Borrelia burgdorferi Lipoprotein–Mediated TLR2 Stimulation Causes the Down-Regulation of TLR5 in Human Monocytes

Erik S. Cabral,[1,a,b] Harald Gelderblom,[2,a,b] Ronald L. Hornung,[4] Peter J. Munson,[3] Roland Martin,[2,b] and Adriana R. Marques[1]

[1]Clinical Studies Unit, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, [2]Cellular Immunology Section, Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, and [3]Mathematical and Statistical Computing Laboratory, Division of Computational Bioscience, Center for Information Technology, National Institutes of Health, Bethesda, and [4]Clinical Services Program, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland

Toll-like receptors (TLRs) trigger innate immune responses via the recognition of conserved pathogen-associated molecular patterns. Lipoproteins from *Borrelia burgdorferi*, the agent of Lyme disease, activate inflammatory cells through TLR2 and TLR1. We show that stimulation of human monocytes with *B. burgdorferi* lysate, lipidated outer surface protein A, and triacylated lipopeptide $Pam_3CysSerLys_4$ results in the up-regulation of both TLR2 and TLR1 but the down-regulation of TLR5, the receptor for bacterial flagellin, and that this effect is mediated via TLR2. TLR4 stimulation had no effect on TLR2, TLR1, and TLR5 expression. Human monocytes stimulated with TLR5 ligands (including p37 or flaA, the minor protein from *B. burgdorferi* flagella) up-regulated TLR5. In addition, TLR2 stimulation rendered cells hyporesponsive to a TLR5 agonist. These results indicate that diverse stimuli can cause differential TLR expression, and we hypothesize that these changes may be useful for either the pathogen and/or the host.

Lyme disease is a multisystem illness caused by *Borrelia burgdorferi* and is the most common vectorborne illness in the United States. It usually begins with erythema migrans, a characteristic rash at the inoculation site. Within several days or weeks, there is hematogenous dissemination of the spirochetes, and patients may present with dermatological, neurological, cardiac, and rheumatological involvement [1]. The mechanisms involved in eliminating the organism versus those contributing to disease and persistent infection are not yet understood. The main inflammatory response against *B. burgdorferi* is directed against spirochetal lipoproteins [2–4].

Toll-like receptors (TLRs) are a family of pattern-recognition receptors that are important in innate immune defense. Signal transduction through TLRs activates NF-κB and the production of cytokines, chemokines, and costimulatory molecules [5]. TLR2 mediates immune responses to a broad range of microbial products and is critical for the recognition of bacterial lipopeptides. It functions in combination with TLR1 to recognize triacylated lipopeptides, such as mycobacterial lipoprotein, or the outer surface protein A (OspA) of *B. burgdorferi* [6, 7]. TLR5 recognizes flagellin [8], the main component of bacterial flagella, which is critically important for bacterial motility.

Received 14 June 2005; accepted 12 October 2005; electronically published 8 February 2006.

Financial support: Intramural Research Program, National Institutes of Health; National Cancer Institute, National Institutes of Health (contract NO1-CO-12400).

Potential conflicts of interest: none reported.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsements by the US Government.

[a] E.S.C. and H.G. contributed equally to the article.

[b] Present affiliations: Stanford School of Medicine, Stanford, California (E.S.C.); Department of Neurology, Charité, Humboldt-University, Berlin, Germany (H.G.); Unitat de Neuroimmunologia Clínica, Hospital Universitari Vall d'Hebron, Universita Autonoma de Barcelona, Barcelona, Spain (R.M.).

Reprints or correspondence: Dr. Adriana R. Marques, Clinical Studies Unit, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bldg. 10, Rm. 11N228, 10 Center Dr., Bethesda MD 20892-1888 (amarques@niaid.nih.gov).

