pCAOGS1 construct by the electroporation with GenePulser (Bio-Rad, Hercules, CA). After G418 selection, stably transfected clones were obtained as described earlier.

**ELISA**

Concentration of TNF-α in cell culture supernatant and serum was determined by a Mouse TNF-α ELISA Ready-SET-Go! kit (eBiosciences), according to the manufacturer’s instructions.

**Endotoxic shock model**

Mice were i.p. injected with sublethal dose of UT12 (5 μg/head), and serum was collected 3 d before and 1 and 3 h after the challenge. Seven days later, RBC-lysed spleen cells were stained and subjected to flow cytometry.

**Results**

**TLR4 and RP105 induce B cell activation without mutual interaction**

In B cells, both TLR4 and RP105 receptors were reported to function in response to LPS (4, 5). To dissect the individual roles of TLR4 and RP105 in LPS-induced B cell activation, we examined the impact of agonistic mAbs against TLR4 (UT12) and RP105 (RP16) on splenocytes from TLR4−/− and PR105−/− mice. UT12 and RP16 both induced proliferation of RP105−/− and TLR4−/− splenocytes, respectively, comparable with that of wild-type (wt) B cells (Fig. 1A). Consistent with this, upregulation of CD69 and CD86 in RP105−/− and TLR4−/− splenic B cells was
induced by UT12 and RP/16, respectively, to a similar degree as seen in wt B6 B cells (Fig. 1B). TLR4−/− and RP105−/− splenocytes were unresponsive to UT12 and RP/16 stimulation. These results indicated that TLR4 and RP105 activated B cells without mutual interaction.

**Impaired TLR4 signaling in BALB B cells compared with that in B6 B cells**

BALB B cells were reported to be less responsive to LPS than B6 B cells (21, 22). To reveal whether the different sensitivity is attributable to TLR4- or RP105-mediated signaling, we compared the response of B6 and BALB B cells with UT12 and RP/16 (Fig. 2A, 2B). UT12-induced proliferation and upregulation of CD69 and CD86 was clearly decreased in BALB B cells. In contrast, RP/16-induced responses of BALB B cells were similar or slightly higher than those of B6 B cells. These findings indicate that strain-dependent differences in LPS responsiveness were due to TLR4-specific signals. Furthermore, we tested B cell responses to a TLR9 agonist, CpG, to reveal whether other TLR signals were also decreased in BALB B cells. We found that CpG was a good stimulator of both B6 and BALB B cells, which resulted in robust proliferation and upregulation of CD69 (Fig. 2C, 2D).

**Impaired cell-surface expression of BALB TLR4**

Considering the comparable responses to CpG of these mouse strains, we thought it unlikely that signaling events that are common among TLRs were impaired in BALB B cells. Therefore, we hypothesized that the low responsiveness of BALB B cells to LPS could be caused by decreased amounts of cell-surface TLR4 molecules. To test this possibility, we compared TLR4 mRNA levels in purified B cells by real-time PCR. However, its expression level in BALB B cells was comparable with that in B6 B cells (Fig. 3A). Next, we compared surface expression levels of TLR4 by flow cytometry. In general, surface TLR4 levels on B cells are known to be too low to detect by flow cytometry. However, among the TLR4 mAbs we tested, TLR4 was clearly detected with UT49 (Fig. 3B). Consistent with our speculation, surface TLR4 in BALB B cells was lower than that in B6 B cells (Fig. 3B). We confirmed the specificity of this staining using TLR4−/− mice to exclude the possibility that this signal resulted from nonspecific binding to other cell-surface molecules (Fig. 3B). Following these observations, we further questioned the dependency of TLR4 surface expression on MD-2 using MD-2−/− mice. It was found that MD-2−/− B cells expressed slightly lower levels of TLR4 on their cell surface compared with wt B6 B cells (Fig. 3B). There was no difference in MD-2 mRNA levels between B6 and BALB B cells (Fig. 3A). These results suggest that surface TLR4 levels were decreased in BALB B cells because of posttranscriptional mechanisms, and that B cells do not necessarily require MD-2 for TLR4 targeting to the cell surface, just as macrophages and dendritic cells do not (11, 13). Because RP105 expression was reported to be controlled by MHC haplotype (22), we also compared its expression level in B cells. However, we were unable to observe the extremely low surface RP105 expression. The level in BALB B cells appeared similar or slightly lower than that in B6 B cells (Fig. 3C).
A V254I mutation in BALB TLR4 impairs surface TLR4 expression

