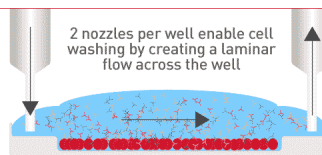


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## Signaling through CD14 Attenuates the Inflammatory Response to *Borrelia burgdorferi*, the Agent of Lyme Disease

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# Signaling through CD14 Attenuates the Inflammatory Response to *Borrelia burgdorferi*, the Agent of Lyme Disease<sup>1</sup>

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Lyme disease is a chronic inflammatory disorder caused by the spirochetal bacterium, *Borrelia burgdorferi*. In vitro evidence suggests that binding of spirochetal lipoproteins to CD14, a pattern recognition receptor expressed on monocytes/macrophages and polymorphonuclear cells, is a critical requirement for cellular activation and the subsequent release of proinflammatory cytokines that most likely contribute to symptomatology and clinical manifestations. To test the validity of this notion, we assessed the impact of CD14 deficiency on Lyme disease in C3H/HeN mice. Contrary to an anticipated diminution in pathology, CD14<sup>-/-</sup> mice exhibited more severe and persistent inflammation than did CD14<sup>+/+</sup> mice. This disparity reflects altered gene regulation within immune cells that may engender the higher bacterial burden and serum cytokine levels observed in CD14<sup>-/-</sup> mice. Comparing their in vitro stimulatory activity, live spirochetes, but not lysed organisms, were a potent CD14-independent stimulus of cytokine production, triggering an exaggerated response by CD14<sup>-/-</sup> macrophages. Collectively, our in vivo and in vitro findings support the provocative notion that: 1) pattern recognition by CD14 is entirely dispensable for elaboration of an inflammatory response to *B. burgdorferi*, and 2) CD14-independent signaling pathways are inherently more destructive than CD14-dependent pathways. Continued study of CD14-independent signaling pathways may provide mechanistic insight into the inflammatory processes that underlie development of chronic inflammation. *The Journal of Immunology*, 2005, 174: 1539–1548.

*Borrelia burgdorferi*, the agent of Lyme disease, is transmitted via the bite of an *Ixodes scapularis* tick (1). Mice, like humans, suffer from multisystemic inflammation predominantly affecting the joints and heart, thus making them an invaluable model for the study of Lyme disease pathogenesis (2). Upon infection, disease severity in mice is influenced by a variety of factors, including the age and genotype of the mouse (3), spirochetal burden (4), and the levels of pro- and anti-inflammatory immunomodulators produced by host cells (5–9). Important to the present study is the fact that infection of disease-susceptible C3H/HeN mice is distinguished from that of disease-resistant C57BL/6 mice by virtue of increased TNF- $\alpha$ , IL-6, and IFN- $\gamma$  secretion by the former genotype and greater IL-10 production by the latter (5).

Unlike typical Gram-negative bacteria, *B. burgdorferi* lack the potent glycolipid LPS (10). They do, however, possess an abundance of membrane-associated polypeptides with an N-terminal

tripalmitoyl-S-glyceryl-cysteine modification (i.e., lipoproteins) (11). Borrelial lipoproteins are thought to represent the principal proinflammatory agonists within the host and are highly immunogenic. Although several in vitro studies have demonstrated the capacity of lipoproteins to stimulate immune cells to secrete immunomodulators and up-regulate adhesion molecules (12–17), little was known regarding their in vivo ability to induce inflammation. Recent flow cytometric and immunohistochemical studies now implicate lipoproteins as a driving force behind the initiation of both innate and adaptive immunity in humans (18–20).

CD14 is a pattern recognition receptor on monocytes/macrophages (M $\phi$ )<sup>3</sup> and polymorphonuclear cells (PMN) capable of binding a diverse array of pathogen-associated molecular patterns (PAMPs) (21). In vitro studies have shown CD14 to be the principal molecule that initiates immune cell activation by *B. burgdorferi* lipoproteins (7, 22–25). CD14 exists as a 55-kDa polypeptide attached to the plasma membrane of cells via a covalent GPI linkage (26), and as a soluble serum protein (soluble CD14) that facilitates activation of cells that lack membrane CD14 (mCD14) (27). Given the inability of mCD14 to transduce signals, it was postulated that a signaling cascade ensues following interaction between a CD14-PAMP complex and a downstream signaling element(s). This conjecture was proven correct by the demonstration that borrelial lipoproteins mediate their immunostimulatory effects through TLR2 (28–34) in complex with TLR1 (35). TLRs represent an ancient family of receptors implicated in host antimicrobial defenses and that have sequence and functional homology to the IL-1R (36). CD14 is thought to play a critical role in enhancing the sensitivity of cells to minute amounts of microbial products

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<sup>3</sup> Abbreviations used in this paper: M $\phi$ , monocyte/macrophage; HPRT, hypoxanthine-guanine phosphoribosyltransferase; KO, knockout; mCD14, membrane CD14; PAMP, pathogen-associated molecular pattern; PEC, peritoneal exudate cell; PMN, polymorphonuclear cell; SFM, serum-free medium; TREM-1, triggering receptor expressed in myeloid cells 1; WT, wild type.

through tethering of certain PAMPs to the plasma membrane and facilitating their interaction with downstream TLRs and perhaps other signaling elements (37). Under certain in vitro circumstances (i.e., high agonist concentration), PAMPs can, however, signal in a CD14-independent fashion (22, 28, 38, 39).

Much of our understanding of the role of CD14 in initiating lipoprotein-mediated immune cell activation derives from in vitro experimentation using purified native lipoproteins or synthetic lipopeptides and discrete cell populations. Although informative, these studies have left unanswered whether CD14 plays a similar role during natural infection and whether whole organisms and their constituent parts share a mechanistic mode of action. To shed light on these issues, we used C3H/HeN mice with a targeted disruption of the *cd14* gene to evaluate host- and pathogen-related factors that influence disease outcome following infection with *B. burgdorferi*. This model system also afforded an opportunity to assess the impact of CD14 expression on the in vitro capacity of live spirochetes and lysed organisms to stimulate immune cells. Contrary to in vitro findings showing that blockade of CD14 diminishes inflammatory responses to some PAMPs (21), we noted that *B. burgdorferi*-infected CD14<sup>-/-</sup> (knockout (KO)) mice develop more severe and persistent inflammation than do CD14<sup>+/+</sup> (wild-type (WT)) mice. Furthermore, it was observed that live spirochetes, but not lysed organisms, are a potent CD14-independent stimulus for proinflammatory cytokine production.

Collectively, our results support the rather provocative notion that engagement of the CD14-TLR2 signaling pathway plays a critical in vivo role in attenuating the innate inflammatory response to *B. burgdorferi* and facilitating disease resolution. Moreover, they demonstrate that intact bacteria are capable of driving an exaggerated proinflammatory response in the absence of CD14 that differs fundamentally from the response triggered by free bacterial components.

