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Overexpression of Toll-Like Receptor 4 Amplifies the Host Response to Lipopolysaccharide and Provides a Survival Advantage in Transgenic Mice

Franck Bihl,*¶§ Laurent Salez,‡ Magali Beaubier,§ David Torres,§ Line Larivière,‡ Line Laroche,‡ Alexandre Benedetto,‡ Dominic Martel,‡ Jean-Martin Lapointe,¶ Bernhard Ryffel,§ and Danielle Malo2*†‡

Toll-like receptors are transmembrane proteins that are involved in the innate immune recognition of microbial constituents. Among them, Toll-like receptor 4 (TLR4) is a crucial signal transducer for LPS, the major component of Gram-negative bacteria outer cell membrane. The contribution of TLR4 to the host response to LPS and to infection with virulent Salmonella typhimurium was studied in four transgenic (Tg) strains including three overexpressing TLR4. There was a good correlation between the level of TLR4 mRNA expression and the sensitivity to LPS both in vitro and in vivo: Tg mice possessing the highest number of TLR4 copies respond the most to LPS. Overexpression of TLR4 by itself appears to have a survival advantage in Tg mice early during infection: animals possessing more than two copies of the gene survived longer and in a greater percentage to Salmonella infection. The beneficial effect of TLR4 overexpression is greatly enhanced when the mice present a wild-type allele at natural resistance-associated macrophage protein 1, another critical innate immune gene involved in resistance to infection with Salmonella. TLR4 and natural resistance-associated macrophage protein 1 exhibit functional epistatic interaction to improve the capacity of the host to control bacterial replication. However, this early improvement in disease resistance is not conducted later during infection, because mice overexpressing TLR4 developed an excessive inflammatory response detrimental to the host. The Journal of Immunology, 2003, 170: 6141–6150.

Despite widespread use of antibiotic therapy, septic shock subsequent to sepsis is responsible for >200,000 deaths annually in the United States alone (1). LPS (1), originally named endotoxin, is a major integral glycolipid component of the outer membrane of Gram-negative bacteria (2, 3). The recognition of LPS by various host cells plays a central role in the outcome of sepsis. LPS alone can induce many of the pathophysiological symptoms that are characteristic of Gram-negative bacterial infection. Cell types, including lymphocytes, macrophages, endothelial cells, and fibroblasts, respond to the proinflammatory and immunostimulatory properties of LPS by production and release of proinflammatory cytokines (TNF, IL-1, IL-6, and IL-12), enhancement of their Ag-presenting and microbicidal capacities, and induction of proliferation (4–6).

LPS binds to a serum protein termed LPS binding protein (LBP)3 that catalyzes the transfer of LPS monomers to a membrane-bound receptor CD14 expressed on the surface of myeloid cells (7–9). This mediates the physical association of the LPS/CD14 complex with the transmembrane receptor Toll-like receptor 4 (TLR4) and MD-2, an extracellular accessory protein (10, 11). The subsequent activation of TLR4 induces the recruitment of MyD88, an intracellular adaptor protein that in turn recruits the IL-1R-associated kinase (12–15). Beside MyD88, another component named MyD88-adaptor-like/Toll-IL-1R domain-containing adapter protein participates in the transduction of this signal that ultimately results in the activation of transcription factor NF-κB and mitogen-activated protein kinases (16–18).

Most inbred strains of mice are susceptible to the immunostimulatory properties of LPS. However, C3H/HeJ, C57BL/10ScNcr, and C57BL/6.KB2-motor neuron disease mice exhibit endotoxin hyporesponsiveness, with macrophages being central in the mediation of this phenotype (4, 19–23). Positional cloning analysis revealed the presence of mutation in alleles of the Tlr4 gene. C3H/HeJ mice present a single missense mutation resulting in a proline-for-histidine substitution at codon 712 within the signaling domain. In C57BL/10ScCr mice, there were no Tlr4 transcripts detected as a consequence of a 75-kb chromosomal deletion encompassing the whole Tlr4 gene. The mutation identified in C57BL/6.KB2-motor neuron disease Tlr4 consists of a complete deletion of exon II, leading to a frameshift resulting in the appearance of a stop codon just downstream of the exon junction. The

1 This work was supported by grants from the Canadian Institutes of Health Research and the Howard Hughes Medical Institute. D.M. is a scholar of the Canadian Institutes of Health Research and an International Research Scholar of the Howard Hughes Medical Institute.

