Toll-Like Receptor 2 Is Required for Innate, But Not Acquired, Host Defense to *Borrelia burgdorferi*[1]

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***Borrelia burgdorferi* lipoproteins activate inflammatory cells through Toll-like receptor 2 (TLR2), suggesting that TLR2 could play a pivotal role in the host response to *B. burgdorferi*. TLR2 does play a critical role in host defense, as infected TLR2$^{-/-}$ mice harbored up to 100-fold more spirochetes in tissues than did TLR2$^{+/+}$ littermates. Spirochetes persisted at extremely elevated levels in TLR2-deficient mice for at least 8 wk following infection. Infected TLR2$^{-/-}$ mice developed normal *Borrelia*-specific Ab responses, as measured by quantity of *Borrelia*-specific Ig isotypes, the kinetics of class switching to IgG, and the complexity of the Ags recognized. These findings indicate that the failure to control spirochete levels in tissues is not due to an impaired acquired immune response. While macrophages from TLR2$^{-/-}$ mice were not responsive to lipoproteins, they did respond to nonlipoprotein components of sonicated spirochetes. These TLR2-independent responses could play a role during the inflammatory response to *B. burgdorferi*, as infected TLR2$^{-/-}$ mice developed greater ankle swelling than wild-type littermates. Thus, while TLR2-dependent signaling pathways play a major role in the innate host defense to *B. burgdorferi*, both inflammatory responses and the development of the acquired humoral response can occur in the absence of TLR2. *The Journal of Immunology*, 2002, 168: 348–355.**

Borrelia burgdorferi is unique in the abundance and number of tripalmitoyl-*S*-glyceryl-cysteine-modified lipoproteins synthesized, as >10% of the genome encodes proteins with a predicted signal peptidase II cleavage site (1, 2). These lipoproteins are potent stimulants of proinflammatory products in numerous cell types, including macrophages, endothelial cells, neutrophils, and B lymphocytes (3–6). Injection of lipoproteins or lipopeptides into joints or skin induces an acute inflammatory infiltrate consisting predominantly of neutrophils (7, 8), suggesting that lipoproteins play a major role in inflammatory induction in vivo. The recent identification of TLR2 as the signal-transducing receptor for bacterial lipoproteins leading to nuclear translocation of the inflammatory transcription factor NF-κB provides a mechanism for the initiation and modification of inflammatory events associated with Lyme disease (3, 9–13).

Toll-like receptors (TLR)[4] are highly conserved throughout evolution and have been implicated in the innate defense to many pathogens; *Drosophila toll* is required for the anti-fungal response (14), while the related *18-wheeler* is involved in antibacterial defenses (15). In mammals, TLR have been implicated in both inflammatory responses and innate host defense to pathogens (16). The best example is the C3H/HeJ mouse, which possess a nonactivating mutation in the gene encoding TLR4, the inflammatory signaling receptor for enterobacterial LPS (17, 18). C3H/HeJ mice are highly resistant to LPS-induced shock while being exquisitely susceptible to the lethal consequences of infection by the LPS-bearing pathogen *Salmonella typhimurium* (19). Interestingly, *B. burgdorferi* does not produce LPS (1, 20), and TLR4 appears to play no physiological role in the host defense to *B. burgdorferi*: C3H/HeJ mice display identical parameters of infection and disease as congenic C3H/HeN mice that possess a wild-type TLR4, including the kinetics of bacterial dissemination, the persistence of spirochetes in tissues, and the pathological severity of arthritis and carditis (21, 22). Furthermore, macrophages from LPS-hyporesponsive C3H/HeJ mice respond strongly to *B. burgdorferi* lipoproteins and sonicated bacteria (5). These findings suggest that TLR2 could play a unique role in the inflammatory response and host defense to infection with *B. burgdorferi*, analogous to that of TLR4 during infection with enterobacterial species. This hypothesis was tested by infecting mice possessing a targeted disruption in the *tlr2* gene with *B. burgdorferi*.

Materials and Methods

Mice

C3H/HeN mice were obtained from the National Cancer Institute, and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR2-deficient mice were generated by replacing a portion of the *tlr2* gene with the neomycin resistance gene, oriented in the opposite reading frame (23). These mice do not produce TLR2 protein, and TLR2 transcripts are larger than wild type due to the presence of the neomycin resistance gene (23). Cells from these mice are functionally deficient in TLR2-mediated signaling (see Fig. 1) (23). The TLR2$^{-/-}$ mutation was on a mixed 129SV × C57BL/6 background (see Figs. 1 and 2*A*) or was backcrossed four generations onto C57BL/6J mice, and heterozygous parents were used to generate experimental TLR2$^{-/-}$, TLR2$^{+/-}$, and TLR2$^{+/+}$ littermates

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[4] Abbreviations used in this paper: TLR, toll-like receptor; OspA, outer surface protein A.

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(see Figs. 2*B* and 3–5 and Tables I–III). Mice were housed in the Animal Resource Center at the University of Utah Medical Center, according to guidelines of the National Institutes of Health for the care and use of laboratory animals.

Mouse macrophages

Bone marrow-derived macrophages from C3H/HeN, C57BL/6, TLR2$^{-/-}$, and TLR2$^{+/+}$ mice were prepared as previously described (24). Briefly, bone marrow cells were cultured in RPMI supplemented with L929-conditioned medium for 7 days at 37°C. Macrophages were recovered with ice-cold PBS and replated in 12-well culture dishes at a density of 5×10^5/well in serum-free medium containing 1% Nutridoma (Roche, Indianapolis, IN). After overnight incubation at 37°C, nonadherent cells were removed, and the indicated agonists were added. Supernatants were harvested from these cultures at the designated times and either assayed immediately (IL-10 and TNF) or stored at −20°C until assayed.