## Materials and Methods

### Reagents

Care was taken during preparation of buffers and reagents to minimize contamination with LPS by using baked (180°C for 4 h) and autoclaved glassware, disposable plastic ware, and LPS-free H<sub>2</sub>O.

### Cultivation of *B. burgdorferi*

Virulent *B. burgdorferi* strain 297 was grown at 34°C in Barbour-Stoenner-Kelly medium, as previously described (24). For some experiments, spirochetes isolated from infected mice were cultivated at 34°C before growth at 23°C. Organisms grown at 23°C then were temperature shifted to 37°C and grown to mid- to late-log phase, as previously described (24), before use in experiments.

### Mice

Four- to 8-wk-old C3H/HeN mice (Taconic Farms) were housed in the Animal Resources Facility at Albany Medical College. CD14 KO mice were generated, as previously described (40), and then backcrossed 10

generations onto a C3H/HeN background. All animal procedures conformed to the Institutional Animal Care and Use Committee guidelines.

### Tick inoculation of mice

Rearing, infection, and infestation of *I. scapularis* ticks were conducted, as previously described (41). Following tick-mediated infection with *B. burgdorferi*, various tissues were isolated from mice and bisected; one-half of each tissue was processed for histopathology or snap frozen in liquid nitrogen and stored at -80°C for subsequent genomic DNA extraction.

### Assessment of inflammation

Joint inflammation was evaluated: 1) grossly by digital caliper measurement of tibiotarsal joint thickness, and 2) histologically by examination of decalcified, paraffin-embedded specimens stained with H&E. Disease severity in joints was assessed on the basis of edema, inflammatory cell infiltration, and thickening of the tendon sheath (42). The amount of polymorphonuclear and mononuclear cell infiltration was graded as 0 (none), 1 (light), 2 (moderate), or 3 (heavy). In addition, the presence or absence of cartilage and/or bone erosion and pannus formation was noted, and the degree of synovial hyperplasia was scored as mild, moderate, or severe. Hearts from the same time points also were evaluated for histopathological alteration. All specimens were scored in a blinded manner.

### Quantification of *B. burgdorferi* DNA by real-time PCR

Quantitative real-time PCR was performed using TaqMan Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Genomic DNA was extracted and quantitative real-time PCR was performed in triplicate using 40 ng of target DNA along with *B. burgdorferi*-specific *flaB* primers (200 nM) and probe (320 nM) or primers (400 nM) and probe (320 nM) directed against the single-copy mouse *nidogen* gene, as described elsewhere (43, 44). Standards were developed to quantify the number of *B. burgdorferi* *flaB* and mouse *nidogen* copies across a range of 10<sup>0</sup>-10<sup>6</sup> copies. Amplification data were acquired and analyzed using the ABI PRISM 7700 Sequence Detection software, version 1.7a (Applied Biosystems), and quantification of target DNA was accomplished, as described elsewhere (43, 45).

### Cytokine measurements

An ELISA kit (BioSource International) was used to measure TNF-α alone, or a Mouse Inflammation Cytometric Bead Array kit (BD Pharmingen) was used for simultaneous measurement of multiple cytokines in serum samples. Two-parameter flow cytometric analysis was performed using a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data were acquired and analyzed using the CBA software, version 1.1 (BD Immunocytometry Systems).

### Analysis of cytokine and TLR gene transcripts

Resident peritoneal cells within WT and CD14 KO mice were stimulated in situ by introducing 1 × 10<sup>7</sup> *B. burgdorferi* (resuspended in 0.5 ml of serum-free RPMI 1640 medium (SFM)) into the peritoneum. Instillation of this number of spirochetes represents an approximate ratio of 10 organisms/cell based upon enumeration of recoverable cells/mouse. Sham-inoculated mice received 0.5 ml of sterile medium. Peritoneal exudate cells (PECs) were collected by lavage, and first strand cDNA was produced by using 5 μg of total RNA and Applied Biosystems RT-PCR reagents. Gene products of interest were amplified using primer pairs shown in Table I, and transcript levels were normalized on the basis of the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene. The intensity of individual ethidium bromide-stained bands was determined using the FluorChem

Table I. Oligonucleotide PCR primers used to assess transcriptional activity

Gene	Forward Primer	Reverse Primer	Size
<i>tnfa</i>	5'-GATCTCAAAGACAACCACTAGTG-3'	5'-CTCCAGCTGGAAGACTCCTCCCAG-3'	254
<i>ifny</i>	5'-TGAACGCTACACACTGCATCTTGG-3'	5'-CGACTCCTTTTCCGCTTCTTGAG-3'	469
<i>il10</i>	5'-GGTTGCCAAGCCTTATCGGA-3'	5'-ACCTGCTCCACTGCCTTGCT-3'	190
<i>tlr2</i>	5'-TTTCCACGGGCTGTGGTAC-3'	5'-TGGGCTTCTCTTGGCC-3'	61
<i>tlr5</i>	5'-TGCCCAGAGCCGAGTC-3'	5'-GACAGCGATCCCACCACC-3'	65
<i>tlr9</i>	5'-TGGAATTGCCGTCGCT-3'	5'-GGGACTTTTGGCCACATTTCTAT-3'	61
<i>trem1</i>	5'-GCCAGAAGGCTTGGCAGAGAC-3'	5'-GGCCTCTGTGTGACCACCA-3'	66
<i>hprt</i>	5'-GTTGGATACAGGCCAGACTTTGTTG-3'	5'-GATTCAACTTGCGCTCATCTTAGGC-3'	162

8000 Imaging system (Alpha Innotech) and expressed as a ratio of integrated density values (cytokine or *tlr* gene/*hprt* gene).

#### Measurement of intracellular cytokines

Resident PECs were isolated by lavage and resuspended in SFM supplemented with 4% autologous serum to a final concentration of  $1 \times 10^6$  cells/ml. It is important to note that all in vitro cell stimulation experiments were conducted using SFM containing 4% autologous serum to preclude the potential effects of FBS-derived soluble CD14 on CD14 KO cell responses (46). Cells were incubated with medium alone or stimulated for 1 h with *B. burgdorferi* using spirochete/cell ratios ranging from 0.1:1 to 100:1 or with repurified *Salmonella minnesota* R5 (Rc mutant) LPS (catalogue L8893; Sigma-Aldrich) at concentrations ranging from 0.1 to 10 ng/ml. Brefeldin A (10  $\mu$ g/ml) from ICN Biomedicals was added for an additional 5 h of incubation, followed by sequential incubation of cells with PE-conjugated rat anti-mouse F4/80 (1/100 dilution) Ab from Caltag Laboratories and FITC-conjugated rat anti-TNF- $\alpha$  Ab (1/20 dilution; Caltag Laboratories), as described elsewhere (47). Cells were washed and analyzed on a FACScan flow cytometer (BD Immunocytometry Systems).