There are six genetic variations between the B6 and BALB TLR4-coding sequences (Fig. 4A) (31). Four of these cause amino acid changes (M209I, V254I, E593D, and R761H). The other two are synonymous mutations (N575N and N715N). We cotransfected either B6 or BALB TLR4 expression constructs with a CD14 construct (as a control to monitor transfection efficiency) into HEK293 cells to reveal whether decreased expression was intrinsic to the TLR4-coding sequence. As shown in Fig. 4B, BALB TLR4-transfected HEK293 cells showed lower expression than B6 TLR4-transfected cells when stained with the UT49 mAb. In contrast, there were no prominent differences in the expression level of surface CD14, indicating similar transfection efficiencies among the transfected cells. This suggests that decreased surface expression of BALB TLR4 was attributable to the TLR4-coding sequence. To reveal which variation(s) in particular affected cellular-surface expression of TLR4, we constructed a panel of mutated TLR4 expression constructs (Fig. 4A) and transfected them into HEK293 cells. As shown in Fig. 4B and Supplemental Fig. 1, expression of B6 TLR4 with the M209I and V254I mutations (BALB/B6 TLR4) was comparable with that of BALB TLR4, whereas expression of B6 TLR4 with the N575N, E593D, N715N, and R761H mutations (B6/BALB TLR4) was clearly higher than that of BALB TLR4. This indicated that either M209I or V254I (or both) was the responsible single nucleotide polymorphism (SNP) for decreased expression of BALB TLR4. Therefore, we further examined the contribution of both M209I and V254I to decreased TLR4 by introducing site-specific mutations into B6 TLR4. The V254I mutation clearly caused decreased B6 TLR4 expression, comparable with BALB TLR4 levels, whereas the M209I mutation did not. Similar findings were obtained in stably transfected HEK293 cells expressing B6, M209I, V254I, and BALB TLR4 (Fig. 4C). These findings suggest that V254I was the mutation responsible for impaired surface expression of BALB TLR4.

Surface expression of and signaling by transfected BALB TLR4 is lower compared with B6 TLR4 even in the presence of MD-2

MD-2 has been reported to enhance cell-surface TLR4 expression (11, 12), although it is dispensable in HEK293 cells (11). MD-2−/− B cells expressed slightly lower surface TLR4 levels (Fig. 3B). Therefore, we performed a transfection study in which MD-2 was transduced into B6 or BALB TLR4 stably transfected cells to clarify whether MD-2 could rescue lower surface BALB TLR4 expression. As previously demonstrated (11), MD-2 transfection enhanced BALB TLR4 surface expression. However, the higher BALB TLR4 level was still lower than B6 TLR4 expression (Fig. 5A, Supplemental Fig. 2). To confirm this, we cotransfected varying amounts of MD-2 constructs together with the B6 or BALB TLR4 construct (Fig. 5B, Supplemental Fig. 3). Cotransfection of MD-2 dose dependently increased BALB TLR4 expression. However, the absolute expression levels of BALB TLR4 remained lower than B6 TLR4 levels at all concentrations of MD-2 construct. The transfection efficiency, monitored by CD14 expression, was consistent in these experiments. In addition, we confirmed that BALB TLR4 expression level is still lower than B6 TLR4 level when they are stably transfected in Ba/F3 cells together with MD-2 and NF-κB reporter gene (Fig. 5C). These findings indicate that MD-2 could not fully rescue surface BALB TLR4 expression to the level seen with B6 TLR4 and support the notion that low expression of TLR4 in BALB B cells is due to the V254I mutation in the BALB TLR4 sequence. Together with these findings, we performed NF-κB reporter assay and revealed that BALB TLR4-transfected Ba/F3 cells were hypo-responsive to LPS stimulation compared with B6 TLR4-transfected cells (Fig. 5D). These results suggest that V254I mutation is a mechanism to account for low LPS responder phenotypes of BALB B cells.