Infection of mice with B. burgdorferi

TLR2$^{-/-}$, TLR2$^{+/+}$, and TLR2$^{+/-}$ littermate controls were infected by intradermal injection with 2×10^3 organisms of a passage five culture of the N40 isolate of *B. burgdorferi* provided by S. Barthold (University of California, Davis, CA) (25).

Measurement of ankle joints

Ankle joint measurements were made as described previously (26). Rear ankle joints were measured at the time of infection and at 2, 4, or 8 wk following infection using a metric caliper, and data were reported as an increase in swelling. Measurements were performed on TLR2$^{-/-}$ mice and littermate controls in a blinded fashion on the thickest portion (anterior-posterior) of the ankle, with the joint extended.

Lesion assessment of ankle joints

Histological analyses were performed on the rear ankle joint that exhibited the greatest swelling from mice sacrificed at 2, 4, or 8 wk postinfection. Joints were fixed in 10% neutral-buffered formalin, decalcified, embedded in paraffin, and sectioned at 5 μm before staining with H&E. Sections were viewed in a blinded fashion and assessed for various lesions of disease, including edema, neutrophil and/or mononuclear inflammation, tendon sheath thickness, and reactive/reparative responses. Each lesion was issued a score ranging from 0 to 5, with 5 representing the most severe lesion and 0 indicating normal tissue. Scores for individual lesions were incorporated into the overall lesion score reported in this study.

Reagents

Sonicated *B. burgdorferi* was prepared from 10-day cultures as previously described (27). The protein content of this preparation was determined by Bradford assay. Recombinant outer surface protein A (OspA) was provided by J. Dunn (Brookhaven National Laboratories). The recombinant OspA and sonicated *B. burgdorferi* contained <0.3 endotoxin units/500 ng as determined by *Limulus* amebocyte assay (Associates of Cape Cod, Cape Cod, MA). Ab and standards for cytokine and Ig ELISAs were obtained from PharMingen (San Diego, CA), and polymyxin B was obtained from Sigma (St. Louis, MO). LPS from *Escherichia coli* D31 m4 (Re) was purchased from List Biological Laboratories (Campbell, CA) and was repurified according to our previous report (28).

Isolation of DNA from infected mouse tissues

One rear ankle joint, the heart, and ear tissues were harvested from experimental animals sacrificed at 2, 4, or 8 wk postinfection, and DNA was prepared as previously described (22). Briefly, individual tissues were incubated in 0.1% collagenase at 37°C overnight before adding an equal volume of 0.2 mg/ml proteinase K (Sigma). After overnight incubation at 55°C, DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Following digestion with 1 mg/ml DNase-free RNase (Sigma), samples were again extracted, and DNA was recovered by precipitation. This precipitate was resuspended in 1.5 ml water, and DNA content was determined by absorbance at 260 nm.

Quantification of B. burgdorferi *by continuous monitoring of PCR*

PCR analyses were performed in a fluorescence temperature cycler (LightCycler LC24, Idaho Technology, Idaho Falls, ID) as previously described (29). Briefly, amplification was performed on 200 ng sample DNA in a 10-μl final volume containing 50 mM Tris (pH 8.3), 3 mM $MgCl_2$, 4.5 μg BSA, 200 μM dNTP, a 1/30,000 dilution of SYBR Green I (Molecular Probes, Eugene, OR), 5 μM of each primer, 0.5 U *Taq* polymerase (Life Technologies, Gaithersburg, MD), and 110 ng TaqStart Ab (Clontech, Palo Alto, CA). Amplification was performed at 40 cycles, with each cycle comprised of heating at 20°C/s to 95°C with a 1-s hold, cooling at 20°C/s to 60°C with a 1-s hold, and heating at 1°C/s to 84°C. This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled product. The cycle at which the product is first detected is used as an indicator of the relative starting copy numbers present in the sample. Copy numbers for mouse *nidogen* and *B. burgdorferi recA* were calculated using the LightCycler software, and *recA* values were corrected by normalization based on *nidogen* copies. The oligonucleotide primers used to detect mouse nidogen were nido.F (5′-CCA GCC ACA GAA TAC CAT CC-3′) and nido.R (5′-GGA CAT ACT CTG CTG CCA TC-3′). The oligonucleotide primers used to detect *B. burgdorferi recA* were nTM17.F (5′-GTG GAT CTA TTG TAT TAG ATG AGG CTC TCG-3′) and nTM17.R (5′-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3′).

Ig quantification

Serum obtained by retro-orbital bleeding of experimental animals was assayed by ELISA to determine Ig content. Microtiter plates were coated with either sonicated *B. burgdorferi* or goat Ab to mouse IgG, IgM, and IgA (Life Technologies). Serum dilutions were added to plates for 90 min at 37°C and bound murine Ig was detected by addition of HRP-conjugated Abs to murine IgG or IgM (Zymed, San Francisco, CA). Ig content was estimated by comparing with standard curves using purified IgG or IgM. *B. burgdorferi*-specific Ig subclasses were assessed by titrating serum samples on plates coated with sonicated *B. burgdorferi* using isotype-specific HRP-conjugated secondary Abs (Zymed).

Western blot analysis

Western blots were prepared by separating 120 μg sonicated N40 isolate of *B. burgdorferi* on 12% SDS-PAGE and transferring to Immobilon (Millipore, Bedford, MA). Blots were incubated with a 1/50 dilution of infected or control mouse sera. OspC and OspA were detected with a 1/500 dilution of rabbit polyclonal anti-OspC or 2 μg/ml monoclonal anti-OspA, provided by T. Schwan (Rocky Mountain Laboratories, Hamilton, MT). Bands were detected with alkaline phosphatase-labeled goat anti-mouse or goat anti-rabbit IgG (Life Technologies).