The Journal of Infectious Diseases 2006; 193:849–59

0022-1899/2006/19306-0016$15.00

In the present study, we show that human peripheral blood mononuclear cells (PBMCs) and monocytes up-regulate TLR1 and TLR2 and down-regulate TLR5 when they are stimulated with *B. burgdorferi* lysate (BL), lipidated-OspA (L-OspA), and $Pam_3CysSerLys_4$ (Pam_3CSK_4, a synthetic lipohexapeptide that mimics the structure of the lipoprotein lipid moiety). These findings are specific for TLR2 stimulation, because TLR4 stimulation does not up-regulate TLR1 and TLR2 or down-regulate TLR5. TLR5 ligands up-regulate TLR5 but have no effect on TLR1 and TLR2. The results of our study indicate a role of the differential expression of certain TLRs during infection with *B. burgdorferi.*

MATERIALS AND METHODS

Samples. PBMCs and monocytes were obtained by apheresis under studies approved by the National Cancer Institute and the National Institute of Allergy and Infectious Diseases Institutional Review Boards. All volunteers provided signed, informed consent.

Stimulus. Low-passage sonicate of *B. burgdorferi* sensu stricto strain B31 was purchased from Biodesign; L-OspA and nonlipidated OspA (nL-OspA) were obtained from SmithKline Beecham Biologicals; Pam_3CSK_4 was purchased from EMC Microcollections; recombinant *B. burgdorferi* p37, a flagellar outer-sheath protein and the *flaA* gene product, was a gift from B. Johnson (Centers for Disease Control and Prevention, Atlanta, GA); purified flagellin isolated from *Salmonella typhimurium* strain 14028 (fliC) was purchased from Alexis Biochemicals. The final endotoxin concentration in medium for all antigens tested was <0.05 EU/mL (Endosafe-PTS) or <0.1 EU/mL (Cambrex Bio Science). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich.

PBMC and monocyte cultures. PBMCs were isolated by Ficoll density-gradient centrifugation (Bio-Whittaker) and resuspended in Iscove's modified Dulbecco's medium (Gibco-BRL) supplemented with 2 mmol/L L-glutamine, 50 μg/mL gentamicin, 100 U/mL penicillin/streptomycin, and 5% human AB serum (Gemini Bioproducts; complete medium [CM]). Cultures of 5×10^6 PBMCs were performed in 24-well plates. Monocytes (>95%) were isolated by countercurrent elutriation from peripheral blood, as described elsewhere [9]. Monocytes (1×10^5) were plated in 96-well flat-bottom microtiter plates in CM. Cells were cultured for different periods with or without stimuli, as described in Results. For the blocking experiments, cells were preincubated for 1 h with medium that contained 10 μg/mL anti–human TLR2 monoclonal neutralizing antibody (aTLR2 MAb 2392; courtesy of Genentech) or medium alone.

Flow-cytometric staining. Staining was done using murine monoclonal antibodies against the following human surface antigens: CD3 (UCHT1, mouse $IgG_1\kappa$), CD14 (M5E2, mouse $IgG_{2a}\kappa$), and CD19 (HIB19, mouse $IgG_1\kappa$; BD PharMingen), in addition to human TLR1 (GD2.F4, mouse $IgG_1\kappa$) and TLR2 (TL2.1, mouse $IgG_{2a}\kappa$; eBioscience). A total of 1×10^6 PBMCs/assay were incubated with the respective antibody at a concentration of 2.5 μg/sample for 30 min at 4°C. After 2 washes, fluorescence intensity was measured in a FACSCalibur device and analyzed using CellQuest (version 3.3) software (both from Becton Dickinson). Results are expressed as median fluorescence intensity (MFI) values after subtraction of the MFI of the isotype antibody control. All experiments were performed at least in triplicate.

Quantitative Western blot. Monocytes (2×10^7) were incubated for 48 h with stimuli or medium alone. Protein from whole cell lysates was loaded onto SDS-PAGE gels at 10 μg/lane, in accordance with the Bradford assay, transferred to polyvinylidene fluoride membranes, and blocked with 5% powdered milk in PBS with Tween. Membranes were probed using rabbit polyclonal antibody against human TLR5 at a dilution of 1:200 (Santa Cruz Biotechnology), followed by a second block and goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (BioRad) at a dilution of 1:5000. Reaction products were visualized with the Opti-4CN Detection Kit (BioRad). Densitometric analysis of bands was performed by Scion Image Beta (version 4.02; Scion). All experiments were performed at least 3 times.