Slightly impaired TLR4 expression and signaling in BALB dendritic cells, but not in macrophages

To extend our findings, we examined cell-surface expression of B6 versus BALB TLR4 on other immune cell types such as BMDCs, BMMs, and resident macrophages. Reduced TLR4 expression level was observed in BALB BMDCs as observed in B cells (Fig. 6A). Consistent with this, the responses of BALB BMDCs to LPS and UT12 were lower than those of B6 BMDCs as assessed by TNF-α secretion (Fig. 6B). In contrast with B cells and BMDCs, we could not see any strain difference in TLR4 expression and responses to LPS and UT12 in resident macrophage and BMM (Fig. 6C–E). Therefore, V254I mutation in TLR4 gene appears not always to confer low responsiveness on all cell types.

BALB mice have similar sensitivity to endotoxic shock but impaired plasma cell differentiation

We examined the sensitivity to endotoxic shock in vivo by administering agonistic UT12 mAb and measuring serum TNF-α. It was found that endotoxic shock was induced with similar degree
between B6 and BALB mice (Fig. 7A). In this experiment, we unexpectedly found that UT12-injected mice have enlarged spleen at least because of the increased B cell and CD138+ plasma cell numbers (Fig. 7B). This massive B cell proliferation was more obvious in B6 mice than in BALB mice, a finding that proves that TLR4 signaling in BALB B cells is still impaired in vivo as demonstrated in vitro.

Discussion
By using agonistic mAbs to TLR4 and RP105, we revealed that susceptibility to LPS in murine B cells was genetically controlled by signals from TLR4, not from RP105. B6 B cells showed stronger responses to agonistic anti-TLR4 mAb than BALB B cells, as assessed by proliferation and upregulation of CD69 and CD86, whereas the responses to agonistic anti-RP105 mAb were slightly lower or similar rather than higher in B6 B cells. We also demonstrated by flow cytometry that the surface TLR4 level in B6 B cells was higher than that in BALB B cells. NF-κB reporter assay showed that BALB TLR4-transfected Ba/F3 cells were hyporesponsive to LPS stimulation than B6 TLR4-transfected cells in concordance with decreased surface expression. Because surface TLR4 levels are related to the magnitude of the LPS response...
FIGURE 5. BALB TLR4 surface expression and signaling are lower than B6 TLR4 in the presence of MD-2. (A) B6 (white columns) or BALB (black columns) TLR4 stably transfected cells established in Fig. 4C were transiently transfected with human CD14 (1.5 μg) and mouse MD-2 (1.5 μg) expression constructs. After 24–48 h cultivation, surface expression of TLR4 and CD14 was determined by flow cytometry using Bio-UT49 and Bio-1B12 followed by PE-stv. Data are represented as MFI in the gated regions (R1 and R2). Percentage of gated cells is depicted in dot plot. Results are representative of three independent experiments (Supplemental Fig. 2). (B) B6 (white columns) or BALB (black columns) TLR4 expression constructs (2 μg) were cotransfected into HEK293 cells with human CD14 (0.5 μg) and mouse MD-2 expression constructs at the indicated amounts. After 24–48 h cultivation, surface expression of TLR4 and CD14 was determined and represented as MFI in the gated regions, as in (A). Results are representative of two independent experiments (Supplemental Fig. 3). (C) Stable Ba/F3-transfected clones expressing B6 or BALB TLR4 with MD-2 and NF-κB reporter gene were analyzed by flow cytometry using UT49 for TLR4 and UT12 for TLR4/MD-2 followed by PE-conjugated anti-mouse IgG Ab. Open (Figure legend continues)
(9, 32, 33), we suggest that differential surface expression levels of TLR4 accounted for at least one mechanism underlying the different sensitivity to LPS between B6 and BALB B cells.