#### Isolation and activation of peritoneal M $\phi$

Proteose peptone-elicited PECs were isolated by lavage and resuspended in SFM containing 4% autologous serum. PECs were dispensed into 24-well tissue culture plates (BD Discovery Labware) at a density of  $2.5 \times 10^5$  cells/ml, and cell monolayers were washed after 3 h to remove nonadherent cells. Peritoneal M $\phi$  were incubated for 6 h with medium alone or increasing numbers of spirochetes (cultured at 34°C or temperature shifted to 37°C). For experiments comparing the stimulatory properties of live and lysed organisms, spirochete cultures were divided into two equal aliquots, one of which was subjected to sonication.

#### Membrane-based microarray analysis of gene activity

GEArray Q Series Mouse Inflammatory Cytokine/Receptor arrays (SuperArray Bioscience) were used to profile the responses of M $\phi$  exposed to *B. burgdorferi*. Proteose peptone-elicited M $\phi$  were incubated in vitro for 24 h with *B. burgdorferi* (100 spirochetes/cell) or medium alone, and total RNAs were isolated and converted to biotinylated cDNAs by reverse transcriptase. Biotinylated cDNA was hybridized to gene-specific cDNA fragments spotted onto four identical, positively charged nylon membranes that then were blocked and incubated with alkaline phosphatase-conjugated streptavidin for detection by chemiluminescent substrate, CDP-Star. Chemiluminescent signals were captured digitally using a Fluorchem 8000 Imaging system (Alpha Innotech), and the relative abundance of transcripts was determined by normalizing the gene's signal intensity to that of the *gapdh* gene using GEArray Analyzer software, version 1.3 (SuperArray Bioscience). Results are presented as a comparison of *B. burgdorferi*-induced fold change in gene expression within CD14 KO vs WT M $\phi$ . Real-time RT-PCR analysis using primer/probe sets specific for select genes (e.g., *il1 $\beta$*  and  *$\beta_2$ -microglobulin*) were used to corroborate microarray results.

#### FACS analysis of surface expression of TLR2

M $\phi$  were isolated, as described above, and incubated with medium alone or stimulated with *B. burgdorferi* (10 organisms/cell) for a period of 24 h in SFM containing 4% autologous serum. After 24 h, cells were gently washed to remove any unbound spirochetes/nonadherent cells and cultured for an additional 72 h. Cells were collected at subsequent 24-h intervals, blocked with mouse anti-mouse CD16/32 (Caltag Laboratories), and incubated with 5  $\mu$ g/ml FITC-conjugated rat anti-mouse TLR2 (clone 6C2) from eBiosciences. Cells stained with FITC-conjugated rat IgG2b isotype-matched Ab served as a control. Cells were washed, fixed, and then subjected to flow cytometric analysis, as described above.

#### Statistics

Results are expressed as a mean  $\pm$  SEM. A two-way ANOVA, followed by Newman-Keuls or planned comparisons using Fisher's least significant difference test (Statistica 6.0; StatSoft), was used to establish whether a significant difference at the  $\alpha = 0.05$  level existed between data from WT and CD14 KO mice.

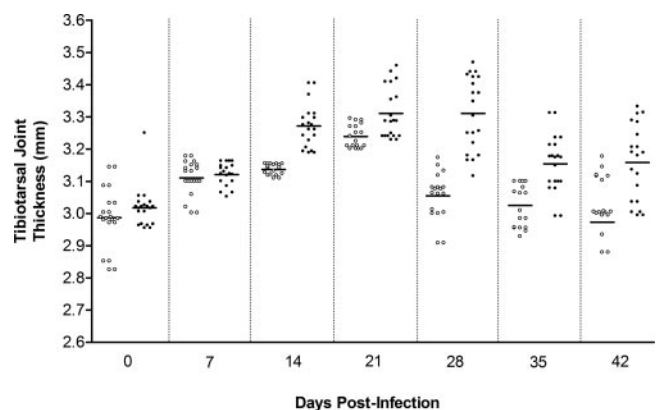
## Results

### *B. burgdorferi*-infected CD14 KO mice exhibit more severe and persistent inflammation

To begin assessing the role of CD14 in Lyme disease pathogenesis, the thickness of tibiotarsal joints was measured at various times

postinfection. As seen in Fig. 1, with the exception of 7 days, joints of CD14 KO mice were significantly thicker than those of their WT counterparts ( $p < 0.05$  for day 21 and  $p < 0.001$  for days 14, 28, 35, and 42). By 35 days, whereas the thickness of joints of WT mice returned to baseline levels, those of the CD14 KO mice remained elevated for the duration of the experiment. Differences in joint inflammation were even more striking at the histological level. As previously observed (48), joints of WT infected mice at 21 days were characterized by a dense PMN infiltrate within the peritendinous sheath, mild synovial hyperplasia (Fig. 2, C and D), and little or no involvement of the tendon and bone (Fig. 2G). In contrast, joints of CD14 KO mice exhibited extensive and highly organized inflammation of the peritendinous sheath, moderate to severe synovial hyperplasia (Fig. 2, E and F), and erosive destruction of the articular cartilage (Fig. 2H). In blinded scoring, lesion severity in joints from WT and CD14 KO mice was  $1.1 \pm 0.4$  and  $2.7 \pm 0.3$ , respectively ( $p < 0.01$ ). At 42 days, WT mice exhibited a persistent moderate to severe inflammation of the peritendinous sheath and some erosion of the articular cartilage (our unpublished data). In contrast, cellular infiltrates in joints from CD14 KO mice were reduced at this time interval. Although discordant with the observation that joints from CD14 KO mice are thicker than those of their WT counterparts at 42 days, it should be noted that edema and lesion severity are two independently regulated traits mapping to distinct genetic loci (49).

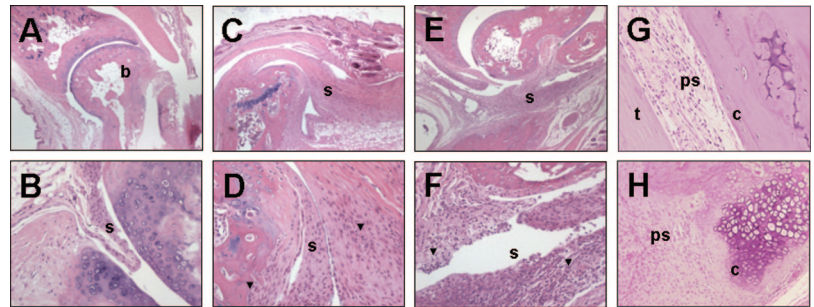
Examination of hearts from WT and CD14 KO mice at 7 and 21 days showed a similar disparity in the degree of inflammation (Fig. 3). Although focal acute and chronic inflammation was only slightly greater in CD14 KO mice than in WT mice at 7 days, a pronounced difference was evident at 21 days. Compared with WT mice, which had moderately diffused inflammation of the epicardium and endocardium with mild focal extension into the myocardium (Fig. 3A), CD14 KO mice had severe and dense epicardial and endocardial inflammation with greater myocardial involvement (Fig. 3B). Furthermore, the predominant infiltrating cell type differed between the two genotypes. Infiltrates in WT mice were predominantly lymphoplasmacytic with scattered M $\phi$  (Fig. 3C, inset), whereas those in CD14 KO mice were typified by a predominance of PMN (Fig. 3D, inset). By 42 days, hearts from both WT and CD14 KO mice showed resolution of inflammation with only mild focal chronic inflammation (Fig. 3, E and F).