Cytokine and chemokine protein secretion assays. Interferon (IFN)–γ, interleukin (IL)–1β, IL-6, IL-8, IL-10, IL-12 (p70), and tumor necrosis factor (TNF)–α concentrations were deter-

Table 1. Quantitative reverse-transcription polymerase chain reaction primer and probe sequences.

	Sequence (5′→3′)		
	Primer		
Target	Sense	Antisense	Probe
TLR1	CCACAACAAGTTGGTGAAGATTTCT	GCATATAGGCAGGGCATCAAATG	ACTGTGAACCTCAAGCACTTG GACCTGTCA
TLR2	TTCAGGATGTCCGCCTCTCG	CCCGTGAGCAGGATCAGCA	ACAGAGCACAGCACATGCCAGAC ACCA
TLR5	GGACTGGGGAAAATGTATGAACC	TGGCTTTTGCTGATGGCATTG	TTCCTGTGATGTCCACTGTCCAGCCATT
TLR4	AAAGCCGAAAGGTGATTGTTGTG	TGCTCAGAAACTGCCAGGTCT	TGTCCCAGCACTTCATCCAGAGCCGC
TLR6	GAAGAAGAACAACCCTTTAGGATAGC	GCTGGATTCTGTTATGGGAAAGTC	CAAAAAGACCTACCGCTGAAAACCAAAGTC
MyD88	CTCCTCCACATCCTCCCTTCC	CGCACGTTCAAGAA CAGAGACA	CCGCACTCGCATGTTGAGAGCAGCCAG

NOTE. MyD88, myeloid differentiation factor 88; TLR, Toll-like receptor.