Differences in surface TLR4 expression are not likely due to the amount of TLR4 produced in B6 versus BALB B cells, because the amount of TLR4 mRNA, and probably of TLR4 protein, produced was similar in these strains of mice. Susceptibility to LPS in B cells was demonstrated to be most closely linked with the locus containing TLR4 (22). Therefore, we examined the impact of polymorphic variations of the TLR4 gene on surface expression of this molecule in HEK293 cells. This cell line expresses transfected TLR4 on its cell surface without the help of MD-2 (11). Consistent with B cells, lower surface expression of BALB TLR4, compared with B6 TLR4, was observed when HEK293 cells were transiently and stably transfected with the TLR4 expression vector. NF-κB activation was less inducible to LPS stimulation in BALB TLR4-transfected Ba/F3 cells than B6 TLR4-transfected cells. These findings suggest that genetic control of surface expression of TLR4 and, therefore, LPS sensitivity is directly attributable to SNP(s) within the TLR4-coding sequence.

Using B6/BALB chimeric TLR4 constructs as well as site-directed mutagenesis, we identified a V254I mutation as responsible for lower expression of BALB TLR4. Aa254, which is located on the ninth LRR in the central domain of TLR4, is included in the B patch, which is critical for TLR4–MD-2 association (34). Proteins associated with TLR4 (PRAT4A) and gp96 have been identified as chaperones controlling TLR4 translocation in mice. PRAT4A interacts with the E24-F54 region of TLR4, which is close to the A patch, and other regions in TLR4 are not important for their interaction (35). Therefore, it is unlikely that the V254I mutation impacts PRAT4A-mediated TLR4 transportation. Rather, the function of gp96 could be influenced by this mutation. Interestingly, Shibata et al. (36) found that PRAT4A−/− macrophages on the BALB background completely lacked surface TLR4, whereas B6 PRAT4A−/− macrophages expressed decreased but detectable levels of TLR4 on their cell surface. These findings allow us to speculate that TLR4 is transported by both PRAT4A-dependent and -independent mechanisms on the B6 genetic background, but that the contribution of the PRAT4A-independent mechanism is limited in the BALB background. The PRAT4A-independent mechanism may not support transport of V254I TLR4. Gp96 may be involved in this PRAT4A-independent mechanism. The TLR4 region required for gp96 interaction has not yet been elucidated. It is possible that other processes, after transcription but before cell surface localization of TLR4, are impaired by the V254I SNP (e.g., protein stability, glycosylation), in addition to TLR4 transport.

In human TLR4, aa 299, located on the 10th LRR, has a critical impact on LPS responsiveness, as the naturally occurring D299E mutation interrupts TLR4-mediated LPS signaling (25). Because this mutated amino acid is relatively close to aa 254, located on the ninth LRR, it is possible that human TLR4 with the D299E mutation has an impaired ability to localize to the cell surface.

MD-2 is another candidate controller of TLR4 expression on the cell surface (6). However, we observed that MD-2 mRNA levels were comparable between B6 and BALB B cells. MD-2 deficiency in B6 B cells showed an only slightly decreased surface TLR4 level; however, this was still higher than that of BALB B cells.

Furthermore, BALB TLR4 expression was low compared with B6 TLR4 with the same amount of cotransfected MD-2 in HEK293 cells. No SNPs between the B6 and BALB MD-2 coding sequences have been reported in the Mouse Genome Informatics database (http://www.informatics.jax.org/). Stable transfection of MD-2 in Ba/F3 cells failed to express BALB TLR4 at comparable levels of B6 TLR4. These findings suggest that MD-2 is not a critical molecular determinant for genetic control of surface TLR4 expression in B cells.

