Impaired host defense to infection and Toll-like receptor 2-independent killing of *Borrelia burgdorferi* clinical isolates in TLR2-deficient C3H/HeJ mice

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Abstract

To investigate the role of Toll-like receptor 2 (TLR2)-mediated signaling in host innate defense and development of Lyme disease, the pathogenicity of *Borrelia burgdorferi* sensu stricto clinical isolates representing two distinct genotypes (RST1 and RST3A) was assessed in TLR2$^{-/-}$ C3H/HeJ mice. All TLR2$^{-/-}$ mice infected with a *B. burgdorferi* RST1 isolate developed severe arthritis. The numbers of spirochetes in heart, joint and ear biopsy specimens were significantly higher in TLR2$^{-/-}$ mice than in wild-type mice similarly infected as determined by real-time quantitative polymerase chain reaction. Interestingly, despite the higher spirochete levels in heart tissues, milder carditis was observed in TLR2$^{-/-}$ than in wild-type mice infected with this RST1 isolate ($P = 0.02$). By contrast, no positive cultures were obtained from any of the blood and tissue specimens from TLR2$^{-/-}$ mice inoculated with two RST3A clinical isolates. The data suggest that there is impaired host innate defense against infection and TLR2-independent killing of *B. burgdorferi* clinical isolates in TLR2-deficient C3H/HeJ mice.

Keywords: *Borrelia burgdorferi*; Lyme disease; Toll-like receptor 2; Innate immunity; Animal disease model

1. Introduction

Borrelia burgdorferi sensu stricto, the etiological agent of Lyme disease in North America, has been classified into three genetically distinct groups based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the 16S-23S ribosomal RNA intergenic spacer, designated ribosomal spacer types (RST) 1, 2 and 3 [1–3]. There is a significantly different distribution of genotypes in clinical isolates obtained from skin compared with blood from patients with early Lyme disease, which suggested an association between specific genotypes of *B. burgdorferi* and hematogenous dissemination in patients with Lyme disease [4]. Recently, the kinetics of dissemination and severity of resultant disease were evaluated for 12 clinical isolates representing these two genotypes of *B. burgdorferi* in a murine model of Lyme disease [5,6]. Ninety-five of 135 (70.4%) blood samples collected from mice infected with six RST1 clinical isolates during the course of infection were positive by culture. In contrast, positive cultures were obtained in only 18 of 144 (12.5%) blood specimens from mice infected with six RST3 clinical isolates. Moreover, mice infected with RST1 isolates developed more severe arthritis and carditis than those infected with RST3 isolates, demonstrating that different genotypes of *B. burgdorferi* vary in their pathogenic potential [5,6]. Interestingly, three of six RST3 clinical isolates (B356, B331 and B418, designated RST3A) showed substantially less disseminative and pathogenic potential. No spirochetes could be recovered from blood or any tissues of mice inoculated with these RST3A isolates

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[5,6]. It is most likely that RST3A isolates are cleared by host innate immunity, since no spirochetes were recovered in skin biopsies from the inoculation sites taken 24–48 h after injection in wild-type C3H/HeJ mice or in severe combined immunodeficient (*scid*) C3H mice lacking functional T and B lymphocytes [5].

Toll-like receptors (TLRs) represent an evolutionarily conserved family of membrane proteins responsible for the recognition of diverse microbial products [7]. TLRs have been implicated in both inflammatory responses and innate host defense to pathogens [7]. To date, 10 TLRs with differential expression in mammalian cells have been described [8]. Of these, TLR2 is involved in the recognition of lipoproteins and other microbial components from a variety of pathogens [7,9]. The genome of *B. burgdorferi* consists of over 150 genes encoding putative lipoproteins [10]. These abundantly expressed outer surface lipoproteins are strong stimuli for activation of nuclear factor-κB [11,12], which can result in release of both pro- and anti-inflammatory cytokines by various host cells [13]. It has been reported that inflammatory signaling by *B. burgdorferi* lipoproteins is mediated by CD14 [14] and TLR2 [15]. Moreover, studies in C57BL/6 mice suggest that TLR2 is crucial for innate host defense against *B. burgdorferi* infection [16]. Nevertheless, elucidation of TLR2 involvement in inflammatory disease development has been hampered by the genetically resistant nature of C57BL/6 mice to *B. burgdorferi* infection [13]. The aim of the present study was, therefore, to assess the impact of TLR2-mediated signaling on host innate defense and susceptibility to infection with *B. burgdorferi* isolates in TLR2-deficient mice with a C3H background.