Results

Strong evidence indicates that bacterial lipoproteins activate murine and human cells through TLR2 (9–11). The prototypic *B. burgdorferi* lipoprotein, OspA, is a strong stimulant of cytokine production in murine bone marrow-derived macrophages and human cell lines, with responses induced by as little as 1–10 ng/ml OspA (9, 13). As expected, macrophages from TLR2$^{-/-}$ mice failed to respond to 500 ng/ml OspA (Fig. 1, *A–C*), while responding strongly to repurified LPS (28, 30). The OspA-stimulated production of the proinflammatory products, IL-6 (Fig. 1*A*), NO (Fig. 1*B*), and TNF-α (Fig. 1*D*), was abolished in TLR2-deficient macrophages, as was the production of the anti-inflammatory cytokine IL-10 (Fig. 1*C*). Thus, lipoprotein signaling through TLR2 is required for both pro- and anti-inflammatory signaling in response to *B. burgdorferi* lipoproteins, as was previously found for the facilitating molecule CD14 (13, 31, 32).

Because *B. burgdorferi* lacks LPS but abundantly produces lipoproteins, it has been hypothesized that the majority of cytokine stimulatory activities of the bacteria result from the lipoproteins (33). Macrophages from TLR2-deficient mice were used to test for the presence of additional, TLR2-independent, stimulatory molecules in a sonicated preparation of *B. burgdorferi*. These experiments demonstrated that the major stimulatory component of *B. burgdorferi* acts through TLR2, with little response from TLR2-deficient macrophages to 0.5 or 5 μg/ml sonicate (Fig. 2*A*). However, 50 μg/ml of sonicated *B. burgdorferi* possessed strong stimulatory activity for macrophages from TLR2$^{-/-}$ mice, although a 10-fold greater concentration of sonicate was required to elicit IL-6 production equivalent to that of macrophages from wild-type

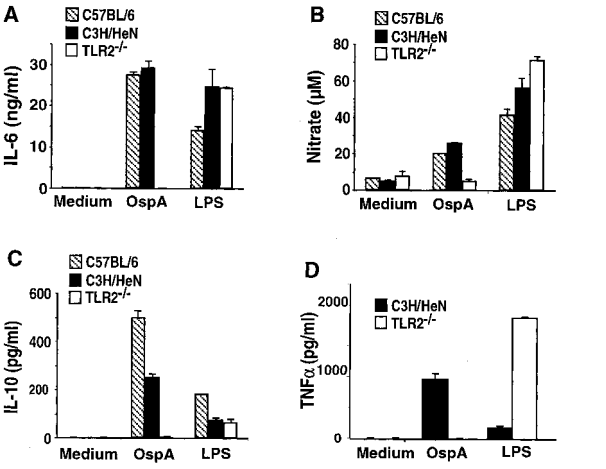

FIGURE 1. *A–D,* Macrophages from TLR2-deficient mice fail to respond to *B. burgdorferi* lipoproteins. Bone marrow-derived macrophages from different mouse strains were stimulated with either recombinant OspA (500 ng/ml) or repurified LPS (100 ng/ml), and 24-h supernatants were assessed for IL-6 (*A*), IL-10 (*C*), and TNF-α (*D*) by ELISA or for nitrate production (*B*) by Greiss reaction. All samples except those with LPS contained 10 μg/ml polymyxin B.

C3H/HeN mice (Fig. 2*A*). The differences in the response of macrophages from C3H/HeN and TLR2-deficient mice (derived from 129SV $\times$ C57BL6/J) to sonicated *B. burgdorferi* (Fig. 2*A*) could have been influenced by other variations besides the specific disruption of the *tlr2* gene. Therefore, macrophages were prepared from TLR2$^{-/-}$ mice and their wild-type littermates for comparison of responses to OspA and sonicated *B. burgdorferi*. Macrophages from TLR2$^{-/-}$ mice did respond to sonicated *B. burgdorferi*, but again required 10- to 100-fold more sonicate for equivalent NO production as macrophages from wild-type littermates (Fig. 2*B*).

The ability of sonicated *B. burgdorferi* to stimulate responses in macrophages from TLR2-deficient mice is not likely to be due to a second receptor for lipoproteins, as these macrophages failed to respond to 5 μg/ml OspA, a 10-fold higher concentration than the 500 ng/ml found to be optimal in our laboratory (Fig. 2*B*). Failure of TLR2-deficient macrophages to respond to 5 μg/ml OspA is shown for NO, the most sensitive indicator of macrophage activation, and similar results were obtained with TNF-α, IL-6, and IL-10. The diminished response by wild-type macrophages to 5 μg/ml OspA relative to the more standard maximum concentration of 500 ng/ml (Fig. 2*B*) was demonstrated to be due to the effects of detergent in the OspA preparation (not shown). These results suggest that the TLR2-independent stimulatory activity in sonicated *B. burgdorferi* is not mediated by lipoproteins, but, rather, involves additional, less potent microbial products. Numerous products associated with Gram-negative bacteria have been found to signal through various TLR, including peptidoglycan (34), glycolipids (35), unmethylated CpG containing DNA (36), and flagellin (36). Interestingly, the contribution of these nonlipoprotein bacterial compounds to *B. burgdorferi* stimulation of murine macrophages was only evident in the absence of TLR2 signaling.