Previously, genetic control of LPS sensitivity has been reported in other immune cells (23, 37–39) in addition to B cells (21, 22, 40). We revealed that surface TLR4 expression was impaired in BALB BMDCs, albeit to a lesser extent, with decreased TNF-α production by LPS and UT12. In contrast, macrophages did not have strain difference in TLR4 expression and signaling. Therefore, the genetic mechanism underlying impaired TLR4 expression in B cells does not always apply to all immune cells. Presumably, the mechanism for TLR4 surface transportation varies on cell types, and thereby the impact of SNPs in TLR4 gene on the mechanism could be different among cell types.

Because systemic responses to LPS are influenced by genetic background (41), we challenged mice with agonistic TLR4 mAb. Serum TNF-α, a causative major mediator of endotoxic shock (42), was equally increased in B6 and BALB mice, a finding suggesting similar sensitivity to endotoxic shock in these strains. Considering that macrophage is a major player for the induction of endotoxic shock (10), this finding is compatible with those of surface TLR4 expression and signaling in macrophages as described earlier. We revealed that UT12 induces enlarged spleen at least because of expansion of B cells, which results in the differentiation to CD138+ plasma cells with significant increase in B6 mice. LPS unresponsive strains with TLR4 P712H mutation have significantly lower natural IgG3 against LPS than wt strains (43). Therefore, we suggest, as in vivo significance of our findings, that natural Ab and/or innate immunity to invaded bacteria of B cells could be impaired by polymorphic variations of TLR4 gene in BALB mice.

Rodo et al. (22) reported that the MHC locus is strongly linked to LPS sensitivity in B cells, possibly through regulation of RP105 expression. However, it may be difficult to explain low-responder phenotypes of BALB B cells only by the MHC haplotype because of its minor contribution as revealed by their congenic strains (22). B cell responsiveness could be controlled by other primary mechanism(s). It is worth noting that A/J mice, another strain with low LPS responder phenotypes of B cells, have different MHC haplotype from but share identical SNPs in TLR4 gene with BALB mice (31). This suggests that polymorphic variation, in particular, V254I, is a primary cause of low LPS sensitivity in these strains. Minor contribution of MHC haplotype may explain moderate difference in B cell responses to LPS as previously demonstrated between BALB and A/J mice (40).

As a possible mechanism for genetic control of LPS sensitivity through MHC haplotype, distinctly lower surface expression of RP105 was shown in BALB B cells (22). However, we were unable to observe such low expression. Surface RP105 levels were similar or slightly lower in BALB B cells compared with B6 B cells. Our findings were consistent with the observation that responses to agonistic anti-RP105 mAb are not impaired in BALB B cells, as histograms represent staining without primary mAbs. Data are summarized as mean ± SD MFI from three independent clones, as in Fig. 4C. Similar results were obtained in two independent experiments. (D) Stable clones in (C) were stimulated with LPS (5, 50, 500 ng/ml) or UT12 (10 μg/ml) for 5 h. Luciferase activity was shown as the mean ± SD fold increase against that of nonstimulated cells in triplicate cultures. Similar results were obtained in two independent experiments.
assessed by proliferation and cell-surface activation marker expression. A recent article by Vale et al. (21) also showed slightly lower surface expression of RP105 in BALB B cells, which is consistent with our findings. The reason why Vale et al. (21) and our findings regarding RP105 expression were not in agreement with a previous report (22) is unclear. Further investigation is required to reach the conclusion about direct contribution of RP105 to strain-dependent LPS sensitivity via the linkage with MHC locus.

In conclusion, the decreased surface TLR4 expression caused by a V254I point mutation accounts for the low LPS responder phenotype of BALB B cells. The results shown in this study reveal

![Figure 6. Slightly impaired TLR4 expression and signaling in BALB dendritic cells, but not in macrophages. (A, C, and D) B6 or BALB BMDCs (A), BMMs (C), and resident macrophages (D) were stained with Bio-UT49 and FITC-conjugated CD11c (A) or CD11b (C, D) mAbs followed by PE-stv. The expression of TLR4 on CD11c⁺ cells (A) and on CD11b⁺ cells (C, D) was analyzed by flow cytometry. Open histogram represents staining with a Bioisotype control. Numbers in histogram indicate the MFI of staining. Results are representative of three independent experiments. (B and E) B6 (white columns) or BALB (black columns) BMDCs (B) and BMMs (E) were stimulated with LPS (1, 10, 100 ng/ml) or UT12 (10 μg/ml) for 21 h. TNF-α in cell culture supernatant was shown as the mean ± SD of triplicate cultures. Similar results were obtained in three independent experiments.