**FIGURE 1.** *B. burgdorferi*-infected tibiotarsal joints from CD14 KO mice exhibit greater and more persistent swelling. WT (○) and CD14 KO mice (●) were infected with *B. burgdorferi*, and tibiotarsal joint thickness was measured at 1-wk intervals. The horizontal bars indicate mean thickness for each group, and the data are representative of two independent experiments.



**FIGURE 2.** *B. burgdorferi*-infected tibiotarsal joints from CD14 KO mice exhibit more extensive and highly organized inflammation. Joints from an uninfected WT (A and B) mouse and a 21-day infected WT (C, D, and G) and CD14 KO (E, F, and H) mouse are shown. The tendon (t), peritendinous sheath (ps), synovium (s), cartilage (c), and bone (b) are indicated. M $\phi$  and PMN are identified by arrowheads in D and F, respectively. Magnifications are as follows: A, C, and E,  $\times 40$ ; B, D, and F,  $\times 200$ ; and G and H,  $\times 400$ .



*B. burgdorferi*-infected CD14 KO mice have higher bacterial burdens and serum levels of proinflammatory cytokines

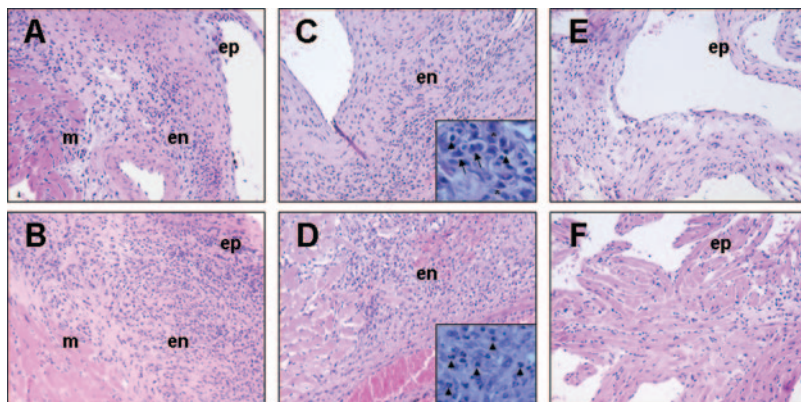
Given the correlation between spirochetal burden and the intensity of the inflammatory response in mice (4), bacterial burdens in WT and CD14 KO mice were compared. As seen in Table II, at 7 days, high spirochetal numbers were observed in bladder, heart, and ear, with considerably fewer organisms present in the joints. Although tissue burdens in CD14 KO mice tended to be higher than in WT mice, the differences reached statistical significance only at 42 days. Thus, lesional development in CD14 KO mice at 21 days, which was greater than in WT mice, preceded the peak in spirochetal burden. This finding suggests that equivalent numbers of spirochetes can elicit a more fulminant inflammatory response in the absence of CD14 than in its presence, a result analogous to the differences in Lyme disease severity observed between C3H/HeN and C57BL/6 mice (3, 50). The repertoire and level of pro- and anti-inflammatory cytokines also are known to influence the intensity of Lyme disease manifestations (51, 52); thus, we compared their production in infected WT and CD14 KO mice. Fig. 4 shows that by 7 days, serum levels of TNF- $\alpha$ , IFN- $\gamma$ , MCP-1, and IL-6 were substantially higher than in uninfected animals ( $p < 0.05$ ), returning to near baseline by 21 days. Remarkably, sera from CD14 KO mice had dramatically higher TNF- $\alpha$ , IFN- $\gamma$ , MCP-1, and IL-6 levels than did sera from WT mice. In contrast, IL-10 and IL-12p70 levels never differed between the two genotypes and were significantly elevated above baseline only at 42 days ( $p < 0.05$ ). Comparable experiments were performed using CD14 KO C57BL/6 mice. C57BL/6 mice are highly resistant to *B. burgdorferi*-induced disease and produce little or no proinflammatory cytokines, despite large numbers of bacteria in infected tissues (3, 53). Nevertheless, as was observed with C3H/HeN mice, CD14 deficiency led to the overproduction of proinflammatory cytokines by infected C57BL/6 mice (our unpublished data). Collectively, these findings suggest that engagement of CD14 is essential for protecting the host against chronic inflammation and that CD14

deficiency can undermine a genetic predisposition toward disease resistance.

*CD14 deficiency alters the kinetics of B. burgdorferi-induced pro- and anti-inflammatory cytokine gene transcription*

To explore the extent to which pro- and anti-inflammatory cytokine production is dysregulated in CD14 KO vs WT C3H/HeN mice, we assessed the transcriptional activity of key immunomodulators in *B. burgdorferi*-activated PECs. First, we used an in situ stimulation model that recapitulates the complex network of interactions that occur when cells encounter pathogens in the mammalian host. Fig. 5A depicts marked differences in both the kinetics and levels of gene transcription seen in WT and CD14 KO PECs. Transcription of *tnfa* was elevated by 1.5 h in both genotypes and peaked at 3 and 6 h in WT and CD14 KO cells, respectively. However, *tnfa* levels at 6 h were higher in CD14 KO than in WT cells. Levels of *ifn $\gamma$*  at 6 h also were higher in CD14 KO cells and peaked at 12 h. Notably, *ifn $\gamma$*  transcripts, unlike those of *tnfa*, were undetectable in WT cells at 12 and 24 h. *il10* transcript levels were more variable, with no consistent difference between WT and CD14 KO cells.

To determine whether a correlation exists between TNF- $\alpha$  mRNA levels and cytokine production, we determined the percentage of F4/80<sup>+</sup> PECs (i.e., M $\phi$ ) expressing TNF- $\alpha$  after stimulation with increasing doses of LPS or *B. burgdorferi* (Fig. 5B). Not surprisingly, when stimulated with 1 and 10 ng/ml LPS, a much lower percentage of CD14 KO M $\phi$  expressed TNF- $\alpha$  compared with WT cells ( $p < 0.05$  and  $p < 0.01$ , respectively) (Fig. 5B, inset). In stark contrast, *B. burgdorferi* at ratios of 10 and 100 organisms/cell induced TNF- $\alpha$  production in a higher percentage of CD14 KO than WT cells. Although more physiologically relevant than using a discrete cell population, this experimental design left unanswered the question of whether the TNF- $\alpha$  production was a direct consequence of stimulation by spirochetes or the influence of activated bystander cells on the M $\phi$ . To clarify this issue



**FIGURE 3.** Hearts from *B. burgdorferi*-infected CD14 KO mice exhibit more acute and extensive carditis. Hearts from 21-day infected WT (A and C) and CD14 KO (B and D) mice and 42-day infected WT (E) and CD14 KO (F) mice are shown. The epicardium (ep), endocardium (en), and myocardium (m) are indicated. Lymphocytes (arrowheads), plasma cells (arrows), and M $\phi$  (asterisks) are identified in C inset, and PMN (arrowheads) are identified in D inset. Magnifications are as follows: A–F,  $\times 200$ ; insets,  $\times 1000$ , oil immersion.