2 Address correspondence and reprint requests to Dr. Danielle Malo, Center for the Study of Host Resistance, Montreal General Hospital, Room L11-144, Montreal, Quebec, Canada H3G 1A4. E-mail address: danielle.malo@mcmill.ca

3 Abbreviations used in this paper: LBP, LPS binding protein; Tlr4, murine Toll-like receptor 4; TLR, Toll-like receptor; Nramp1, murine natural resistance associated macrophage protein 1; BAC, bacterial artificial chromosome; Tbp, murine TATA binding protein; PFGE, pulse field gel electrophoresis; Tg, transgenic; PMN, polymorphonuclear cell.
putative Tlr4 mutation protein is equivalent to the first 31 N-terminal residues of its wild-type counterpart (23–26). Subsequent inactivation of the gene confirmed the role of Tlr4 in mediating LPS biological activities (27). Another important manifestation of altered LPS responsiveness of C3H/HeJ and C57BL/10ScNcr mice is their enhanced susceptibility to the Gram-negative bacteria *Salmonella enterica* serovar *typhimurium* (*S. typhimurium* infection). In these mice, replication of *Salmonella* in the spleens and livers is uncontrolled and results in premature death, compared with C3H/HeN and C57BL/10Sn wild-type animals (4, 22, 28).

In addition to Tlr4, another gene termed natural resistance-associated macrophage protein 1 (*Nrampl*), recently renamed solute carrier family 11 member 1 (because of its membership to a family of solute carriers) has been shown to play an important role in innate immunity, especially in *S. typhimurium* infection (29). In mice, *Nrampl* is a phagosomal protein exclusively expressed in phagocytic cells that controls the replication of certain intracellular pathogens during the early phase of infection (30–33).

In this paper, we investigate the effect of Tlr4 expression by generating four strains of transgenic (Tg) mice. We show a good correlation between the number of integrated copies of Tlr4, its RNA expression, the LPS-induced proliferation of splenocytes in vitro and the sensitivity to septic shock after LPS challenge in vivo in each strain. Furthermore, all the Tg mice were more resistant to *S. typhimurium* infection in terms of survival or bacterial loads in reticuloendothelial organs. Finally, we demonstrate the existence of a genetic epistatic interaction between Tlr4 and *Nrampl* with respect to the host response to infection with *S. typhimurium*.

### Materials and Methods

#### Generation of Tg mice

Tg mice were generated using the bacterial artificial chromosome (BAC) 152C16 that contained Tlr4 (129/Sv library; Research Genetics, Huntsville, AL). BAC 152C16 isolation and sequencing were reported previously (25). The preparation of the linearized BAC DNA has been adapted from several previous publications dealing with yeast artificial chromosome transgenesis (35–38). Briefly, BAC 152C16 was purified using QiAmp Plasmid Mega or Giga kits (Qiagen, Valencia, CA), digested with NotI (New England Biolabs, Beverly, MA), loaded onto a 1% low melting point agarose (SeaPlaque GTG; FMC Bioproducts, Chicago, IL) gel without ethidium bromide and submitted to pulse field gel electrophoresis (PFGE) in 0.5× TBE (Tris-boric acid-EDTA) buffer. Electrophoresis was performed at 6 V/cm for 16 h at 14°C with initial and final switching times of 5 s and with an included angle of 120°. After migration, small strips (2 cm) on each side of the gel were cut and submitted to ethidium bromide staining, while the other strips were cut and submitted to ethidium bromide staining, while the center part was stored in fresh migration TBE buffer. After reassembling the gel, the slice that contained the 150-kb band of DNA was cut out, stripped into 1-cm pieces, pre-equilibrated in soaking buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA) and digested with β-agarase (New England Biolabs) using conditions suggested by the supplier. Agarased DNA solution was dialyzed 4 h against the microinjection buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA) using pre-equilibrated filters (VMWP02500; Millipore, Bedford, MA) in petri dishes. The final concentration was estimated after the migration of a sample of the dialyzed DNA fragment with known quantities of the initial digested BAC onto a 1% agarose (SeaKem GTG; FMC Bioproducts) PFGE gel. Under these conditions, starting with 10 μg of digested BAC, we obtained ~500 μl of purified construct at a concentration of 5 ng/μl. This construct was microinjected directly into F1 (C3H/HeN × C57BL/6N) host eggs that were transferred into pseudopregnant CD1 mice. Tg animals were subsequently backcrossed toward a C57BL/10ScNcr background.

#### Genotyping of Tg mice

Founders and subsequent littermates were genotyped by PCR. Genomic DNA was extracted from the tails of mice as described previously (39). We used one primer specific for the vector (pBeloBAC11) of the Bac library and one primer located in the insert, at each extremity. The first fragment (SP6 end) was 170 bp in length, using primers 5′-CTCAGTATTTCTCTTATACAG-3′ and 5′-gCCAAgCTATTTAggTACAC-3′; the second fragment (T7 end) was 192 bp in length, using primers 5′-gCACTCTCAAAACCTGCTTG3′ and 5′-ATACgACTCTAATTAGCAA-3′. To exclude any internal deleting recombinational event, a third PCR amplification was done using primers 5′-TACCGTGATTTCTGTCAG3′ and 5′-AACtgAgAAATAgAAgTACg-3′. The fragment amplified with these two primers (~140 bp) is located in the Tlr4 promoter and contains a (CA)n repeat microsatellite marker that is polymorphic between the C57BL (n = 28), C3H (n = 30), and 129N (n = 32) strains of mice, allowing us to follow the transgene. *Nrampl* genotyping was realized using DJ Mcdq4 microsatellite (34). All these amplifications were performed in a final volume of 20 μl using standard cycling conditions. [γ-32P]ATP end-labeled primer allowed visualization by polyacrylamide gel electrophoresis followed by autoradiography.