2. Materials and methods

2.1. B. burgdorferi *isolates*

Low passage (passage 3–5) *B. burgdorferi* clinical isolates B515, B356 and B331 were used in this study. These isolates were recovered from skin biopsy specimens of early Lyme disease patients with erythema migrans. Previously, infection of wild-type C3H/HeJ mice with *B. burgdorferi* RST1 isolate B515 resulted in disseminated infection and severe disease, whereas no or only mild disease was observed in mice inoculated with the two RST3A isolates B356 and B331 [5,6]. *B. burgdorferi* strains were grown in BSK-H medium (Sigma, St. Louis, MO, USA) supplemented with 6% rabbit serum (Sigma) at 33°C [5].

2.2. Wild-type and TLR2$^{-/-}$ C3H/HeJ mice

TLR2-deficient mice were provided by Tularik (South San Francisco, CA, USA) and generated by Delatgen (Menlo Park, CA, USA) [17]. The TLR2$^{-/-}$ mutation was backcrossed five generations onto C3H/HeJ mice

and maintained as homozygous TLR2$^{-/-}$. Five-week-old wild-type C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained at the Animal Resource Center of the University of Utah, following the NIH guidelines for care and use of laboratory animals.

2.3. Infection of mice with B. burgdorferi

Wild-type and TLR2$^{-/-}$ C3H/HeJ mice were randomly divided into three groups with five mice in each group. Each mouse was inoculated intradermally with 1×10^4 of either *B. burgdorferi* RST1 isolate B515, RST3A isolate B331 or RST3A isolate B356 in 50 μl of BSK-H medium in the shaven back. Additionally, two TLR2$^{-/-}$ and one wild-type mouse were inoculated with the same volume of BSK-H medium and used as negative controls. Approximately 100 μl of whole blood was collected from each mouse on day 7 for culture of *B. burgdorferi*. The diameters of rear ankle joints of mice were measured with a metric caliper (Dolla Eastern, Long Island City, NY, USA) before inoculation (day 0) and on days 7 and 21 after infection as previously described [18]. Mice were killed on day 21 after inoculation. Whole blood (approximately 100 μl), ear biopsy (ca. 2 mm) and urinary bladder specimens were collected for cultivation of *B. burgdorferi*. Heart and ankle joint specimens were collected for histological and quantitative PCR analysis.

2.4. Preparation of DNA from mouse samples

DNA from mouse ear, heart and joint specimens was prepared using a commercial nucleic acid extraction kit (Isoquick; Orca Research, Bothell, WA, USA) as previously described [5]. Two to four microliters of diluted DNA preparations (with 20–40 ng of total DNA) were used for quantitative PCR.

2.5. Histopathology

One ankle joint and half of the heart from each mouse were coded randomly and subjected to histological analysis using semiquantitative scoring criteria as previously described [5]. Three parameters were assessed and scored separately for heart (myocarditis, valvulitis and aortitis) and joint tissues (synovitis, joint capsule inflammation and myositis). Scores of 0 (absent), 10 (mild), 20 (moderate), or 30 (severe) were assigned to each parameter, and the average scores of all three parameters were used to reflect the overall severity of carditis and arthritis for each group.