Table II. *Spirochetal burdens in tissues from CD14 KO mice are significantly greater than in tissues from WT mice<sup>a</sup>*

		Bladder	Heart	Ear	Joint
Day 7	WT	2,482 ± 726	4,442 ± 930	7,186 ± 1,424	195 ± 58
	CD14 KO	2,840 ± 1,079	8,495 ± 1,887	11,710 ± 2,348	222 ± 46
Day 21	WT	365 ± 81	143 ± 22	401 ± 87	768 ± 238
	CD14 KO	306 ± 78	208 ± 38	479 ± 89	850 ± 109
Day 42	WT	217 ± 48	142 ± 29	112 ± 15	350 ± 51
	CD14 KO	645 ± 146*	455 ± 122**	290 ± 39*	1,437 ± 224***

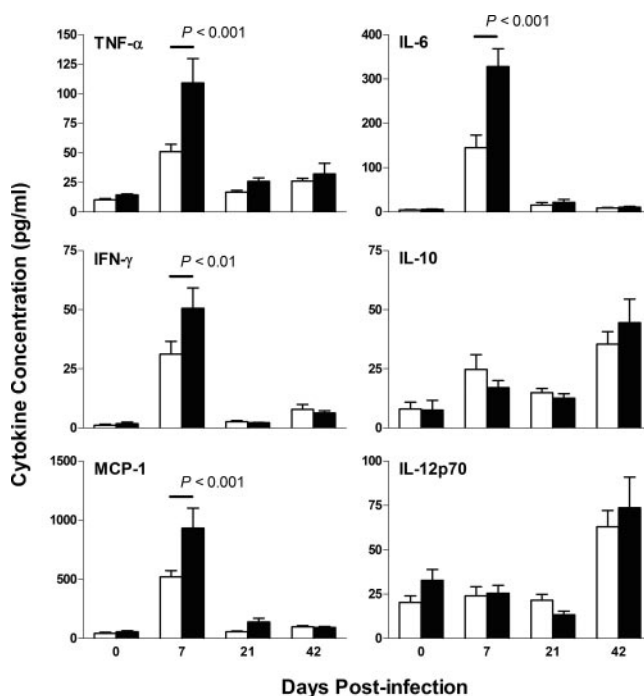
<sup>a</sup> Mice ( $n = 10$ – $15$ ) were sacrificed at 1, 3, and 6 wk post-tick inoculation, and DNAs were isolated from the indicated tissues for quantitative real-time PCR analysis. Results represent the mean ± SEM of copies of *B. burgdorferi* *flaB* for groups of mice from three independent experiments. Differences between the WT and CD14 KO groups at 42 days were considered significant (\*,  $p < 0.01$ ; \*\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ).

and explore whether viable spirochetes and borrelial lysates differed in their capacity to stimulate cells, purified peritoneal M $\phi$  were incubated in vitro with live temperature-shifted spirochetes or an equivalent number of lysed organisms (Fig. 5C). Temperature-shifted spirochetes were used because their lipoprotein expression pattern more closely reflects that observed on organisms infecting the mammalian host (54–56). We show that *B. burgdorferi* directly stimulate CD14 KO M $\phi$  to release substantially more TNF- $\alpha$  than their WT counterparts. In contrast, an equivalent number of lysed spirochetes was a much poorer stimulus of TNF- $\alpha$  production and exhibited a greater dependence on CD14. The validity of this comparison is predicated upon the efficient lysis of bacteria and the addition of equivalent amounts of live and lysed *B. burgdorferi* to the cell cultures. To ensure this to be the case, dark-field microscopy was used to verify 100% lysis of bacteria after sonication. Furthermore, after splitting a bacterial culture and sonicating one of the two aliquots, equivalent amounts of intact or lysed bacteria were subjected to SDS-PAGE, followed by silver staining; only results from experiments in which the intensity of silver staining for intact and lysed bacteria was equivalent are shown. These results demonstrate a clear-cut dichotomy in cellular response as a function of CD14 expression and the pathogen's structural integrity that may be central to immune recognition of microbes.

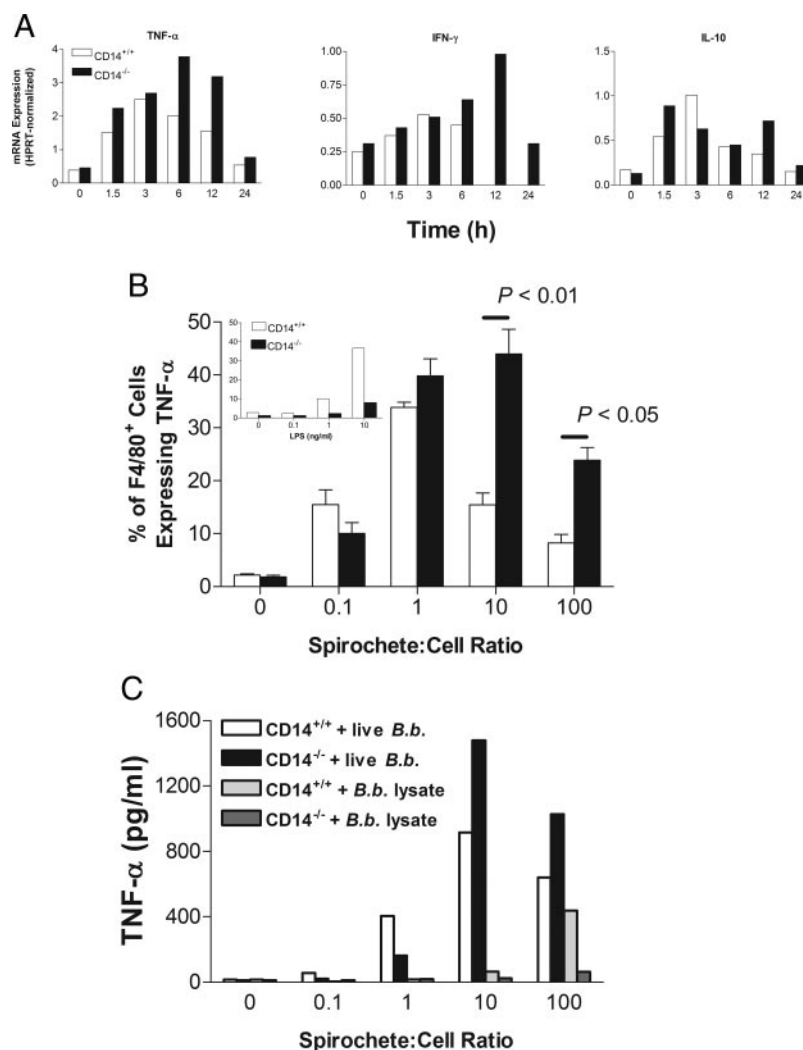
Next, we extended our profiling of the responses of WT and CD14 KO M $\phi$  to live spirochetes using microarrays. Although transcription of a majority of the inflammatory cytokine/receptor genes interrogated remained unchanged or changed less than 2-fold in response to *B. burgdorferi* (Fig. 6A), transcription of nearly a dozen genes was altered dramatically and differed between WT and CD14 KO M $\phi$  (Fig. 6B). Large-scale changes in gene activity were overrepresented in CD14 KO M $\phi$  and while genes encoding CCL3 (MIP-1 $\alpha$ ), IL-1 $\beta$ , IL-1R type-1, and CXCL15 (lungkine) were up-regulated in CD14 KO M $\phi$ , others, such as CCL4 (MIP-1 $\beta$ ), CCL1 (TCA-3), CCL21 (SLC), and the  $\alpha$ -subunits of IL-2R, IL-10R, and IL-13R were down-regulated. Molecules that were up-regulated are generally involved in the recruitment and/or activation of innate immune cells, whereas those that were down-regulated are involved in recruitment and/or activation of T cells or down-modulation of M $\phi$  effector functions. In particular, IL-1 $\beta$ , a potent proinflammatory cytokine, was dramatically up-regulated in CD14 KO M $\phi$  (~39-fold) compared with only a ~7-fold increase in WT cells ( $p < 0.001$ ). In contrast, spirochetes induced a 10- to 25-fold decrease in the transcription of genes encoding IL-2R $\alpha$ , IL-10R $\alpha$ , and IL-13R $\alpha$ 2 in CD14 KO M $\phi$ . This pattern of gene activity suggests a heightened capacity on the part of CD14 KO M $\phi$  to exacerbate rather than mitigate inflammation when activated by *B. burgdorferi*.