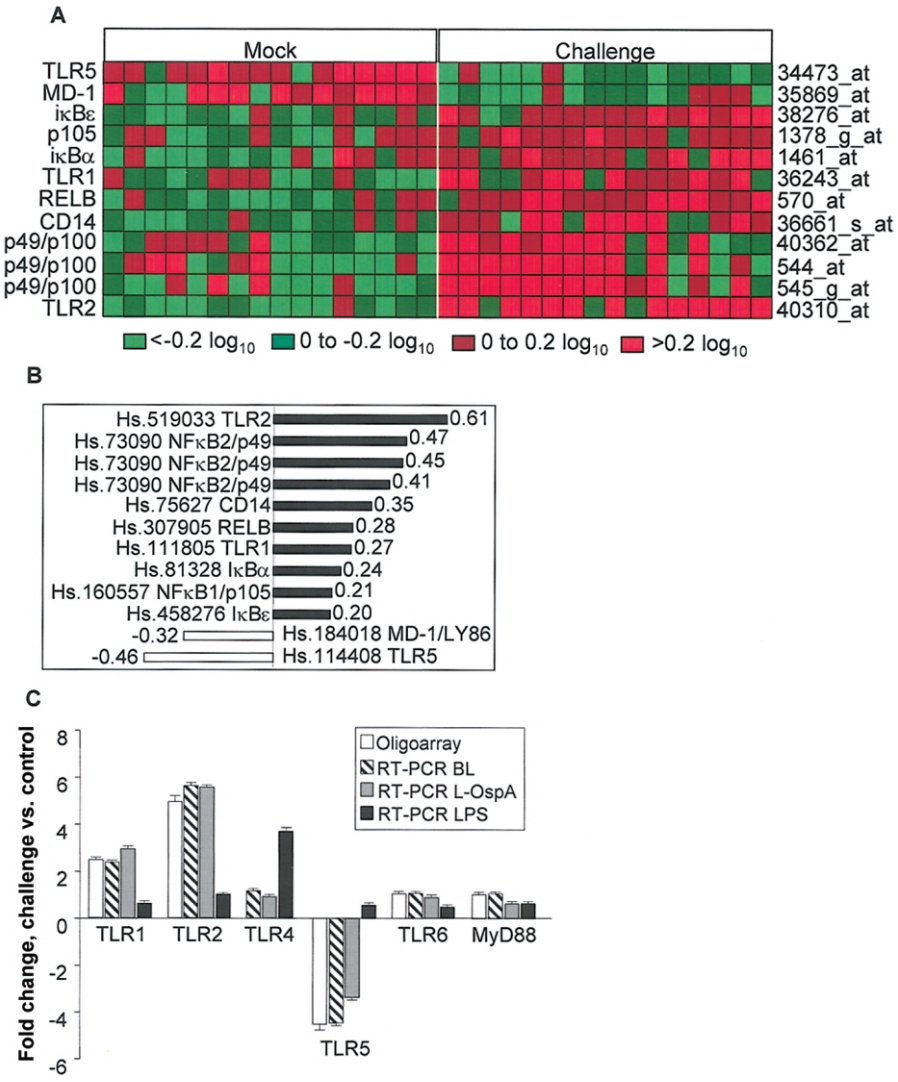

Figure 1. *Borrelia burgdorferi* stimulation and differentially expressed Toll and NF-κB pathway genes. *A,* Heat map of genes differentially expressed across peripheral blood mononuclear cells (PBMCs) from 16 individuals, stimulated with *B. burgdorferi* lysate (BL) at 1 μg/mL (challenge), compared with unstimulated PBMCs (mock). Each column represents 1 individual and each row represents a gene, with the gene abbreviation and its Affymetrix U95A probe set no. indicated. The values are the change in expression intensity, going from mock to challenge condition, and are expressed in a symmetric adaptive transform scale based on the original average difference values. *B,* Expression values for the displayed genes. The *X*-axis shows the $\log_{10}$ fold change in challenge cells, relative to mock control cells. Positive fold-change values indicate that the transcript is present at a higher level in PBMCs stimulated with *B. burgdorferi,* whereas negative values indicate that the transcript is more abundant in mock control cells. *C,* Toll-like receptor (TLR)–1, TLR2, TLR5, TLR6, and myeloid differentiation factor 88 (MyD88) mRNA quantification by oligoarray and quantitative reverse-transcription polymerase chain reaction (RT-PCR). PBMCs from 5 individuals were stimulated with either 1 μg/mL BL or with medium only and were analyzed by oligoarray or stimulated with 1 μg/mL BL, 100 ng/mL bacterial lipopolysaccharide (LPS), and 0.1 μg/mL lipidated outer surface protein A (L-OspA) for 48 h and analyzed by RT-PCR. RT-PCR mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. The fold change for both array and RT-PCR data was calculated as mRNA expression of a specific gene in response to challenge, compared with cultures in medium only. The quotients predicted by array quantification consistently agreed with values subsequently measured by RT-PCR. TLR4 stimulation with LPS had no effect on TLR1, TLR2, TLR5, TLR6, and MyD88 expression but up-regulated TLR4 expression as measured by RT-PCR. TLR4 is not included in the Affymetrix U95A array.

mined with a Luminex100 cytometer (Luminex) using BioPlex Manager software (version 3.0, build 282; BioRad) and LINCOplex Cytokine Kits (Linco Research), in accordance with the manufacturer's instructions. All experiments were performed at least 3 times, and each supernatant was measured in triplicate.

RNA isolation and quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from cells by use of the RNeasy Kit (Qiagen) and reverse transcribed to cDNA with random hexamers by use of the Taqman Reverse Transcription Reagents, in accordance with the man-

ufacturer's instructions (PerkinElmer). RT-PCR was performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for sample normalizations. Primers and probes for myeloid differentiation factor 88 (MyD88), TLR1, TLR2, TLR4, TLR5, and TLR6 (Synthegen) are shown in table 1. The quantification of gene expression relative to GAPDH was calculated using the $\Delta\Delta Ct$ method. All experiments were performed at least 3 times.

Oligonucleotide array hybridization. After 48 h of culture, PBMCs were harvested, and total RNA was isolated (Qiagen). The total RNA (15 μg) was reverse transcribed (Invitrogen) with a T7-polyA primer. In vitro transcription of cDNA was performed in the presence of biotinylated ribonucleotides (ENZO Diagnostics). Hybridization to Human U95A GeneChip (Affymetrix) and scanning were performed in accordance with the manufacturer's protocols; 16 of 17 original samples yielded satisfactory hybridizations for both "mock" and "challenge" conditions and were retained in the study.