2.6. Quantitation of B. burgdorferi *DNA in mouse tissues*

The spirochete loads in heart, joint and ear biopsy specimens of infected and control mice were determined by a

real-time multiplex quantitative PCR (qPCR) assay in 96-well microplates in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). *B. burgdorferi*-specific chromosomally encoded *fla* and mouse-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified and quantitated simultaneously for each sample with the use of external standard sets for each gene. To quantify the number of copies of *B. burgdorferi* DNA in mouse samples, an external standard set containing $10–10^5$ copies of *B. burgdorferi* genomic DNA was run in each PCR plate [5]. For quantitation of the mouse gene, 10-fold serial dilutions of total mouse genome DNA (purchased from the Jackson Laboratory, stock # 000659) that containing $10–10^5$ copies of the GAPDH gene were included in each PCR run. It is estimated that there are approximately 1.6×10^4 copies of GAPDH per μl of mouse genomic DNA at a concentration of 50 μg ml^{-1} based on OD$_{260}$, assuming a size of 2800 Mb for the mouse genome and a single copy of the GAPDH gene per genome [19].

Real-time multiplex qPCR was carried out in 25-μl reaction mixtures containing $1 \times$ TaqMan universal PCR master mix (200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 400 μM dUTP, 0.01 U of uracil-*N*-glycosylase per μl, 2.5 mM MgCl$_2$, and 0.025 U of Ampli*Taq* Gold per μl, and a reference dye, 6-carboxy-X-rhodamine, Applied Biosystems), primers and probes for *B. burgdorferi fla* (1 μM of primer FL-571F (5′-GCAGC TAATG TTGCA AATCT TTTC-3′), FL-677R (5′-GCAGG TGCTG GCTGT TGA-3′) and 200 nM of TaqMan™ probe FL-611P (5′-AAACT GCTCA GGCTG CACCG GTTC-3′), size of amplicon: 107 bp) [20] and mouse *GAPDH* (100 nM of forward and reverse primers and 200 nM of probe, size of amplicon: 177 bp, from TaqMan™ rodent GAPDH control reagents, P/N 4308313, Applied Biosystems) and 2–4 μl of external standard DNA or mouse template DNA. The amplification program started at 50 °C for 2 min, then 95 °C for 10 min, followed by 40–45 cycles at 95 °C for 15 s and 60 °C for 60 s. The numbers of spirochetes and copies of mouse gene in each PCR reaction were calculated by comparing the cycle number of threshold (C_t) of the sample with those of the standards using the ABI Sequence Detection Systems Software (SDS 2.0, Applied Biosystems).

2.7. Detection of B. burgdorferi-*specific antibodies*

Plasma samples from infected and control mice were taken at day 21 and analyzed for *B. burgdorferi*-specific antibodies (IgM+IgG+IgA) by Western immunoblot with a commercial kit, in which the whole cell lysates from in vitro cultured *B. burgdorferi* isolate B31 were used as antigens (MarDx, Carlsbad, CA, USA) [5].

3. Results

3.1. Culture of B. burgdorferi *from blood and tissue specimens*

Results for cultivation of *B. burgdorferi* from blood and tissue specimens of mice are summarized in Table 1. For mice infected with *B. burgdorferi* RST1 isolate B515, positive cultures were obtained in 14 of 20 (70%) and 16 of 20 (80%) blood and tissue samples taken from wild-type and TLR2$^{-/-}$ C3H/HeJ mice, respectively ($P > 0.05$). In contrast, blood and tissue specimens collected from wild-type or TLR2$^{-/-}$ C3H/HeJ mice inoculated with RST3A isolates B356 and B331 did not yield any positive culture. The results indicate that host defense efficiently cleared RST3A organisms, even in the absence of TLR2-mediated innate immunity.