#### Quantification of the transgene by TaqMan PCR

Genomic DNA levels of murine Tlr4 and TATA-binding protein (Tbp) genes were quantitated by real-time PCR using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) and the Brilliant Quantitative PCR Core Reagent kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Amplification was achieved using an initial cycle of 20°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Tlr4 genomic DNA levels during the linear phase of amplification were normalized against Tbp controls. Determinations were made in triplicate and mean ± SD was determined. Primers (forward and reverse) and probes were designed using the Primer Express (Applied Biosystems) software and are as follows: Tlr4, forward, 5′-ACACgTgTgAAACAATACAATgTg-3′, reverse, 5′-AAATCCAGCgACTgAATgCTgTgA-3′, and probe, 5′-TACCAgATgTCAgATCACg-3′; Tbp, forward, 5′-TgAtggCTTcCCAgCTAgATgTg-3′, reverse, 5′-CTggTggTgCgAACA-gg-3′, and probe, 5′-TCCCCACCATgTgTCTgAgCTgATAgTCTgA-3′ (40). Amplicon sizes were 201 and 104 bp, respectively. Reporter and quencher dyes were FAM and TAMRA for Tlr4 probe, and VIC and TAMRA for Tbp probe (Applied Biosystems).

RT-PCR

Total spleen RNA from Tg and non-Tg mice was extracted using TRIzol (Life Technologies, Rockville, MD) according to the manufacturer’s conditions. First-strand cDNA synthesis was conducted using 2 μg of total RNA of 4 h, 50°C for 30 min, and 200 U of RNase H and 1000 U of M-MLV reverse transcriptase (Life Technologies) in a total volume of 20 μl. An aliquot was used for each independent PCR. Tlr4 expression was monitored following 35 cycles with the primers 5′-CTCTgCATgAggAggTAgCTCgTCA-3′ and 5′-g-agarose gel TAAgCCTAGCTATgTACg-3′. PCR products were resolved on a 1% and blotted onto a nylon membrane. An internal primer end-labeled with γ-32P]ATP polymerase (32). The membrane for 16 h at 55°C in aqueous solution (6× SSC, 5× Denhardt’s solution, 1% SDS, and 200 μg/ml herring sperm DNA). The membrane was washed to a final stringency of 0.1× SSC and 0.1% SDS for 20 min at 50°C. The amount of cDNA was quantitated after exposure in a STORM 860 PhosphorImager with the help of the Image Quant software (Molecular Dynamics, Sunnyvale, CA). To normalize for the amount of RNA, the same procedure was conducted for GAPDH (25 cycles of amplification).

#### FACs analysis

Thioglycolate-elicited peritoneal macrophages were obtained from mice injected with 4 ml of 2% thioglycolate i.p. (Boivall, Morne La, Vallée, France). The cells were collected 4 days postinjection and double-stained with anti-CD11b mAb conjugated with PerCP (BD Pharmingen, San Diego, CA) and anti-Thi1/MD-2 mAb conjugated with PE (Mabtech, Santa Cruz Biotechnology, Santa Cruz, CA). Cells were analyzed on a BD Biosciences (Mountain View, CA) LSR apparatus.

#### Spleen cell mitogenic response

Spleen cell suspensions were prepared from C57BL/10SnJ and C57BL/10ScNcr, Tg and non-Tg animals as previously described (23). Briefly, organs were removed aseptically and homogenized, and splenocytes were resuspended in 5 ml of cold complete RPMI 1640 (Life Technologies). Cells were loaded on a density separation medium (Lymphocyte-M; Cedar Lane Laboratories, Hornby, Ontario, Canada) and 12×10^6 to 40×10^6

The host response to LPS and *Salmonella* infection in Tlr4 Tg mice
viable cells were obtained per mouse spleen. Single-cell splenocytes suspensions were prepared at a final concentration of 2 × 10⁶ cells/ml in RPMI 1640 medium containing 20% heat-inactivated FBS (Clontech, Palo Alto, CA). Cells were cultured (100 μl/well) in 96-well plates, stimulated with increasing concentrations of LPS (E. coli K235; Sigma-Aldrich, St. Louis, MO) or 1.5 μg/ml Con A (Sigma-Aldrich) in RPMI 1640 medium and further incubated for 48 h at 37°C. During the last 6 h of incubation, 1 μCi of [H]thymidine was added per well. Cells were harvested, and [H]thymidine incorporation was measured by scintillation counting. For each treatment, triplicate cultures were assayed.

**In vivo injection of LPS**

All procedures involving animals were performed in accordance with the regulations of the Canadian Council on Animal Care. C57BL/10SnJ and C57BL/10ScNCR, Tg and non-Tg mice were each injected with 0.5 ml of PBS containing increasing amounts (10 μg to 1 mg) of LPS (E. coli K235; Sigma-Aldrich) i.p. Animals were monitored twice a day and moribund animals were sacrificed for ethical reasons.