Analysis of oligoarray results. Scanned images were processed with Affymetrix Microarray Suite software (version 4.0). Data from each oligoarray were normalized in accordance with the manufacturer's protocol. Subsequently, we used a quantile normalization procedure, which forces the normalized average difference distribution to be identical on every chip. Data were then transformed using a "symmetric adaptive transform" [10–12] to remove the inherent dependence of variability of a measured value on the mean of the values across arrays. The paired Student's *t* test was applied to the transformed data comparing the "mock" and "challenge" sample from each subject, with a required false-discovery rate of <10% [13]. We also required expression levels to have changed by >0.5 on the transformed scale (approximately equivalent to requiring a >2-fold change on the original measurement scale) and that at least 40% of the samples show a "present call" in 1 of the 2 groups.

RESULTS

***Differential expression of TLRs by cells stimulated with* B. burgdorferi.** Multiple receptors and proteins involved in the TLR and NF-κB pathways were differentially expressed in the 16 individual human PBMCs stimulated with 1 μg/mL BL ("challenge"), compared with the corresponding nonstimulated cells ("mock") (figure 1*A* and 1*B*). Exposure to BL caused the up-regulation of TLR1 and TLR2 (0.28 and 0.61 $\log_{10}$ expression intensity), whereas TLR5 was down-regulated by 0.46 $\log_{10}$ expression intensity. There were no changes in TLR3, TLR6, and MyD88 (data not shown). MD-1 was down-regulated by 0.32 $\log_{10}$ expression intensity, whereas CD14 was up-regulated by 0.35 $\log_{10}$ expression intensity. There was induction of NF-κB2 and NF-κB1, as well as of the NF-κB–related inhibitory molecules IκBα and IκBε.