3.2. Clinical course of infection and disease severity in wild-type and TLR2$^{-/-}$ mice

Ankle joint swelling was observed in all wild-type and TLR2$^{-/-}$ mice after 2 weeks of infection with *B. burgdorferi* RST1 isolate B515. However, no clinically apparent arthritic disease was seen in any wild-type and TLR2$^{-/-}$ mice inoculated with the two RST3A isolates B356 and B331. This is consistent with the increase in average diameter of ankle joints among groups of mice as measured with a caliper before inoculation and on day 21 post infection (Fig. 1). The increase of average ankle joint diameter was significantly higher in wild-type and TLR2$^{-/-}$ mice infected with RST1 isolate B515 than in mice inoculated with RST3A isolates ($P < 0.001$). No significant difference was noted in the increased diameter between wild-

Table 1
Cultivation of *B. burgdorferi* from blood and tissues of wild-type (WT) and TLR2$^{-/-}$ C3H/HeJ mice

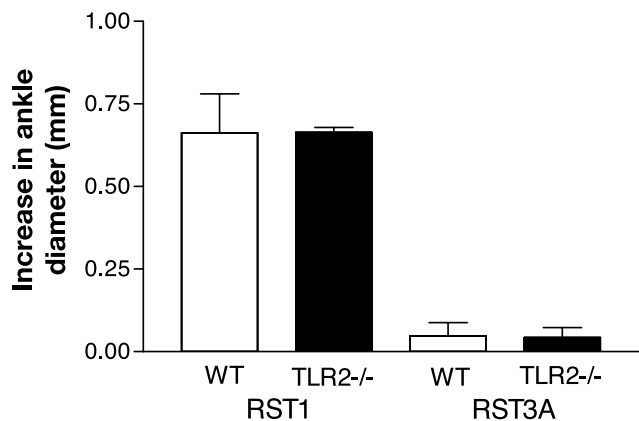| *Borrelia* isolate (genotype)	Mice	Blood		Ear	Bladder
		Day 7	Day 21	Day 21	Day 21
B515 (RST1)	WT	4/5	0/5	5/5	5/5
	TLR2$^{-/-}$	5/5	1/5	5/5	5/5
B356 (RST3A)	WT	0/5	0/5	0/5	0/5
	TLR2$^{-/-}$	0/4	0/4	0/4	0/4
B331 (RST3A)	WT	0/5	0/5	0/5	0/5
	TLR2$^{-/-}$	0/5	0/5	0/5	0/5

Fig. 1. Increase of ankle joint diameters in wild-type (WT) and TLR2$^{-/-}$ C3H/HeJ mice infected with *B. burgdorferi* clinical isolates. The increase in ankle joint diameter was an average of five RST1 or 10 RST3A isolate-inoculated mice as measured by caliper before inoculation and on day 21 after infection.

type and TLR2$^{-/-}$ mice infected with RST1 isolate B515 ($P > 0.05$).

To reflect more accurately the disease severity between wild-type and TLR2$^{-/-}$ mice infected with different subtypes of *B. burgdorferi*, the average scores of carditis and arthritis were assessed using a semi-quantitative histological approach. All wild-type mice infected with RST1 isolate B515 developed moderate to severe carditis (average score of 23). However, the severity of carditis was significantly milder in TLR2$^{-/-}$ mice than in wild-type mice infected with RST1 isolate (Fig. 2A, average scores of 9 vs. 23, $P = 0.02$). Of five TLR2$^{-/-}$ mice infected with isolate B515, only three mice had notable carditis (scores of 7, 17 and 20, respectively). The severity of arthritis in B515-infected wild-type mice (average score of 26) and TLR2$^{-/-}$ mice (average score of 23) was comparable (Fig. 2A, $P = 0.53$). No or only mild carditis and arthritis was observed in both wild-type and TLR2$^{-/-}$ mice inoculated with the two RST3A isolates (carditis: average score of 0.3 for wild-type and 0.7 for TLR2$^{-/-}$ mice, $P > 0.05$; arthritis: average score of 0.3 for wild-type and 0.7 for TLR2$^{-/-}$ mice, $P > 0.05$).

3.3. Quantitative analysis of spirochete burdens in tissues

The number of spirochetes in heart, joint and ear specimens was analyzed by a real-time qPCR assay and normalized to 10^4 mouse GAPDH gene copies (Fig. 2B). Significantly higher spirochete loads were observed in heart and joint tissues of TLR2$^{-/-}$ than in wild-type mice (approximately three-fold for heart, $P < 0.001$; and six-fold for joint, $P < 0.01$). The number of spirochetes in ear biopsy specimens was approximately 70-fold higher in TLR2$^{-/-}$ mice than that in wild-type mice infected with isolate B515 ($1.3 \times 10^4 \pm 5.2 \times 10^3$ vs. $1.9 \times 10^2 \pm 1.1 \times 10^2$ per 10^4 copies of mouse gene, $P < 0.001$).