**Infection with S. typhimurium**

Each mouse was infected with 0.2 ml of PBS containing 10⁵ CFU of S. typhimurium in the caudal vein. For survival curves, each animal was monitored twice a day, and moribund animals were sacrificed for ethical reasons. To determine the growth rate of the bacteria within the reticuloendothelial organs, mice were sacrificed 5 days postinfection, and their spleens and liver were recovered aseptically. One-half of each organ was homogenized in 2 ml (spleen) or 5 ml (liver) of isotonic saline with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Serial dilutions of each homogenate were plated on trypticase soy broth agar to enumerate the CFU within each organ. The remaining one-half was snap frozen in liquid nitrogen.

**Histopathology**

Two naive or infected mice per group were sacrificed 5 days postinfection, and their spleens, livers, hearts, and kidneys were recovered aseptically, fixed in 10% buffered formaldehyde, and embedded in paraffin. Sections (4 μm thick) were cut and stained with hematoxylin-phloxin-saffron. All sections were examined by the same pathologist.

**Results**

**Generation of Tlr4 Tg strains of mice**

To study the effect of Tlr4 overexpression in vivo, Tg strains of mice carrying BAC 152C16 (129/Sv library; Research Genetics) were generated. As shown previously by ourselves and others (25, 41), this BAC contains the entire Tlr4 gene. Strategically, we wanted to microinject a linearized construct containing the insert of interest but with minimal vector pBeloBAC11 sequence. For this purpose, we initiated a first screening to identify rare cutter restriction enzymes that cleave only the vector and release the whole insert. Among the various enzymes used (NotI, EagI, BstEII, KpnI, NarI, SphI, SalI, and BssHII), BAC genomic DNA digested with NotI revealed the presence of two bands of ~150 and 7 kb after PFGE (data not shown). According to the vector restriction map, the 7-kb band corresponded to most of the vector (expected size of 6877 bp), whereas the 150-kb fragment corresponded to the whole insert plus 383 and 247 bp of the vector at the T7 and Sp6 extremities, respectively (Fig. 1). Once isolated and purified (as described in Materials and Methods), this 150-kb fragment was directly microinjected into F1, (C3H/HeJ × C57BL/6J) frozen eggs that were subsequently transferred into pseudopregnant CD1 mice. Founders and subsequent litters were genotyped by PCR. To follow the inserted construct through generations, we used one primer specific for the vector (pBeloBAC11) of the BAC library and one primer located in the insert for each extremity (Fig. 1). Furthermore, to exclude any internal deleting recombinational event, a third PCR amplification was done using primers amplifying a (CA)n repeat microsatellite marker located in the Tlr4 promoter and polymorphic between the C57BL (n = 28), C3H (n = 30), and 129/Sv (n = 32) inbred strains of mice (Fig. 1). Under this procedure, 3 of 14 founders were positive for all the three markers used and were named 388, 390, and 394. A fourth founder, called 382, was only positive for the SP6 and promoter microsatellite amplifications. All four Tg animals were subsequently backcrossed to a C57BL/10ScNCR Tlr4 mutant background and successfully transmitted the inserted transgene.

**Estimation of the integrated number of copies**

It is well known that transgenesis usually generates the insertion of multiple copies (1–100) of the injected foreign DNA at a single chromosomal locus. At each integration site, these copies are arranged primarily in a head-to-tail array (42). To estimate the number of integrated copies in the four Tg strains of mice, we have used quantitative PCR. In our study, we analyzed the genomic DNA of the C57BL/10ScNCR and C57BL/10SnJ, Tg and non-Tg strains. The Tlr4-specific amplicon was normalized to the Tcp amplicon, an endogenous gene that is present in 2 copies in the genome of all the strains studied. As expected, no amplification was detected in the C57BL/10ScNCR strain that harbors the complete Tlr4 gene, and a similar amount of Tlr4 and Tcp was confirmed in C57BL/10SnJ wild-type control strain (Fig. 2). The Tlr4 Tg strains presented in their genomes various amounts of integrated Tlr4 copies: the 382 strain had only 1 copy, the 388 had 3 copies, the 390 had 6 copies, and finally, the 394 strain contained ~30 copies of Tlr4.
Estimation of the expression by RT-PCR

To determine whether the number of integrated copies correlates with the level of expression of the Tlr4 gene, we extracted total RNA from the spleen, liver, heart, and lung of C57BL/10SnJ, Tg and non-Tg strains of mice. After a first step of reverse transcription, cDNAs were submitted to amplification with Tlr4 cDNA and Gapdh specific primers in nonsaturating conditions. After normalization, and as expected, we did not detect any noticeable Tlr4 expression in the organs of non-Tg animals (data not shown). Furthermore, there was a gradient of expression extending from the 382 strain to the 394 strain of mice (Fig. 3). The level of Tlr4 expression is well correlated with the number of integrated copies in each strain. It should be noted that the level of expression of Tlr4 in strain 382 is under the level detected in the C57BL/10SnJ wild-type strain, as expected by its unique estimated integrated copy (data not shown).