#### CD14 deficiency alters the kinetics of *B. burgdorferi*-induced *tlr* gene transcription and surface expression of TLR2

Next, we asked whether this dysregulated transcriptional program in *B. burgdorferi*-stimulated CD14 KO M $\phi$  extends to the TLR signaling molecules. As shown in Fig. 7, *tlr2* transcription was up-regulated in WT and CD14 KO PECs within 3–6 h of in situ stimulation by *B. burgdorferi*. Interestingly, prolonged exposure of WT cells, but not their CD14 KO counterparts, to spirochetes reduced *tlr2* transcription below unstimulated baseline levels. To determine the extent to which *B. burgdorferi* regulates other cell signaling elements, we assessed the activity of genes encoding the receptors for bacterial flagellin and DNA (i.e., TLR5 and TLR9, respectively). The pattern of *tlr5* transcription was nearly identical with that of *tlr2*, whereas *tlr9* levels in WT PECs barely rose above baseline, and although they dropped by 24 h, the reduction was not as dramatic as was observed for *tlr2* and *tlr5*. As a control, we chose to study the triggering receptor expressed in myeloid cells 1



**FIGURE 4.** Sera from *B. burgdorferi*-infected CD14 KO mice have higher levels of proinflammatory cytokines. At 7, 21, and 42 day post-tick inoculation, WT (□) and CD14 KO (■) mice ( $n = 10$ ) were sacrificed and serum cytokine levels were measured. Uninfected mice served as a negative control. Results represent the mean ± SEM of cytokine levels measured for each group of mice, and are representative of three independent experiments.



**FIGURE 5.** CD14 deficiency alters the kinetics of *B. burgdorferi*-induced pro- and anti-inflammatory cytokine gene transcription and expression. **A**, WT and CD14 KO mice received an i.p. injection of  $1 \times 10^7$  *B. burgdorferi*. Sham-inoculated mice (0) received medium alone. At the indicated times, PECs were isolated; total RNA then was purified and subjected to RT-PCR analysis to determine cytokine transcript levels. **B**, Resident PECs were incubated in vitro for 6 h with increasing doses of LPS (*inset*) or *B. burgdorferi*, and the percentage of F4/80<sup>+</sup> cells staining positive for intracytoplasmic TNF-α was determined. **C**, Purified peritoneal Mφ were incubated in vitro for 6 h, with increasing doses of viable temperature-shifted *B. burgdorferi* and an equivalent number of lysed organisms, and TNF-α levels were determined. All of the data are representative of three independent experiments.

(TREM-1), a molecule unrelated to the TLR family expressed on Mφ and PMN, which mediates acute inflammatory responses to LPS in conjunction with TLRs (57). Induction of *trem1* transcription in both cell types was identical and showed that the ability of *B. burgdorferi* to down-modulate receptor expression in WT cells is selective.

Finally, surface expression of TLR2 on WT and CD14 KO Mφ was assessed following in vitro stimulation by *B. burgdorferi* for 24 h. Fig. 8 presents changes in TLR2 surface expression relative to a baseline mean fluorescence intensity, which was set at 100%. TLR2 expression was markedly up-regulated on WT and CD14 KO Mφ by 24 h, and the mean fluorescence intensity remained elevated up to 72 h. By 96 h, TLR2 expression began to decline; however, expression on the surface of CD14 KO cells was substantially higher than on WT cells. Furthermore, while expression on CD14 KO cells remained elevated at 96 h, levels on WT cells dropped to 73% of their baseline level.