Of mice inoculated with the two RST3A isolates, no *B. burgdorferi* DNA was detectable in any tissues from mice inoculated with isolate B331. Similarly, no *B. burgdorferi* DNA was detected in animals inoculated with B356, except for very low spirochete number (12 organisms per 10^4 copies of mouse gene) in heart tissue from a single wild-type mouse (Fig. 3).

3.4. Host humoral responses to infection with B. burgdorferi

The overall antibody response to *B. burgdorferi* whole cell antigens in mice inoculated with different genotypes of *B. burgdorferi* isolates is shown in Fig. 4. TLR2 deficiency did not affect host humoral immunity as demonstrated by Western immunoblot with sera from mice infected with RST1 isolate B515. However, no systemic humoral immune response was observed in mice inoculated with RST3A isolates, suggesting that these RST3A isolates were killed by host innate immunity in the early stage of infection.

4. Discussion

Our previous studies with wild-type C3H/HeJ mice demonstrated that there is a significant difference in pathogenicity between *B. burgdorferi* RST1 and RST3A clinical isolates [5,6]. RST3A clinical isolates are likely to be killed
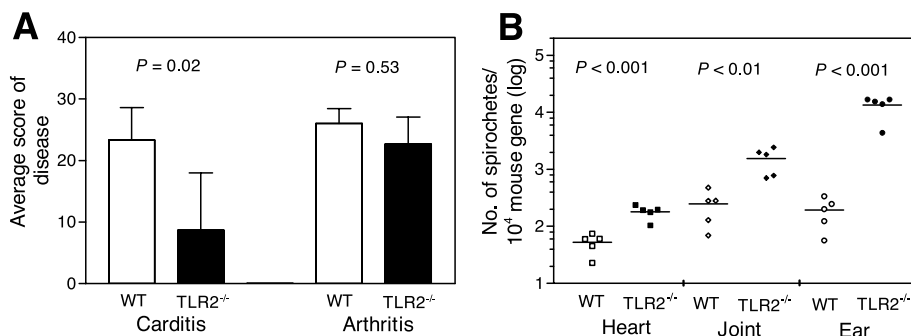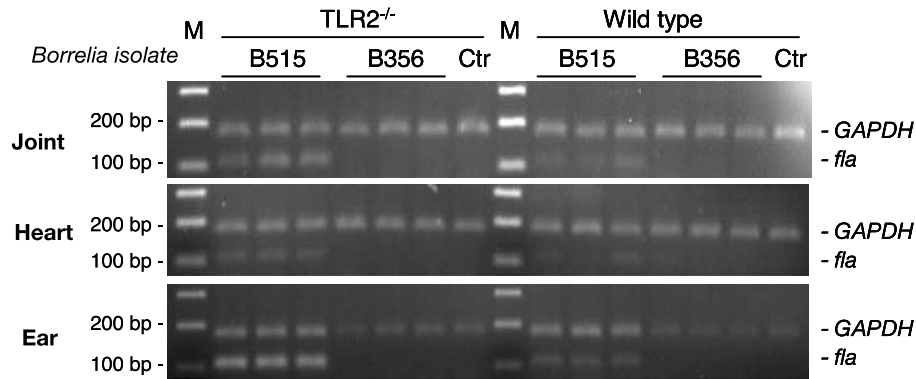

Fig. 2. Histological analysis of disease severity (A) and spirochete loads in heart, joint and ear biopsy specimens (B) of wild-type (WT) and TLR2$^{-/-}$ C3H/HeJ mice infected with *B. burgdorferi* RST1 clinical isolate B515. Severity of disease was expressed as the average scores of carditis and arthritis of each mouse group. *P* values were based on comparison between wild-type and TLR2$^{-/-}$ mice.