Estimation of the cell surface expression of Tlr4 by FACS analysis

The cell surface expression of Tlr4 was assessed with a Tlr4 mAb, MTS510, that specifically recognize the Tlr4/MD-2 complex (43). Thioglycolate-elicited peritoneal macrophages from the four Tg Tlr4 strains were able to express Tlr4/MD-2 complex, as shown in Fig. 4. Interestingly, a gradient of expression was observed as reflected by the intensity of fluorescence, extending from strain 382 to strain 394. These data clearly show that the level of expression...
of Tlr4-MD-2 on the cell surface of thioglycolate-elicited peritoneal macrophages is well correlated with the number of Tlr4 integrated copies in the genome and the level of Tlr4 transcription.

Mitogenic response of splenocytes to LPS stimulation in vitro

We next tested the proliferative ability of splenocytes derived from Tlr4 Tg strains to respond to mitogenic LPS stimulation in vitro. C57BL/10ScNCR and C57BL/10SnJ strains of mice were used as internal controls. No proliferation was detected using the C57BL/10ScNCR splenocytes at any concentrations of LPS used, ranging from 1 pg/ml to 10 μg/ml (Fig. 5). In contrast, C57BL/10SnJ splenocytes began to proliferate in the presence of 1 μg/ml of LPS, but the level of incorporated [3H]thymidine remained low. Interestingly, all Tg animals showed a higher mitogenic response than those exhibited by these wild-type controls. Among them, splenocytes derived from the 382 strain were the least sensitive to the LPS stimulus. Those derived from the 388 and 390 strains presented a similar but intermediate response. Finally, those derived from the 394 strain incorporated the highest amount of [3H]thymidine. Furthermore, whereas the 382 and C57BL/10SnJ splenocytes showed a similar sensitivity to LPS stimulation, those derived from 388, 390, and 394 Tg mice responded to a 10-fold lower LPS stimulus (100 ng/ml). Therefore, there is a clear correlation between levels of Tlr4 expression and the cellular ability to sense LPS and to mediate mitogenic response.

In vivo response to LPS injection

To test the in vivo responsiveness of Tlr4 Tg animals to LPS-induced septic shock, we injected increasing amounts of LPS (10 μg to 1 mg i.p.) in the animals and monitored their survival. C57BL/10ScNCR mice were completely resistant to LPS-induced lethality, whereas C57BL/10SnJ animals became sensitive after the injection of 1 mg of LPS (Table I). Interestingly, all Tg animals exhibited a severe sensitivity to the 1-mg LPS dose, with almost 100% mortality in all four Tg strains. Furthermore, strains 388, 390, and 394 were more sensitive to the lethal effect of LPS and showed 75–100% mortality with 100 μg of LPS. Finally, all strains were resistant to a challenge with 10 μg of LPS. Thus, the number of Tlr4 copies and the level of Tlr4 expression appear to have an impact on the host response to LPS: strain 382 shows a similar behavior to that of wild-type control mice (C57BL/10SnJ) at low dose, and the three other Tg animals were sensitized to LPS at a lower dose. These observations are also in agreement with the in vitro ability of the splenocytes to proliferate after LPS exposure, although a plateau was reached with three copies of the transgene in vivo. The unexpected greater sensitivity of strain 382 to a high dose of LPS in vivo compared with that of wild-type C57BL/10SnJ mice may be explained by an effect of the position of the transgene in the genome and/or a background effect. Despite the fact that C57BL/10SnJ and C57BL/10ScNCR have similar origin, they have been kept separate for >50 years and may have accumulated subspecies-specific mutations, as seen between C57BL/10ScCr and C57BL/10ScNcr mice (44).

Experimental infection with S. typhimurium

Initially, we examined the host response of Tlr4 Tg and non-Tg littermates following infection with virulent S. typhimurium. We monitored survival to infection over a 2-wk period. Regardless of the Tg strain used, non-Tg animals (Nramp1<sup>Asp169/Tg</sup>) died within 4–5 days, whereas Tg animals (Nramp1<sup>Asp169/Tg</sup>) died within 5–7 days (Fig. 6). This protection delay is statistically significant in all four strains studied (p < 0.05 for strain 382; p < 0.01 for strains 388, 390, and 394) using a Kaplan-Meier analysis. Therefore, the presence of the transgene and the expression of Tlr4 conferred protection to the lethality induced by S. typhimurium.

Table I. In vivo response to LPS injection

<table>
<thead>
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<th>1 mg</th>
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<tr>
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<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
</tr>
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</table>

* Number of dead mice/total number of mice. The percentage of dead mice is shown in parentheses.

FIGURE 5. Mitogenic response of splenocytes to LPS stimulation. Spleen cells were isolated from C57BL/10SnJ and C57BL/10ScNcr, Tg and non-Tg, animals. They were stimulated with various concentrations of LPS, and their responses were measured by determining the incorporation of [3H]thymidine over a 16-h period. The values are means ± SEM based on three mice for each group. B10, C57BL10.