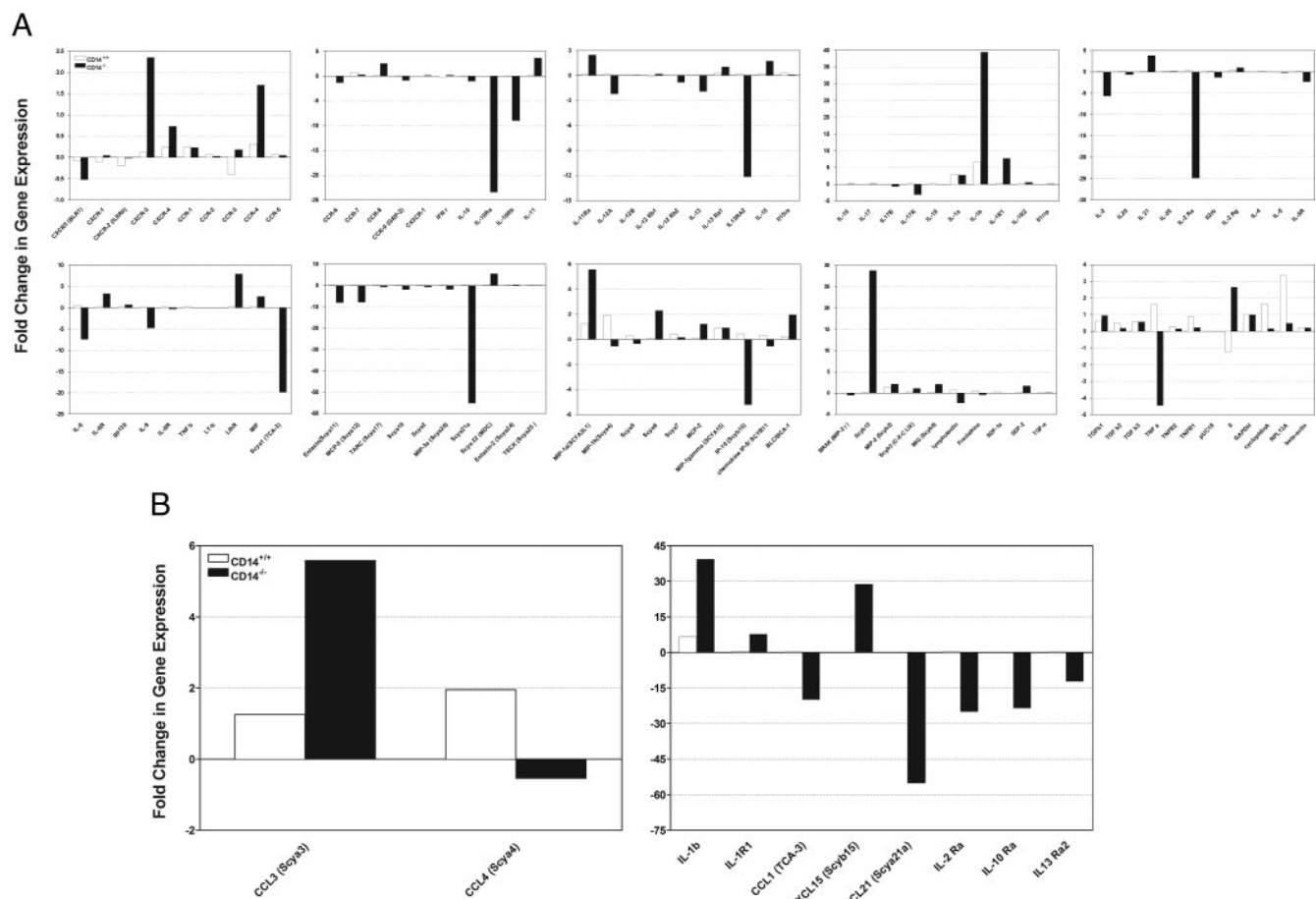
## Discussion

In this study, we define an operational role for CD14 during natural infection with *B. burgdorferi*. Based on in vitro studies demonstrating a critical role for CD14 in lipoprotein-mediated immune cell activation and subsequent immunomodulator release (7, 22–24), CD14 KO mice were expected to lack a significant inflammatory response. Contrariwise, CD14 KO mice suffered from an exaggerated inflammatory response and diminished capacity to

control bacterial replication and/or clear organisms from infected tissues. Given this finding, we revisited the issue of CD14-dependent macrophage activation via lipoproteins and explored how cellular responses are influenced by the context in which PAMPs are presented. Comparing live and lysed spirochetes, intact organisms (at high spirochete/cell ratios) showed a striking capacity to elicit TNF-α production in a CD14-independent fashion, whereas lysates were more reliant on CD14 and, when used at an equivalent spirochete/cell ratio, stimulated very little TNF-α. These findings engender the provocative question: what distinguishes whole bacteria from their component parts, allowing the former to elicit a fulminant inflammatory response independent of CD14?

The ability and/or efficiency with which PAMPs engage TLRs may depend on the topological orientation of molecules in the bacterial membrane. PAMPs distributed across the bacterial surface may more efficiently cluster signaling components within lipid-rich microdomains on the cell's surface or in phagolysosomal vesicles following ingestion of bacteria (58, 59). In contrast, free PAMPs (e.g., lysates or purified components) might require higher concentrations to achieve comparable signal integration. Additionally, live spirochetes may engage alternative phagocytic/signaling receptors on Mφ lacking CD14 that are not available to lysates or purified components. This notion is supported by the observation that intact *Escherichia coli*, but not purified LPS, can stimulate TNF-α secretion by CD14 KO Mφ via interaction with CD11/CD18 (46). Furthermore, the capacity of whole *E. coli* to stimulate





**FIGURE 6.** CD14 deficiency alters *B. burgdorferi*-induced inflammatory gene activity in peritoneal M $\phi$ . Purified peritoneal M $\phi$  from WT and CD14 KO mice were incubated in vitro with medium alone (0) and *B. burgdorferi* (100 spirochete/cell) for 24 h. Total RNA was isolated from cells, reverse transcribed into cDNA, and used to probe a GEMMA Q series Mouse Inflammatory Cytokine/Receptor Gene array. Chemiluminescence signal was subjected to *puc18* background subtraction and *gapdh* normalization. Changes in gene activity are shown for the entire data set (A) and for a subset of genes whose activity was altered from 6- and 60-fold (B). Data shown are representative of three independent experiments.

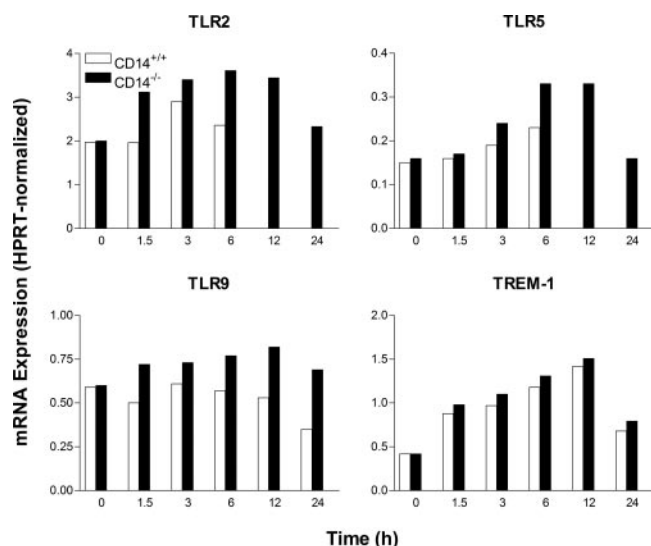
CD14-independent, but not -dependent, production of TNF- $\alpha$  requires cytochalasin B-sensitive phagocytosis (46). These results suggest that intact *E. coli* can initiate CD14-dependent signaling events at the cell surface, whereas CD14-independent signaling must be initiated from an intracellular location. Another point to consider is that as *B. burgdorferi* is degraded within the phagolysosomal compartment, the sequential recognition of PAMPs (e.g., lipoproteins, flagellin, and DNA) by their cognate TLRs (60) may initiate a signaling cascade distinct from that triggered by simultaneous exposure to all spirochetal components or exposure to select, highly purified components. The intracellular compartment(s) to which spirochetal constituents are directed also may influence subsequent inflammatory responses. Owing to their small size, molecules in lysates or purified lipoproteins/lipopeptides may be subject to nonreceptor-mediated (e.g., macropinocytotic) uptake into compartments having a diminished capacity to support CD14-dependent signaling.