![Figure 7. BALB mice have similar sensitivity to endotoxic shock but impaired plasma cell differentiation compared with B6 mice. (A) Five B6 (open circles) or BALB (black circles) mice were i.p. injected with UT12 (5 μg), and serum was collected 3 d before (Pre) or 1 and 3 h after sublethal challenge. Serum TNF-α was shown as the mean ± SD. Similar results were obtained in two independent experiments. (B) Seven days after challenge of B6 (white columns) or BALB (black columns) mice with UT12 mAb (5 μg, n = 5) or PBS (n = 3), the numbers of CD3⁺ T cells, CD19⁺ B cells, and CD138⁺CD19⁺ plasma cells, as well as the ratio of plasma cells to total B cells in spleen, were determined by flow cytometry using the combinations of FITC-CD19/PE-CD3 and of FITC-CD19/PE-CD138 mAbs. Results were shown as the mean ± SD of cell number in spleen or percentage of plasma cells in B cells. Representative dot plots of plasma cell staining were shown with the percentage of gated cells in total spleen cells. *p < 0.05, Student t test. Similar results were obtained in two independent experiments. ]
the genetic control of LPS sensitivity and also help elucidate the mechanism of directing TLR4 to the proper subcellular compartment and innate immunity by B cells. These data are also useful information for immunologists, because LPS is a widely used mitogen, and B6 and BALB mice are the most frequently used model strains, because they are Th1 and Th2 biased, respectively.

Acknowledgments
We thank Dr. S. Akira for providing TLR4/−/− mice.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental figure legends

Supplemental figure 1. *A V254I mutation in BALB TLR4 impairs surface TLR4 expression.*

(A, B) TLR4 constructs shown in Fig. 4A were transfected into HEK293 cells with (A) or without (B) a construct expressing human CD14. Surface expression of TLR4 and CD14 was determined by flow cytometry and represented as the MFI as in Fig. 4B. Percentage of gated cells was depicted in dot plot. Two independent experiments showed similar results to that in Fig. 4B.

Supplemental figure 2. *BALB TLR4 surface expression is lower than B6 TLR4 in the presence of MD-2.*

B6 (open column) or BALB (black column) TLR4 stably-transfected cells established in Fig. 4C were transiently transfected with human CD14 and mouse MD-2 expression constructs. Surface expression of TLR4 and CD14 was determined by flow cytometry and represented as MFI in the gated regions (R1 and R2) as in Fig 5A. Percentage of gated cells was depicted in dot plot. Two independent experiments showed similar results to that in Fig. 5A.

Supplemental figure 3. *BALB TLR4 surface expression is lower than B6 TLR4 in the presence of MD-2.*

B6 (open column) or BALB (black column) TLR4 expression constructs were co-transfected into HEK293 cells with human CD14 and mouse MD-2 expression constructs. Surface expression of TLR4 and CD14 was determined and represented as
the MFI as in Fig. 5B. Result was similar to that in Fig. 5B.
Supplemental figure 1

(A) Exp 1

TLR4 construct: TLR4 construct:

(B) Exp 2

TLR4 construct: TLR4 construct:
Supplemental figure 2

Exp 1

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Exp 2

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Supplemental figure 3

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- MFI (CD14)
- MFI (TLR4)

- FSC
- TLR4
- CD14

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| (-) | 0.005 | 0.05 | 0.5 | (-) | 0.005 | 0.05 | 0.5 |

- MFI (CD14)