FIGURE 6. Survival of mice following infection with S. typhimurium. Effect of the Tlr4 transgene and Nramp1 genotype on survival of mice infected with S. typhimurium. For strain 382, 31 mice were followed with at least 3 mice per group. For strain 388, 32 mice were followed with at least 3 mice per group. For strain 390, 33 mice were followed with at least 5 mice per group. For strain 394, 22 mice were followed with at least 4 mice per group. • and ○, Nramp1<sup>Asp169/Gly169</sup>; △ and ©, Nramp1<sup>Gly169/Asp169</sup>; ● and ©, Tg<sup>−/−</sup>; and ▲ and △, Tg<sup>+</sup>.
However, the intensity of the protection was apparently not correlated with the level of \( Tlr4 \) expression. Because of the small impact of \( Tlr4 \) transgene on resistance to infection in our model using C57BL10ScNCR mice (Nramp1\(^{Asp169} \)) as background strain and the known importance of Nramp1 in the control of \( Salmonella \) infection, we investigated the possible interaction of the \( Tlr4 \) and Nramp1 genes. We created a congenic strain possessing the wild-type resistant allele at Nrampl (Nramp1\(^{Gly169} \)) onto the Nrampl- and \( Tlr4 \)-deficient C57BL10ScNCR background through five consecutive backcross generations. These mice were crossed to \( Tlr4 \) Tg animals to obtain a combination of littermates that were heterozygous at Nrampl\(^{Gly169}\)Nrampl\(^{Asp169} \) (the Nrampl\(^{Gly169} \) allele is fully dominant) and either possessing or not possessing the \( Tlr4 \) transgene. The mice were infected i.v. with \( 10^5 \) CFU \( S. \) typhimurium. Non-Tg animals that were Nrampl\(^{Gly169}\)Nrampl\(^{Asp169} \) (Nrampl\(^{Gly169} \)/Tg) succumbed within 6–9 days postinfection instead of 4–5 days as observed for Nrampl\(^{Asp169}\)/Tg- Tg mice (Fig. 6). This effect is constant over the four Tg strains studied. As expected, Nrampl is playing a major role in protecting the mice during the infection. The most dramatic effect in survival after infection was observed in mice carrying a wild-type allele at Nrampl and the \( Tlr4 \) transgene (Nrampl\(^{Gly169}\)/Tg\(^{+} \)). The protection conferred by Nrampl and \( Tlr4 \) was more pronounced in mice carrying at least three copies of the transgene. The most resistant mice were from strain 388 with 100% survival at day 14 postinfection. Animals were grouped according to their \( Tlr4 \) copies and their genotype at Nrampl\(^{Gly169}\)/Asp169 or Asp169/Asp169). C57BL10/SnJ (\( Tlr4 \)^\(^{+} \), Nrampl\(^{Asp169}\)/Asp169) and B10/Nrampl (\( Tlr4 \)^\(^{+} \), Nrampl\(^{Gly169}\)/Asp169) mice were used as controls.

Significant inflammatory reactions, characterized by infiltration with predominantly polymorphonuclear cells (PMN) and fewer macrophages, were visible in both the spleen and the liver of all Nrampl\(^{Asp169}\)/Asp169 mice 5 days postinfection (Fig. 8, A–E). The lesions in \( Tlr4 \) Tg mice (Fig. 8, C–E) were comparable to those observed in non-Tg littermates (Fig. 8, A and B) and C57BL/10SnJ mice (data not shown). The presence of \( Tlr4 \) and the number of \( Tlr4 \) copies had little impact on the severity of the pathological changes in infected Tg mice on the Nrampl\(^{Asp169}/Asp169 \) background. No difference was observed with respect to site, number, morphology, and cell population involved in inflammatory foci. Only a slight improvement was observed in Tg lines 388, 390, and 394 with respect to the degree of necrosis in the liver and the severity of lymphocyte depletion in the spleen compared with those of non-Tg littermates. In the groups of Tg mice carrying a wild-type allele at Nrampl\(^{Gly169} \) (Nrampl\(^{Gly169} \)/Asp169) (Fig. 8, F–J), the lesions were less evident and the inflammatory \( Salmonella \) foci were reduced in size and number compared with those observed in \( Tlr4 \) Tg Nrampl\(^{Gly169}/Asp169 \) mice (Fig. 8, C–E). In the liver, the lesions consisted of randomly distributed foci of hepatocyte degeneration and necrosis, infiltrated equally by PMN and macrophages. In the spleen, the red pulp showed a progressively increasing expansion by a mixture of macrophages and PMN. In this case also, the presence of \( Tlr4 \) and the number of \( Tlr4 \) copies appear to have little impact on the pathology induced by \( S. \) typhimurium 5 days postinfection. The presence of both \( Tlr4 \) and Nrampl1 has a clear impact on limiting tissue damage after infection with \( Salmonella \).