The contribution of TLR2 to the development of Lyme disease was determined by infecting TLR2$^{-/-}$ mice with *B. burgdorferi* and assessing arthritis severity at 4 wk postinfection, the peak of arthritis development. TLR2$^{-/-}$ mice displayed a striking increase in rear ankle swelling compared with their TLR2$^{+/+}$ and TLR2$^{+/-}$ littermates (Table I). Mice heterozygous for the TLR2 deficiency were similar to homozygous wild-type littermates. The increase in

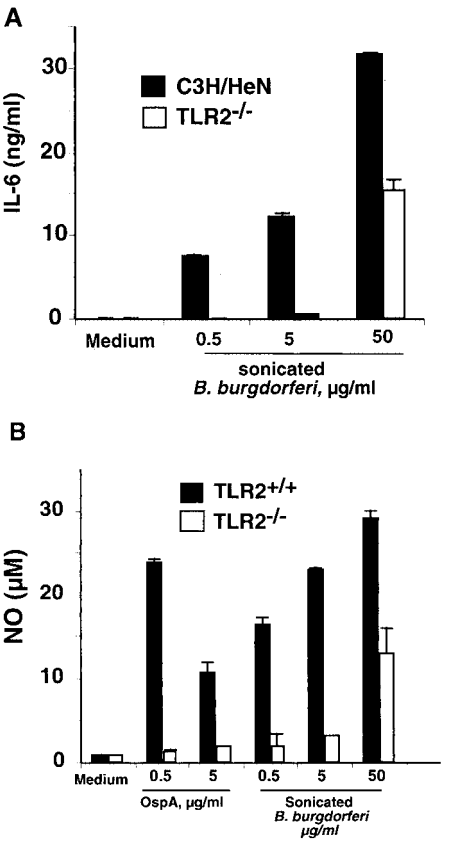

FIGURE 2. Effect of TLR2 deficiency on macrophages responses to sonicated *B. burgdorferi*. *A,* Bone marrow macrophages from TLR2$^{-/-}$ mice and C3H/HeN mice were incubated with the indicated concentrations of OspA or sonicated N40 *B. burgdorferi*. IL-6 was assayed by ELISA in supernatants collected at 24 h. *B,* Bone marrow macrophages were prepared from TLR2$^{-/-}$ mice and wild-type littermates and stimulated with the indicated concentrations of OspA or sonicated N40 *B. burgdorferi*. Supernatants collected at 24 h were assessed for nitrate production by the Greiss reaction. All samples received 10 μg/ml polymyxin B.

ankle swelling in TLR2$^{-/-}$ mice was also remarkable, as both rear ankle joints displayed uniformly increased swelling, whereas infection of arthritis-susceptible immunocompetent mice typically results in one rear ankle joint being more severely affected (21). Ankle swelling gives only a partial picture of joint pathology in murine Lyme disease, providing information primarily on the degree of edema within the ankle tissue. At sacrifice, the most severely swollen ankle joint was taken for histological analysis to obtain a more complete picture of joint pathology. The C57BL/6 mouse strain typically develops mild to moderate arthritis when

Table I. *Effect of TLR2 deficiency on ankle pathology in* B. burgdorferi*-infected mice*

Genotype[a]	Ankle Measurement[b] (mm)	Histopathology[c]
tlr2$^{+/+}$	0.52 $\pm$ 0.40	2.9 $\pm$ 1.4
tlr2$^{+/-}$	0.49 $\pm$ 0.44	2.4 $\pm$ 1.3
tlr2$^{-/-}$	1.39 $\pm$ 0.20[d]	3.0 $\pm$ 1.0

[a] Groups consisted of at least five mice of each genotype. Both male and female mice, 5–7 wk of age, were used.

[b] Rear ankle measurements reflect increases in ankle measurement during the 4 wk of infection.

[c] Overall lesion score for ankle samples scored blinded on a scale of 0 (no lesions) to 5 (severe lesions).

[d] Significantly different from *tlr2*$^{+/-}$ *and tlr2*$^{+/+}$ *by Student t* test ($p < 0.01$).

infected with *B. burgdorferi*, with histopathology peaking at about 4 wk following infection. We expected that TLR2 deficiency could result in less severe arthritis, and therefore, its effect might not be dramatic on this mouse background. Lesions within ankle joints were evaluated for disease parameters, including neutrophil infiltration, mononuclear cell infiltration, sheath thickness, reactive and reparative responses, and overall lesion severity. Joints from $TLR2^{-/-}$ mice received slightly higher scores for mononuclear cell infiltration and tendon sheath thickness than did $TLR2^{+/+}$ and $TLR2^{+/-}$ littermates, However, the scores for these individual parameters and for overall lesion severity for $TLR2^{-/-}$ mice were not statistically different from those for the other two genotypes (Table I). Therefore, on this relatively arthritis-resistant background, TLR2 deficiency did not significantly influence parameters of lesion severity. These findings suggest that the ankle swelling measurements were primarily monitoring edema. The finding that ankle swelling was more dramatically influenced by deletion of TLR2 than was histopathologically scoring of arthritic lesions is consistent with our previous observation that these two traits are regulated independently by genetic loci mapping to distinct locations (37).