Fig. 3. Analysis of real-time multiplex PCR products using template DNA prepared from different tissues of wild-type and TLR2$^{-/-}$ C3H/HeJ mice inoculated with *B. burgdorferi* RST1 isolate B515 and RST3A isolate B356. PCR was performed on an ABI 7900HT Sequence Detection System as described in Section 2. Ctr: control mouse. Lane M: 100-bp DNA ladders (MBI Fermentas).

readily by host innate defense in the early stage of infection. Given that *B. burgdorferi* lipoprotein-induced host inflammatory signaling is mediated by TLR2 [15], this signaling pathway is expected to play a major role in the innate host defense to *B. burgdorferi* infection [16]. It was, therefore, of interest to assess the susceptibility and severity of disease in TLR2-deficient hosts after infection with distinct genotypes of *B. burgdorferi*.

The data suggest that TLR2-mediated signaling plays a dual role in host defense and induction of inflammatory responses to *B. burgdorferi* infection. In TLR2$^{-/-}$ mice infected with *B. burgdorferi* RST1 isolate B515, the innate host defense was impaired, as evidenced by the significant increase in the number of spirochetes in various tissues (Fig. 2B). Such a quantitative difference in spirochete levels is most likely a result of impairment in host immunity, which would limit spirochete dissemination and facilitate the clearance of bacteria from target tissues under normal conditions. On the other hand, despite significantly higher spirochetes in heart tissue, TLR2$^{-/-}$ mice developed relatively milder inflammatory carditis than wild-type C3H/HeJ mice infected with isolate B515 (Fig. 2A). This suggests that the TLR2-mediated signaling is also involved in pro-inflammatory responses and disease evolution. Earlier studies have shown a predominance of macrophages among infiltrated host cells in myocardium of mice experimentally infected with *B. burgdorferi* [21]. As TLR2 is highly expressed on the surface of mammalian monocyte/macrophages [8], the absence of TLR2 and TLR2-dependent signaling could significantly impair the host inflammatory response mediated by macrophages. This, in turn, would result in reduced inflammatory carditis in TLR2$^{-/-}$ mice. It is noteworthy that no significant difference in the severity of arthritis between wild-type and TLR2$^{-/-}$ mice infected with isolate B515 was observed, despite a higher spirochete level in joints of TLR2$^{-/-}$ mice. Dissociation in severity of Lyme carditis and arthritis in murine models has been reported previously, suggesting a potential difference in pathogenesis of these two major clinical outcomes [22]. Alternatively, it is pos-

sible that only a limited number of spirochetes is required for initiating host inflammatory signaling in target tissues. Once the spirochete density in joints reaches the required threshold or fluctuates within a certain range, inflammatory signaling could be activated and may result in severe arthritis in mice regardless of the absolute spirochete number.

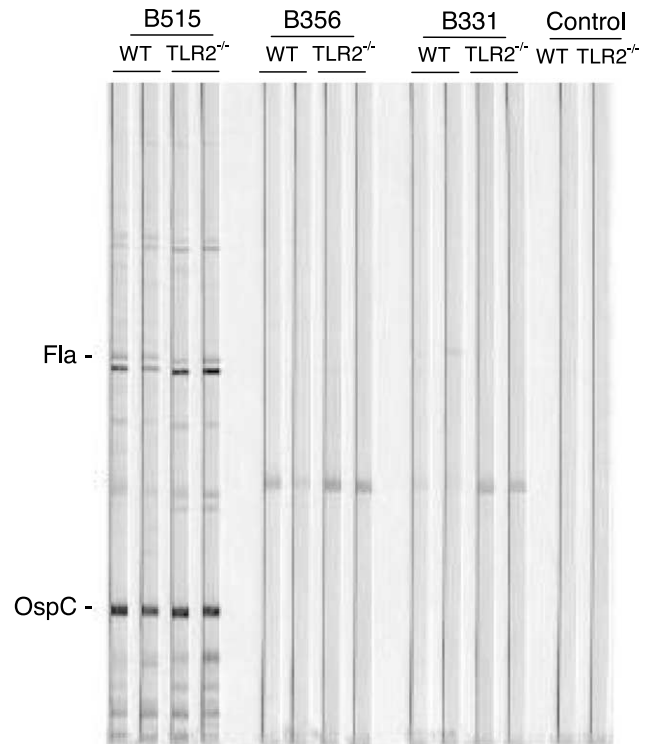No spirochetes could be cultivated from any blood or