All Tg animals from line 388 and a subset from line 394 carrying Nrampl\(^{Gly169}/Asp169 \) survived infection for a period extending 14 days. These \( Salmonella \)-infected Tg mice developed ascites associated with severe hepatosplenomegaly by 14 days after infection. The lesions in the spleen and the liver were different from those observed 5 days postinfection (Fig. 8, K and L). A severe inflammatory response characterized by massive infiltration of macrophages into the liver and spleen was apparent. This inflammatory process coincides with a disorganization and obstruction of

**FIGURE 7.** Bacterial loads in reticuloendothelial organs of \( Tlr4 \) Tg mice after infection with \( S. \) typhimurium. To determine the growth rate of the bacteria within the reticuloendothelial organs, mice were sacrificed 5 days postinfection, and their spleens and liver were recovered aseptically. For each group, four to six mice were analyzed. Tg\(^{+} \), \( Tlr4 \) Tg mice; Tg\(^{-} \), non-Tg littermates; r, Nrampl\(^{Gly169}/Asp169 \); and s, Nrampl\(^{Asp169}/Asp169 \).
hepatic sinusoids that could explain the observed ascites. Additional lesions were found only in the heart and consisted of multifocal myonecrosis with mineralization (Fig. 8M).

Discussion
In this study, we generated four strains of mice that possessed various numbers of Tlr4 gene copies on a C57BL/10ScNCR Tlr4 mutant background. This background was derived from a C57BL/10Sn progenitor strain that is LPS responsive, indicating that the mutant phenotype was acquired after 1953 (45). It has been shown that the LPS unresponsiveness exhibited by these C57BL/10ScNCR mice is due to a null mutation of Tlr4 corresponding to a 75-kb genomic deletion encompassing the Tlr4 locus (26, 46).

All of our experiments used the C57BL/10ScNCR mice, because these mice have a normal IL-12 response as measured by IFN-γ response when inoculated with various pathogens. This is not the case with another LPS-unresponsive C57BL/10 substrain (C57BL/10ScCr) that is not responsive to IL-12 due to a point mutation in the IL-12R gene (44).

Using our protocol of BAC transgenesis, we obtained 4 of 14 (29%) positive founders that led to the generation of four independent Tg strains expressing various functional copies of Tlr4. In the initial genotyping procedure that we used, three of four Tg strains (388, 390, and 394) were positive for the SP6/T7 extremities of the transgene and for the internal polymorphic microsatellite marker located in the Tlr4 promoter. However, the 382 strainFIGURE 8. Histology of Salmonella-induced lesions in Tlr4 Tg mouse strain 388. Spleen (A, C, D, F, H, and I) and liver (B, E, G, and J) tissue samples were analyzed from mice that presented different genotypes at Tlr4 (Tg⁺, Tlr4 Tg mice; Tg⁻, non-Tg littermates) and Nramp1 on day 5 postinfection. Group 1 (A and B), Tg⁺ and Nramp1Asp169/Asp169; group 2 (C, D, and E), Tg⁺ and Nramp1Asp169/Gly169; group 3 (F and G), Tg⁻ and Nramp1Asp169 background; and group 4 (H, I, and J), Tg⁺ and Nramp1Gly169/Asp169 background. Spleen (K), liver (L), and heart (M) tissue sections were analyzed in Tlr4 Tg mice with a Nramp1Gly169/Asp169 background on day 15 postinfection. Tissue sections were stained in hematoxylin-phloxin-saffron and photographed at magnifications of ×10 (A–C, E–H, and J), ×20 (M), and ×40 (D, I, K, and L).
was negative for the T7 amplicon, suggesting that a recombinational event occurred during the integration step of the transgene. Furthermore, considering the presence of a unique copy of the transgene in this strain, it is tempting to speculate that this event could have impeded the subsequent tail-to-head integrations. A finer investigation of the molecular structure of the T7 extremity of the transgene in this strain should clarify this point. However, and despite this event, all the Tg animals selected in our study were able to respond to the various biological properties of LPS and to the infection with *S. typhimurium*.

Using regression analysis, we showed that *Tlr4* mRNA expression levels are linearly correlated to the logarithm of the number of *Tlr4* copies integrated in the genome in spleen ($R^2 = 0.75$), liver ($R^2 = 0.85$), and lung ($R^2 = 0.84$). The increase in the number of integrated *Tlr4* transgenes results in an increase of the level of *Tlr4* expression; however, over a certain number of copies, the transcriptional machinery seems to reach saturation in spleen, liver, and lung. The situation appears to be different in the heart where the level of expression of *Tlr4* is linearly correlated to the number of integrated copies ($R^2 = 0.87$). The levels of *Tlr4* protein expression parallel the levels of mRNA expression and determine the ability of splenocytes to sense LPS and to respond to its mitogenic properties. A major increase in the host response of LPS was seen in vivo in mice carrying three and more copies of the *Tlr4* transgene: overexpression of *Tlr4* confers hypersensitivity to the lethal effects of LPS. These experiments support a crucial role of *Tlr4* in the signaling pathway induced after LPS exposure with intensities of biological responses to endotoxin being strongly dependent on the level of *Tlr4* expression.