Regardless of the precise mechanism(s) whereby *B. burgdorferi* triggers innate immunity, our results suggest that signaling through a CD14-independent pathway(s) elicits an inflammatory response that is inherently more destructive and less subject to negative regulatory feedback. This notion is supported by other animal models of infection that have assessed the role of CD14 in pathogenesis. For example, treatment of rabbits with anti-CD14 mAb exacerbates tissue injury and undermines control of bacterial

growth in models of *Shigella flexneri* (61) and *E. coli* infection (62). The picture in mice is more complex, with the role of CD14 depending upon inoculum size and whether intact organisms or purified PAMPs are used (40, 63–66). CD14 KO mice challenged with either *E. coli* 0111 or its LPS or with *Salmonella typhimurium* are resistant to their lethal effects and produce very low amounts of proinflammatory cytokines compared with their WT counterparts (40, 67). In contrast, Haziot et al. (64) have shown that CD14 plays no major role in the control of bacterial growth or shock induced by *Staphylococcus aureus* infection of mice. Of note, while IL-6 levels were equivalent in CD14 WT and CD14 KO mice exposed to *S. aureus*, the latter produced significantly more TNF- $\alpha$ , a difference observed upon in vitro incubation of cells with live organisms as well. In contrast, TNF- $\alpha$  secretion by M $\phi$  exposed to purified cell walls or peptidoglycan demonstrated a clear reliance on CD14. Similarly, CD14 deficiency does not alter granuloma formation, TNF- $\alpha$  and IFN- $\gamma$  mRNA levels, or bacterial burdens associated with mycobacterial infection (68). Only thioglycolate-elicited peritoneal M $\phi$  from the two genotypes exhibited CD14-dependent differences in TNF- $\alpha$  and IL-6 secretion. It is, however, important to note that the dependence on CD14 for cytokine production was overcome by increasing the inoculum size or the duration of coinoculation of bacteria and cells.

The present study adds a new and important dimension to our understanding of Lyme disease pathogenesis by demonstrating that

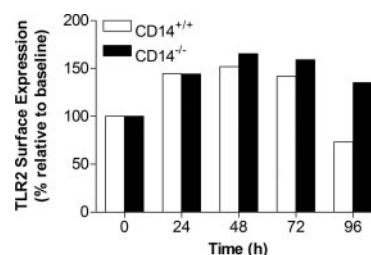




**FIGURE 7.** CD14 deficiency alters the kinetics of *B. burgdorferi*-induced *tlr* gene transcription. WT and CD14 KO mice received an i.p. injection of  $1 \times 10^7$  *B. burgdorferi*. Sham-inoculated mice (0) received medium alone. At the indicated times, PECs were isolated. Total RNA was purified and subjected to RT-PCR analysis to determine receptor transcript levels. The data are representative of three independent experiments.

CD14 signaling is responsible for maintaining a complex balance of pro- and anti-inflammatory mediators and for regulating TLR expression that ultimately may combine to produce disease resistance or susceptibility. The use of microarrays to profile gene activity revealed striking differences between WT and CD14 KO M $\phi$  insofar as transcription of genes involved in tempering their proinflammatory responsiveness or mediating the transition from innate to adaptive immunity either was unchanged or down-regulated in CD14 KO cells. Some of these genes encode chemokines that orchestrate migration of T cells and their subsequent activation, while others would sensitize M $\phi$  to the down-modulatory effects of cytokines such as IL-10 and IL-13; coordination of these adaptive and innate events is essential for bacterial clearance and disease resolution. Given this scenario, it is not surprising that spirochetal replication goes unabated and the nature of the tissue damage is more severe and acute from a cellular perspective in CD14 KO mice. The notion of mice being trapped in an acute inflammatory state is perhaps best exemplified by the preponderance of PMN in the hearts of CD14 KO mice, whereas the hearts of their WT counterparts were infiltrated by lymphocytes and scattered M $\phi$ .

Another reflection of this bias toward acute inflammation is the persistent transcription of *tlr* genes in CD14 KO M $\phi$ . Characterization of the capacity of TLR agonists to modulate transcription of their cognate receptor or those of other agonists has engendered several conflicting reports (69–74). Nevertheless, it is generally accepted that *B. burgdorferi* and its lipoproteins (75), as well as bacterial lipopeptides (76), transiently up-regulate and then down-regulate TLR2 mRNA levels. This observation now has been confirmed and extended by showing that *B. burgdorferi* has the capacity to impact transcription of *tlr2*, *tlr5*, and *tlr9* in a CD14-dependent fashion. The inconsistent findings alluded to above center around whether a correlation exists between changes seen at the mRNA level and surface expression of the TLRs. All studies to date have assessed agonist-induced changes in transcription and surface expression over the same time period, generally 24 h. Recognizing the potential for these two events to be temporally dissociated, we chose to stimulate M $\phi$  with *B. burgdorferi* for 24 h



**FIGURE 8.** CD14 deficiency alters *B. burgdorferi*-induced surface expression of TLR2 on peritoneal M $\phi$ . Purified peritoneal M $\phi$  from WT and CD14 KO mice were incubated in vitro for 24 h with medium alone (0) and *B. burgdorferi* (10 spirochetes/cell). Cells then were washed to remove unbound spirochetes and cultured for an additional 72 h, collecting cells at subsequent 24-h intervals. Cells were stained with FITC-conjugated rat anti-mouse TLR2 or an IgG2b isotype control Ab, and TLR2 expression was assessed by flow cytometry. The data are representative of three independent experiments.

and follow changes in TLR2 surface expression over a subsequent 3-day period. The same difference in *tlr2* transcription observed between WT and CD14 KO M $\phi$  also was evident with respect to the amplitude and duration of TLR2 surface expression, albeit offset by several days. It is worth noting that higher level expression of TLR2 on CD14 KO M $\phi$  immediately precedes the 7-day postinfection time point at which CD14 KO animals exhibit higher serum cytokine levels and more severe histopathology than their WT counterparts. Although circumstantial, it is intriguing to speculate whether persistent expression of TLR2 might prolong the responsiveness of CD14 KO mice to spirochetal lipoproteins.

The results presented in this work have important implications for the evolution of Lyme disease. They suggest that early in the disease process when bacterial burden is relatively low, live spirochetes will engage both CD14-dependent and -independent pathways to induce inflammation. Simultaneous engagement of these pathways is critical to the control of bacterial growth and regulation of the intensity and duration of the inflammatory response. Later during infection, unless bacterial growth is kept in check by CD14-dependent mechanisms, the local concentration of PAMPs may exceed a threshold at which point CD14-independent signaling predominates, thus driving an inflammatory response that is inherently more destructive and less easily controlled by regulatory feedback. Continued use of CD14 KO mice to explore the immunopathogenesis of Lyme disease may help delineate further the qualitative and quantitative differences between acute and chronic responses to *B. burgdorferi* and will facilitate development of strategies for therapeutic intervention in chronic Lyme disease and other chronic inflammatory disorders.

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