The impact of TLR2 on host defense to *B. burgdorferi* was determined by quantification of spirochetes in DNA prepared from tissues of infected mice 2 and 4 wk postinfection. Hearts, rear ankle joints, and ears were analyzed, as these tissues consistently harbor the highest concentrations of persistent bacteria. All $TLR2^{-/-}$ animals possessed elevated numbers of spirochetes in tissues at both time points tested, ranging up to 100-fold higher in individual $TLR2^{-/-}$ mice than in $TLR2^{+/+}$ and $TLR2^{+/-}$ littermates (Fig. 3). The impairment to host defense was greatest in rear ankle joints of mice at 2 wk postinfection, with $TLR2^{-/-}$ mice harboring an average of 40-fold more spirochetes than $TLR2^{+/+}$ mice ($p < 0.000001$). *B. burgdorferi* rapidly colonize the skin of infected mice, exhibited by increased numbers in ear tissues from $TLR2^{+/+}$ and $TLR2^{+/-}$ control mice at 2 wk compared with 4 wk. In contrast, $TLR2^{-/-}$ mice failed to clear the high concentrations of spirochetes from ear tissue by 4 wk, harboring an average of 100-fold more bacteria than littermate controls. Spirochete levels were also much greater in hearts of mice sacrificed at 2 wk than at 4 wk postinfection regardless of the TLR2 genotype. However, at both time points hearts from $TLR2^{-/-}$ mice displayed ~10-fold more bacteria than the $TLR2^{+/+}$ or $TLR2^{+/-}$ littermate controls. To determine whether overwhelming systemic infection was underlying the increased presence of tissue spirochetes, the levels of *B. burgdorferi* in spleens of infected animals were determined. Spleen and blood generally harbor very low numbers of *B. burgdorferi*, and blood levels are difficult to quantify by PCR. The concentration of *B. burgdorferi* in the spleens of wild-type mice was extremely low even at the 2 wk peak of tissue spirochetes, (average, 0.05 *B. burgdorferi recA* genes/1000 copies of the mouse gene *nidogen*). Although spirochete levels in TLR2-deficient mice (average, 0.34 *B. burgdorferi recA* genes/1000 *nidogen* genes) were greater than those in wild-type mice, they were much lower than those found in the three other tissues assayed at 2 wk. Thus, the previous observation that blood and blood-filtering tissues are not major reservoirs for this pathogen (21) is true even in TLR2-deficient mice harboring extremely high levels of bacteria in other tissues. These quantitative differences in spirochete levels in numerous tissues from $TLR2^{-/-}$ mice and littermate controls unequivocally point to a crucial role for TLR2 in the host defense to *B. burgdorferi*.

The effect of TLR2 deficiency on the long term persistence of *B. burgdorferi* was assessed by following a small group of infected mice for 8 wk (Fig. 4). Quantitative PCR revealed little change in spirochete levels in hearts, rear ankles, and ears by 8 wk following infection. Thus, the extremely high levels of *B. burgdorferi* found in tissues from TLR2-deficient mice at 4 wk following infection persisted at 8 wk following infection. Levels in wild-type mice

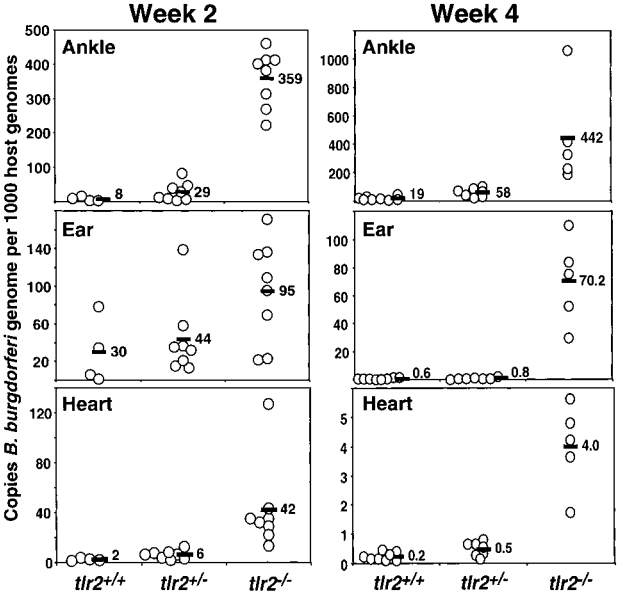

FIGURE 3. The host defense to *B. burgdorferi* is highly dependent on TLR2. Mice were injected with 2×10^3 *B. burgdorferi* organisms (N40 isolate) intradermally into the shaven back. Tissues were assessed for *B. burgdorferi* DNA levels in mice sacrificed at 2 or 4 wk postinfection by continuous monitoring PCR (29). Values reflect the number of *B. burgdorferi recA* gene copies normalized per 1000 copies of a single copy murine gene (*nidogen*); each circle represents an individual animal tissue, while the number beside the bar indicates the average for the group.

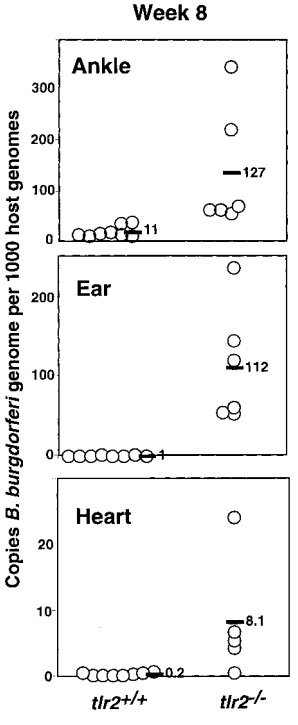

FIGURE 4. Spirochetes levels at 8 wk in TLR2-deficient mice. Mice were infected as described in Fig. 3 and were sacrificed 8 wk postinfection. *B. burgdorferi* DNA was quantified as explained in Fig. 3.

were also similar at 4 and 8 wk following infection. These findings suggest that clearance of spirochetes primarily occurs between 2 and 4 wk, and that spirochetes in wild-type mice may persist at a relatively constant, low level after 4 wk of infection.