The picture observed after infection with virulent *S. typhimurium* is clearly different. There is a good correlation between the level of expression of *Tlr4* and spleen is similar in all Tg strains tested on a C57BL10ScNCR gene(s) as measured by survival analysis and bacterial load in liver ($R^2 = 0.85$), and lung ($R^2 = 0.84$). The increase in the number of integrated *Tlr4* transgenes results in an increase of the level of *Tlr4* expression; however, over a certain number of copies, the transcriptional machinery seems to reach saturation in spleen, liver, and lung. The situation appears to be different in the heart where the level of expression of *Tlr4* is linearly correlated to the number of integrated copies ($R^2 = 0.87$). The levels of *Tlr4* protein expression parallel the levels of mRNA expression and determine the ability of splenocytes to sense LPS and to respond to its mitogenic properties. A major increase in the host response of LPS was seen in vivo in mice carrying three and more copies of the *Tlr4* transgene: overexpression of *Tlr4* confers hypersensitivity to the lethal effects of LPS. These experiments support a crucial role of *Tlr4* in the signaling pathway induced after LPS exposure with intensities of biological responses to endotoxin being strongly dependent on the level of *Tlr4* expression.

The nature of the interaction between *Nrampl* and *Tlr4* is not clear at the moment but may be at the level of the phagosome. During phagocytosis, TLR are also recruited to the phagosome where they sense the nature of the pathogen (59). There is growing evidence that LPS internalization (60–62) and colocalization with *Tlr4* is necessary for activation of different cell types (63, 64), pointing out the importance of cellular internalization and cytoplasmic trafficking in the host response to LPS. In intestinal epithelial cells, LPS and *Tlr4* reside in the Golgi apparatus (63). It is possible to envision that the interaction between *Nrampl* and *Tlr4* reside in a possible effect of *Nrampl* on the cellular distribution of LPS/Tlr4 during the infectious process by preventing the transfer of LPS/Tlr4 to the phagosome from the Golgi. The high level of *Tlr4* expression in *Nrampl*+G1/G1 mice confers an advantage in the innate immune response to infection with *S. typhimurium* early during infection, but the important inflammatory response to the bacterial pathogen in these mice strains has detrimental effects later during infection and leads to the development of liver insufficiency and cardiac failure. The lesions observed in the spleen, liver, and heart later during infection are not characteristic of multiple organ failure due to shock. There was no macrophage infiltration in the kidney and heart nor evidence of abnormal cells in circulation, suggesting a local reaction at the level of the liver and spleen. The pathology is probably mediated not only by *Tlr4* but also by *Nrampl*. In a chronic model of *Salmonella* infection, we have observed that cellular infiltration in the reticuloendothelial system is predominantly PMNs in *Nrampl*−/− mice compared with macrophages in *Nrampl*+/+ mice (65). The massive cellular infiltration in the spleen and liver may result from excessive release of cytokines acting on macrophage recruitment at the site of infection and persistence of infection in these two target organs. It is not clear at the moment whether *Tlr4* overexpression has a causative or exacerbating role in cardiac pathology. The heart is a major site of *Tlr4* expression both in mouse (25) and in humans (66) and LPS was reported to increase *Tlr4* expression in cardiac myocytes (66). In addition, our study clearly show that *Tlr4* expression in heart of Tg mice parallel linearly the number of integrated copies. Accumulation of LPS/Tlr4 regulated cytokines such as TNF and IL-1β, and expression of NO synthase 2 and TLR (Toll-like receptor 2 and Tlr4) have been...
reported to be increased in hearts of patients with heart failure regardless of etiology and in the hearts of animals with experimental cardiac dysfunction (66–68). The potential involvement of Tlr4 in inflammatory processes has been documented in humans and is in agreement with the present study. Genetic variants at TLR4 (Asp299Gly and Thr991Le) are associated with hyperresponsiveness to LPS in humans (69) and higher susceptibility to acute bacterial infections (70). In humans, the Asp299Gly TLR4 allele has a protective effect in the development of cardiovascular disease such as atherosclerosis (70). A more comprehensive examination of the mice surviving infection will be necessary to determine the exact mechanisms leading to the pathologic changes in the reticuloendothelial system and in heart.

A recent paper reports the creation of two other Tlr4 Tg strains (71). Although these strains were generated on a different genetic background (C57BL/10ScCr), the degree of LPS response in vivo and in vitro correlates well with the levels of Tlr4 mRNA expression as observed by ourselves. In addition, our study first demonstrated the correlation between levels of Tlr4 expression and the host response to Gram-negative infection and the functional interaction between two critical innate immune genes, Tlr4 and Nramp1 in mice. In conclusion, our data show that Tlr4 overexpression can elicit a systemic inflammatory response aimed at elimination of the invading pathogen; however, once triggered, this intense reaction follows an uncontrolled progression leading to serious tissue damage on the host. Tlr4 Tg mouse strains constitute a powerful tool in the analysis of the function of Tlr4 receptor in vivo during infection and inflammation. Selective modulation of Tlr4-induced inflammation to preserve its role in host defense while eliminating the associated self-injury would be a major breakthrough in the clinical management of sepsis.

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