Fig. 4. Western immunoblot for detection of *B. burgdorferi*-specific antibodies in wild-type (WT) and TLR2$^{-/-}$ C3H/HeJ mice inoculated with RST1 isolate B515 and RST3A isolates B356 and B331. Two representative blots for each group of mice are shown. The whole cell lysates of cultured *B. burgdorferi* isolate B31 were used as antigens in each blot. Plasma samples were collected on day 21 and diluted at 1:160 for immunoblotting analysis. Antibodies against *B. burgdorferi* flagellin (Fla) and OspC are indicated on the left.

tissue specimens collected during the course of infection from TLR2$^{-/-}$ mice inoculated with RST3A isolates B356 and B331. Except for heart tissue from one mouse, no spirochete DNA was detectable in any other tissues from mice inoculated with these two RST3A isolates. The negative PCR results in tissues from RST3 isolate-inoculated mice is unlikely due to either the presence of PCR inhibitors or the input of insufficient mouse template DNA as simultaneous amplification of the mouse GAPDH gene could be demonstrated by multiplex qPCR analysis. Taken together, the findings based on histology, qPCR and serology indicate that RST3A isolates were not able to establish infection in TLR2$^{-/-}$ mice. It is most likely that RST3A spirochetes were cleared by host defense mediated by a TLR2-independent pathway. A previous study found that sonicated *B. burgdorferi* could stimulate responses in cultured macrophages from TLR2$^{-/-}$ mice, although a significantly higher concentration of bacteria was required [16]. This TLR2-independent response could possibly be mediated by non-lipoprotein components of the spirochetes such as flagellin, CpG and glycolipid [7,8]. Alternatively, the failure to establish infection in either *scid* or TLR2-deficient mice may be a fundamental property of these RST3A isolates (e.g., these organisms are non-infectious). Although this possibility cannot be completely ruled out, the currently available data do not support this alternative explanation. These RST3A isolates were recovered from patients with clinical disease and were cultured in vitro for only three to five passages. All plasmids that have been reported so far to be associated with virulence of *B. burgdorferi* were preserved among these isolates [23]. Moreover, in initial experiments, RST3A isolates were able to adapt and grow in dialysis membrane chambers (DMCs) implanted in rats. Such a culture system is thought to mimic the in vivo environment, but block the effect of host defenses [24]. It will be interesting to evaluate the infectivity and pathogenicity of these RST3A isolates after growth in rat DMCs. Further studies with individual TLR knockout mice will also be helpful for elucidation of the potential roles of other TLRs during *B. burgdorferi* infection.

Host acquired immune responses are reported to be crucial for clearance of *B. burgdorferi* from target tissues and resolution of clinical disease [13]. In C57BL/6 mice, TLR2 is required for innate, but not adaptive, host defense to *B. burgdorferi* [16]. The present study confirms this finding in C3H/HeJ mice. Given that the C3H/HeJ mouse strain also has a functional defect in TLR4 [25], the data imply that production of specific antibodies against *B. burgdorferi* is not dependent on the presence of either TLR2 or TLR4.

In summary, this study provides evidence for the involvement of TLR2-mediated signaling in both host defense against and inflammatory response to *B. burgdorferi* infection. Absence of TLR2-mediated signaling could impair host defense, resulting in higher levels of spirochetes in heart, ankle joint and ear biopsy specimens in TLR2$^{-/-}$ mice infected with RST1 clinical isolates. Nevertheless, TLR2-independent signaling pathways may also play a role in host defense and be responsible for clearance of RST3A clinical isolates in mice.

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