The observation that spirochete concentrations in tissues drop between 2 and 4 wk of infection in immunocompetent mice (as seen in Fig. 3 for TLR2$^{+/-}$ and TLR2$^{+/+}$ mice) has been attributed to the appearance of acquired immunologic defenses, particularly Ab directed to surface exposed proteins of *B. burgdorferi*. Importantly, in mice with the *scid* mutation, spirochete numbers remain elevated at later time points, and passive transfer of Abs can reduce spirochete concentrations in tissues, supporting the role of acquired defenses in clearing infection and resolving disease (38–40). We hypothesized that the persistently elevated concentrations of spirochetes in tissues from TLR2$^{-/-}$ mice could result from defective development of an Ab response. Serum was collected from infected mice at 2, 4, and 8 wk following infection, and the amount of *B. burgdorferi*-specific IgM and IgG was determined by capture ELISA. Surprisingly, TLR2$^{-/-}$ mice produced similar levels of anti-*B. burgdorferi* IgM at 2 wk and of IgG at 4 and 8 wk as their littermates controls (Table II). Infection of mice with *B. burgdorferi* has been associated with an increase in total circulating IgG (26). Uninfected mice possess 1–2 mg/ml circulating IgG, while in infected mice the level of total serum IgG increased 5- to 10-fold, but was similar for all genotypes of infected mice at both 4 and 8 wk (Table II).

The absence of a particular subclass of Ab could result in the poor control of spirochetes in tissues. To assess this possibility, isotype distribution of *Borrelia*-specific Ab was determined in sera collected 4 wk following infection. IgG1, IgG2b, and IgG3 isotypes are produced by C57BL/6 mice, while IgG2a is not (41). As shown in Table III, there was no major shift in the distribution of IgG isotypes in mice of different genotypes, although IgG2b was somewhat under-represented in sera from infected TLR2$^{-/-}$ mice. However, the animal-to-animal variation within groups for *Borrelia*-specific IgG2b was larger than the average differences between genotypes, resulting in a lack of statistical significance. This strongly argues against under-representation of a particular subclass of anti-*Borrelia* IgG in TLR2$^{-/-}$ mice that could be responsible for the increased numbers of spirochetes in tissues. The lack of influence on isotype is in sharp contrast to the dramatic effect of IL-4 gene disruption on IgG1 production in *B. burgdorferi*-infected mice, a finding detected using this assay (42).

The effect of TLR2 deficiency on the spectrum of *B. burgdorferi*-specific Ags recognized by the humoral immune response was assessed by Western blot analysis, using a preparation of the N40 culture. The complexity of Ags recognized by sera from TLR2$^{-/-}$ mice was similar to that from infected TLR2$^{+/+}$ mice (Fig. 5). In fact, the number of bands detected and the intensity of staining were actually greater with sera from TLR-deficient mice than with those from wild-type littermates at both 4 and 8 wk postinfection (Fig. 5, *A* and *B*). This is likely to reflect the much greater levels of spirochetes, and therefore Ags, present in numerous tissues from TLR2-deficient mice. The presence of Abs against two well-characterized *Borrelia* Ags, OspA and OspC, was also assessed by Western blot analysis. Control lanes were developed with monoclonal or polyclonal Abs directed against OspA or OspC, respectively, and indicated that these Ags were present in the Ag preparation (Fig. 5). Infected mouse sera from both genotypes of mice contained Ig that reacted with proteins migrating in the same range as OspA and OspC. However, when the control and infected mouse sera were used in Western blots using recombinant OspA and OspC, reactivity was detected with the control anti-OspA or anti-OspC Abs, but not with the infected mouse sera (not shown). Although cultured bacteria do not represent the totality of Ags expressed by spirochetes within the mammal, this sampling of Ags is likely to reveal major alterations in Ags recognized by TLR2-deficient mice. The Western blot results indicate that the complexity of the humoral response is not reduced by deficiency in TLR2, and that gross absence of Ab directed to immunological proteins is not responsible for the observed failure to clear the spirochetes.

Discussion

B. burgdorferi are atypical Gram-negative bacteria in that they lack LPS and abundantly express Pam_3Cys-modified proteins. We and others previously demonstrated that TLR2 is the signaling receptor for bacterial lipoproteins, including the prototypic *Borrelia* lipoprotein OspA (9–11). It is well documented that LPS-hyporesponsive mice with a mutant TLR4 are more susceptible to lethal infection with *S. typhimurium* while being resistant to LPS-induced shock (19) This suggested that TLR2-mediated signaling could play a similarly complex role in both the inflammatory response and the host defense to *B. burgdorferi*. This hypothesis was tested in mice with a targeted disruption in *tlr2*. A deficiency in TLR2 greatly impaired the host defense to *B. burgdorferi*, resulting in up to 100-fold more spirochetes in tissues than those in wild-type littermates. An increased presence of *B. burgdorferi* was found in three different tissues (ankle joints, ears, and hearts) at three different time points (2, 4, and 8 wk) following infection (Figs. 3 and 4). A fourth tissue, the spleen, was assayed at 2 wk following infection, and TLR2$^{-/-}$ mice were found to harbor ~7-fold more spirochetes than wild-type littermates. These findings present a comprehensive picture of a host severely compromised in its ability to regulate spirochete levels in tissues.

These results provide hints about the normal mechanism of host control of spirochetes in tissues. In the infected wild-type mice, levels of tissue spirochetes peak at about 2 wk following infection

Table II. *Effect of TRL2-deficiency on the humoral immune response in* B. burgdorferi-*infected mice*

	2 wk	4 wk		8 wk	
Genotype[a]	*B. burgdorferi*-specific IgM[b] (μg/ml)	*B. burgdorferi*-specific IgG[c] (μg/ml)	Total IgG[c] (mg/ml)	*B. burgdorferi*-specific IgG[c] (μg/ml)	Total IgG[c] (mg/ml)
tlr2$^{+/+}$	8.5 ± 1.52	18.9 ± 4.42	8.9 ± 1.87	15.352 ± 3.4	4.26 ± 1.0
tlr2$^{+/-}$	8.9 ± 2.15	18.4 ± 4.71	7.6 ± 1.77	NT[d]	NT[d]
tlr2$^{-/-}$	12.4 ± 1.6[e]	21.0 ± 3.16	9.6 ± 3.18	13.09 ± 4.4	4.86 ± 0.30

[a] Mice were as described in Table I.
[b] Sera were harvested from mice at 2 wk postinfection and assessed *B. burgdorferi*-specific IgM using sonicated *B. burgdorferi* bound to ELISA plates.
[c] Sera were harvested from mice at 4 or 8 wk postinfection and assessed for both total and *B. burgdorferi*-specific IgG by ELISA.
[d] NT, Not tested.
[e] Significantly different from *tlr2*$^{-/-}$ *and trl2*$^{+/+}$ *by Student t* test ($p < 0.01$).