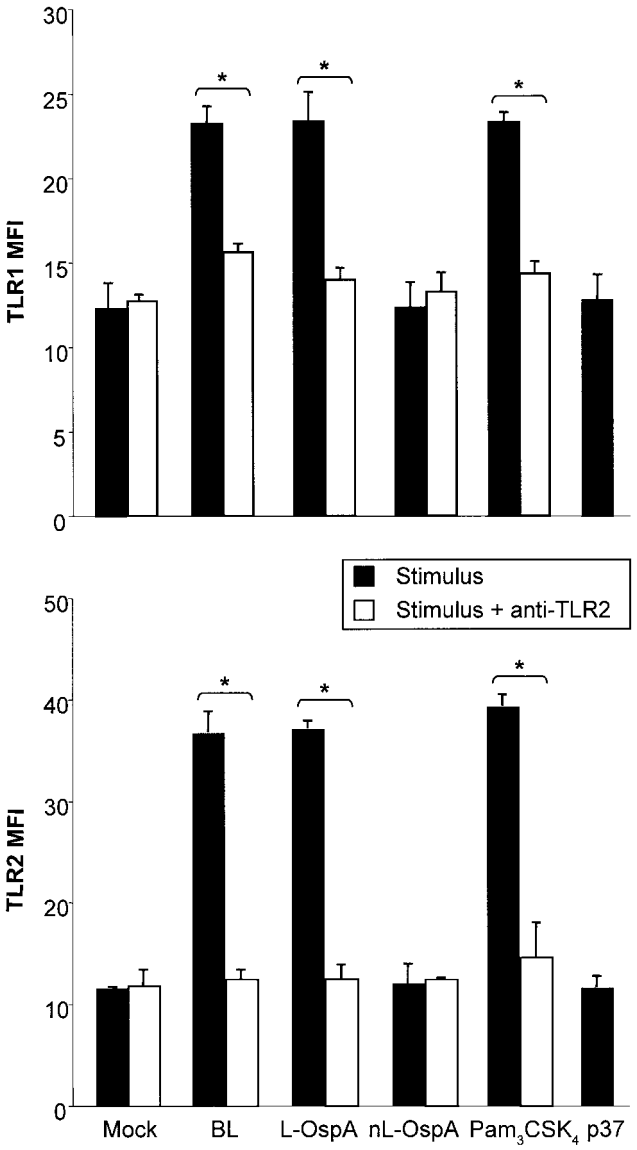

Figure 2. Increased Toll-like receptor (TLR)–1 and TLR2 surface expression by TLR2 agonists. Human monocytes were stimulated for 48 h with *Borrelia burgdorferi* lysate (BL; 1 μg/mL), lipidated outer surface protein A (L-OspA; 0.1 μg/mL), $Pam_3CysSerLys_4$ (Pam_3CSK_4; 0.01 μg/mL), nonlipidated (n) L-OspA (0.1 μg/mL), or the bacterial flagellin antigen p37 (1 μg/mL), and TLR1 and TLR2 surface expression was measured by flow-cytometric analysis. Data are the mean and SD of 2 experiments. The significance of the difference between the means was analyzed using the *t* test for correlated samples, and 1-sided values of $P < .05$ are shown (*). All 3 TLR2 ligands enhanced TLR1 and TLR2 surface expression, whereas nL-OspA and p37 stimulation had no effect. Preincubation of anti-TLR2 antibody with BL (1 μg/mL), L-OspA (0.1 μg/mL), or Pam_3CSK_4 (0.01 μg/mL) down-regulated TLR2 and TLR1 surface expression, compared with that in cells treated with antigen alone. There was no significant difference in TLR2 and TLR1 expression in cells stimulated with nL-OspA, mock-treated cells (with or without anti-TLR2 antibody), and cells stimulated with p37. MFI, mean fluorescence intensity.