Table III. *Distribution of* Borrelia*-specific Ig isotypes in TLR2-deficient and wild-type mice*

Genotype[a]	IgG1[b] ng/ml (*B. burgdorferi*-specific)	IgG2b[b] μg/ml (*B. burgdorferi*-specific)	IgG3[b] μg/ml (*B. burgdorferi*-specific)
tlr2$^{+/+}$	9.3 ± 5.3	2.5 ± 0.4	0.25 ± 0.06
tlr2$^{+/-}$	4.4 ± 2.2	2.3 ± 0.4	0.16 ± 0.08
tlr2$^{-/-}$	14.8 ± 5.5	1.5 ± 0.7[c]	0.28 ± 0.09

[a] Mice were as described in Table I.
[b] Sera were harvested from mice at 4 wk postinfection and assessed for isotypes of *B. burgdorferi*-specific IgG by ELISA.
[c] Difference between *tlr2*$^{+/+}$ *and tlr2*$^{-/-}$ *was not significant by Student t* test ($p = 0.07$).

(43), and drop in heart and ears by 4 wk following infection. Spirochete levels in ankles of wild-type mice remained relatively constant at all three time points: 8, 19, and 11 spirochetes/1000 host genomes. These findings are dramatically demonstrated by the data presented in Figs. 3 and 4. In TLR2-deficient mice, the 2 wk levels of tissue spirochetes were even more elevated than in wild-type mice and remained abnormally high at 4 and 8 wk following infection. Several studies have demonstrated a critical role of *Borrelia*-specific Ig in the protective host defense to *B. burgdorferi* (38–40). These results are consistent with those findings, as the anti-*B. burgdorferi* humoral response at 2 wk is characterized by low level of IgM (26). By 4 wk following infection higher concentrations of *Borrelia*-specific IgG appear (21) (Table II). This suggests that the drop in spirochete number seen in wild-type mice between 2 and 4 wk following infection is due to the appearance of IgG Abs isotypes.

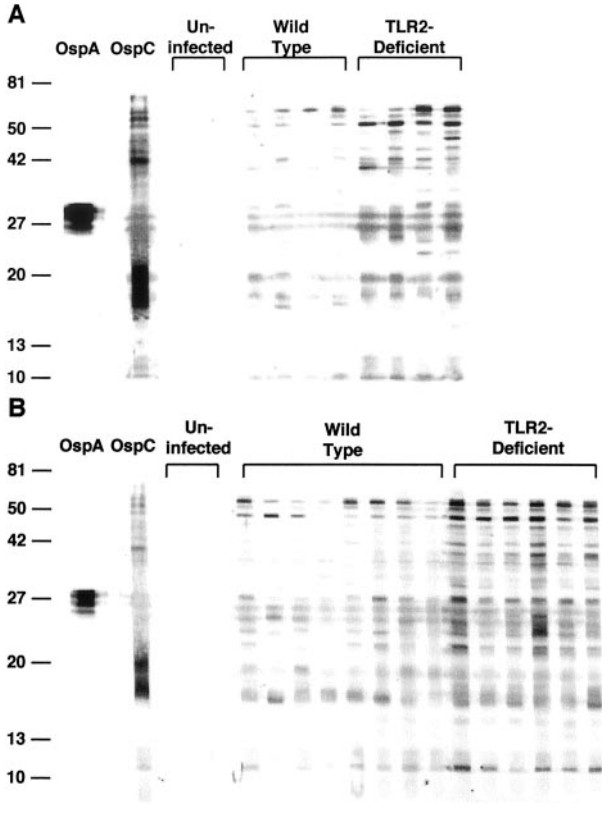

FIGURE 5. Western blot analysis of sera from TLR2-deficient and wild-type mice. Abs directed against *B. burgdorferi* Ags were detected by Western blot analysis as described in *Materials and Methods*. The positions of OspA and OspC were detected with monoclonal anti-OspA and rabbit polyclonal anti-OspC, respectively. Sera were collected from mice sacrificed at 4 wk (*A*) or 8 wk (*B*) postinfection. *A*, Sera were from two uninfected mice (one each, TLR2$^{+/+}$ and TLR2$^{+/-}$), four of the 4 wk infected TLR2$^{+/+}$ mice, or four of the 4 wk infected TLR2$^{-/-}$ mice, randomly selected from those shown in Fig. 3. *B*, Sera were from two uninfected mice (one each, TLR2$^{+/+}$ and TLR2$^{+/-}$) or from all of the 8 wk infected TLR2$^{+/+}$ mice and TLR2$^{-/-}$ mice shown in Fig. 4. The positions of molecular mass markers are indicated.

B. burgdorferi lipoproteins have been reported to possess potent B cell mitogenic properties capable of stimulating polyclonal activation of proliferation and Ig production in vitro (5). These properties as well as the adjuvant properties of lipoprotein vaccines are dependent on the Pam_3Cys modification (44, 45). We hypothesized that the humoral response would be altered in TLR2$^{-/-}$ mice, and that this was responsible for the failure to control tissue spirochetes. A complete analysis of the Ab response revealed virtually no difference between TLR2$^{-/-}$ mice and TLR2$^{+/+}$ littermates; the kinetics of appearance of IgM and class switching to IgG appeared to be normal, the magnitude of IgM and IgG response to *B. burgdorferi* Ags was similar, the relative distribution of IgG isotypes was the same, and the complexity of Ags recognized was similar. In fact, the findings presented in Tables II and III and Fig. 5 fail to support any compromise in the humoral response to *B. burgdorferi* in TLR2-deficient mice and indicate that the increased number of spirochetes found in tissues of TLR2-deficient mice cannot be attributed to a defective humoral immune response.

The finding that the host defense to *B. burgdorferi* in TLR2-deficient mice is severely compromised even though anti-spirochete IgG levels are normal argues that effective elimination of *B. burgdorferi* requires not only Ab, but also a TLR2-expressing effector cell. Phagocytic cells, such as neutrophils and macrophages, are probably TLR2-expressing cells types that could mediate the Ab-dependent clearance of *B. burgdorferi* (46). Neutrophils have been shown to play a critical role in spirochete clearance (47), and they may be very important in controlling numbers of Ab-opsonized *B. burgdorferi* in TLR2-deficient mice. The complete lack of in vitro response of cells from TLR2$^{-/-}$ mice to purified lipoproteins (Figs. 1 and 2) (23) suggests that TLR2-mediated activation of inflammatory cells is critical to the control of spirochete numbers in tissues. Interestingly, there was some clearance from the hearts between 2 and 4 wk in TLR2$^{-/-}$ mice that was not observed in ear tissues and ankle joints. Others have suggested that the inflammatory infiltrate in hearts of infected mice is predominated by monocytic cells, whereas the infiltrate in joints is predominately neutrophil (48). Thus, understanding the TLR2-dependent control of host defense could provide insight into the tissue-specific control of *B. burgdorferi*.

An unexpected finding from this study was the ability of macrophages from TLR2-deficient mice to respond to nonlipoprotein components of *B. burgdorferi*. The TLR2-lipoprotein interaction clearly dominates the macrophage response to this spirochete; however, the unique situation of the TLR2$^{-/-}$ mouse allows detection of the effects of other stimulatory molecules. Although the significance during infection of TLR2-independent signaling events during infection remains to be determined, the fact that

environmental regulation of lipoprotein gene expression is certainly involved in the persistence and pathogenesis of this organism suggest potential importance during selective stages of infection. The finding that the humoral response to *B. burgdorferi* was normal in TLR2-deficient mice argues that TLR2-independent signaling supports B cell activation in mice. This was surprising due to the complete dependence of the OspA vaccine on the Pam_3Cys modification (44). Whether the humoral response requires signals from other TLR or reflects Ag uptake and processing pathways distinct from TLR cannot be determined from these studies. The possible involvement of other TLR is suggested by the wide variety of microbial products that are able to mediate macrophage activation. Several of these signal through TLR2 (12) and therefore should not contribute to the TLR2-independent activation. Some of these may signal through TLR4, such as cellular glycolipids (35), although the in vivo significance of TLR4 signaling is questionable, as C3H/HeJ mice develop disease that is indistinguishable from that in C3H/HeN mice (21, 22). One candidate for nonlipoprotein stimulatory activity in sonicated *B. burgdorferi* is unmethylated CpG DNA, which has recently been shown to activate murine cells through TLR9 (36). Additionally, flagella from two diverse bacteria, *Listeria monoctyogenes* and *S. typhimurium*, have recently been found to signal through TLR5, suggesting that *Borrelia* flagellin could also contribute to the TLR2-independent response (49). The identification of spirochetal products responsible for TLR2-independent signaling will require further studies, as will determination of the relative physiological importance of such host-pathogen interactions. The complexity of TLR involvement has been recently observed in the macrophage response to *Staphylococcus aureus*, suggesting that the integration of multiple signaling receptors may be a common theme in bacterial pathogenesis (50).

Although TLR2-independent responses require significantly higher concentrations of bacteria than do TLR2-dependent responses in cell cultures, these stimuli could be important in vivo, where localized concentrations of bacteria can be relatively high. For example, ankle joints from $TLR2^{-/-}$ mice not only harbor an average of 10-fold more spirochetes than joints from wild-type mice, but also display increased ankle swelling relative to littermate controls. This finding clearly demonstrates that a pattern recognition receptor independent of TLR2 can contribute to *B. burgdorferi*-induced lesions in vivo. A more complete picture of the involvement of TLR2 in inflammatory arthritis development will require studies in which the TLR2 deficiency has been crossed onto a mouse strain that develops more severe Lyme arthritis.

The results presented in this study give a clear picture of TLR2 involvement in control of spirochete levels in tissues, but not in the development of the acquired humoral response. This suggests that the lipoprotein-TLR2 interaction plays a crucial role in activating cells of the innate defense for infiltration, phagocytosis, or killing of spirochetes, and that spirochete ligands for additional TLRs cannot compensate for this interaction. In contrast, the Ab response appeared normal, suggesting that TLR2-independent activation of B lymphocytes functions efficiently in the absence of TLR2. It has been reported with human B lymphocytes that TLR2 is expressed by activated B lymphocytes within germinal centers, but is not expressed by resting B lymphocytes (46, 51). It has also been reported that accessory cells are required for lipoprotein-mediated polyclonal activation of B lymphocytes (52). These results suggests that B cell activation by lipoproteins may be subsequent to activation of accessory cells, which may express numerous TLR. Our results demonstrate that innate defenses to *B. burgdorferi* are more severely compromised by TLR2 deficiency than are acquired defenses and suggest a new paradigm for host-pathogen interactions.

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