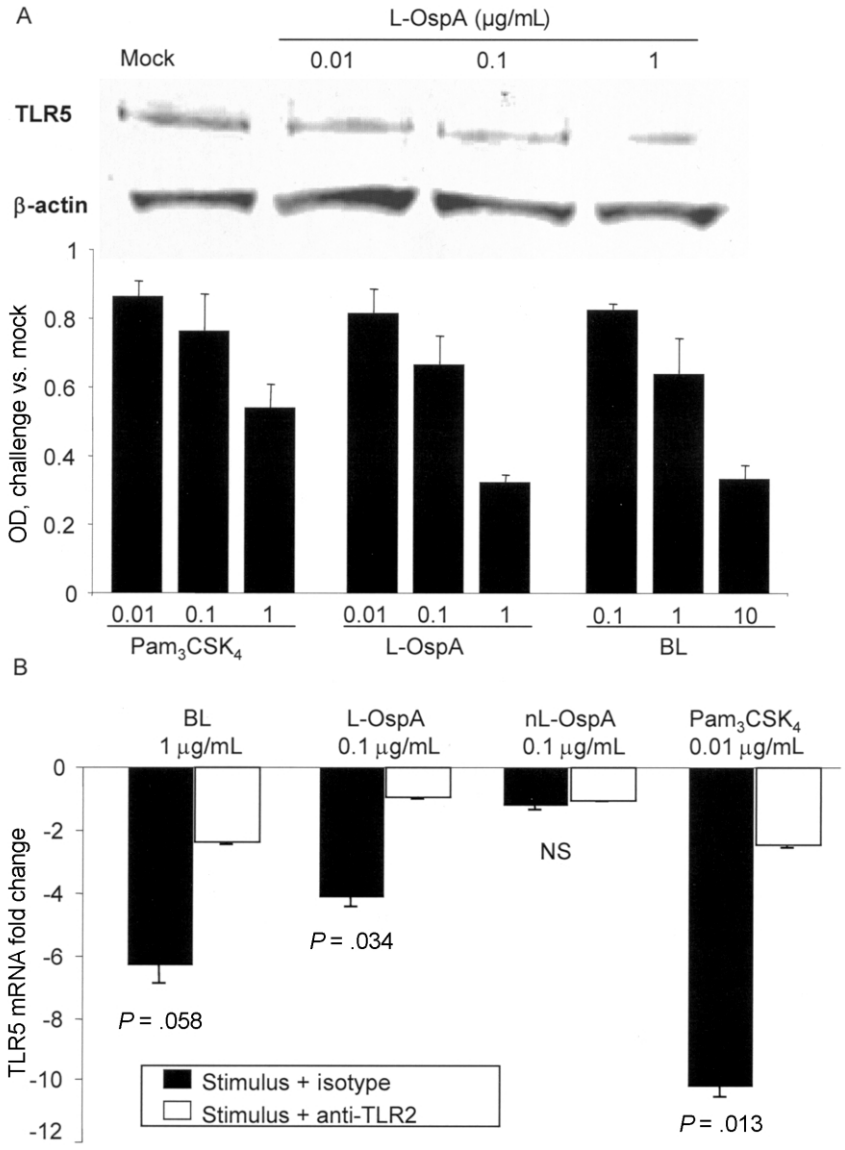

Figure 3. Toll-like receptor (TLR)–5 decreased by TLR2 ligands. *A,* After 48 h of incubation with the different TLR2 agonists, human monocytes were harvested, and protein lysates were isolated. Equal amounts of protein (10 μg) were subjected to Western blot and probed for TLR5. Decreasing optical density (OD) values for TLR5 were observed in response to increasing amounts of *Borrelia burgdorferi* lysate (BL), lipidated outer surface protein A (L-OspA), and $Pam_3CysSerLys_4$ (Pam_3CSK_4). Data are the mean and SD of 3 experiments. *B,* Human monocytes were stimulated for 48 h with the different TLR2 agonists, with or without anti-TLR2 antibody. Data are the mean and SD of 2 experiments, and 2-sided *P* values were calculated using the *t* test for correlated samples. Blocking TLR2 with anti-TLR2 antibody significantly reversed the down-regulation of TLR5 mRNA as measured by quantitative reverse-transcription polymerase chain reaction. nL-OspA, nonlipidated L-OspA.

The up-regulation of TLR1 and TLR2 and the down-regulation of TLR5 were confirmed by RT-PCR in PBMCs (figure 1*C*) and monocytes (data not shown) by use of BL (1 μg/mL) and L-OspA (0.1 μg/mL). Neither stimulus had any effect on TLR6 and MyD88. BL and L-OspA caused no change in TLR4 mRNA expression as determined by RT-PCR (TLR4 is not included in the Affymetrix U95A array). As a comparison, we investigated the effect of LPS, which signals through TLR4, by RT-PCR. PBMCs cultured with LPS (100 ng/mL) for 48 h did not alter TLR1, TLR2, TLR5, and TLR6 or MyD88 expression, but they induced TLR4. Altogether, these results suggest that the appropriate TLR ligands are required to cause transcriptional induction of TLR expression.

Enhanced surface expression of TLR1 and TLR2 in monocytes caused by TLR2 stimulants. To test which PBMC subset displayed enhanced TLR expression, we analyzed $CD3^+$, $CD14^+$, and $CD19^+$ gated populations for TLR1 and TLR2 surface expression. Subpopulations of mock-incubated PBMCs did not

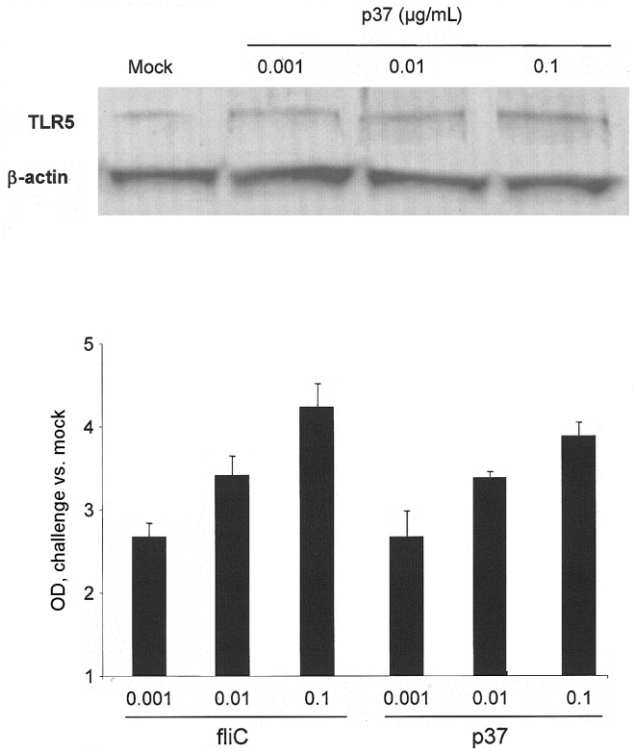

Figure 4. Induction of Toll-like receptor (TLR)–5 by TLR5 agonists. After 48 h of incubation, human monocytes stimulated with bacterial flagellin antigens p37 and fliC were harvested, and protein lysates were isolated. Equal amounts of protein (10 μg) were subjected to Western blot and probed for TLR5. Data are the mean and SD of 3 experiments. A dose-dependent increase in TLR5 protein levels was observed in response to increasing amounts of p37 and fliC.

stain for either TLR1 or TLR2. Coculture with BL (1 μg/mL) induced TLR1 and TLR2 expression mainly on $CD14^+$ cells (71.8%). Less than 1% of $CD3^+$ cells stained positive for both TLR1 and TLR2, and they were undetectable on $CD19^+$ cells. These results led us to focus on $CD14^+$ cells for the subsequent quantification of TLR1, TLR2, and TLR5 in response to TLR ligands.

To further investigate the increase in TLR1 and TLR2 surface expression, we stimulated monocytes with various concentrations of BL, L-OspA, nL-OspA, Pam_3CSK_4, p37, and fliC. Compared with that in controls, TLR1 and TLR2 surface expression on monocytes stimulated with BL, L-OspA, and Pam_3CSK_4 increased significantly between 24 and 48 h. Expression levels remained steady or began decreasing at 72 h. On the basis of the dose- and time-curve results (data not shown), the 48-h time point and the following doses were chosen for further experiments: 1 μg/mL BL, 0.1 μg/mL L-OspA, and 0.01 μg/mL Pam_3CSK_4. As expected, nL-OspA (0.1 μg/mL) had no effect on either TLR1 or TLR2 expression. Similarly, neither p37 nor fliC (tested in a concentration range of 0.01–1 μg/mL) enhanced TLR1 or TLR2 expression on monocytes at 24, 48, or 72 h (data not shown). These results suggest that an appropriate TLR2 agonist is required for the induction of TLR1 and TLR2 surface expression on monocytes.

TLR1 and TLR2 expression reduced by TLR2 blocking. We used neutralizing anti-TLR2 antibody to demonstrate the role of TLR2 in mediating *B. burgdorferi*–induced TLR1 and TLR2 surface expression. Monocytes were incubated with or without anti-TLR2 antibody before coculture with BL (1 μg/mL), L-OspA (0.1 μg/mL), nL-OspA (0.1 μg/mL), or Pam_3CSK_4 (0.01 μg/mL) for 48 h. Compared with stimulation by the ligands alone, preincubation with anti-TLR2 antibody reduced the TLR2 surface expression induced by Pam_3CSK_4, BL, and L-OspA by 63%, 66%, and 66%, respectively ($P = .02$, $P = .027$, and $P = .004$, paired t test) (figure 2). The presence of anti-TLR2 antibody resulted in a 40%, 39%, and 33% reduction in TLR1 surface expression in cells stimulated by L-OspA, Pam_3CSK_4, and BL, respectively ($P = .02$, $P = .03$, and $P = .044$, paired t test). There was no significant difference in TLR2 and TLR1 expression between mock-treated cells and those incubated with anti-TLR2 antibody alone, with nL-OspA (with or without anti-TLR2 antibody), and with p37 (1 μg/mL). Taken together, these results show that the increase in TLR2 and TLR1 surface expression caused by BL, L-OspA, and Pam_3CSK_4 in human monocytes is a result of TLR2 ligation.

TLR5 protein expression down-regulated by* B. burgdorferi *and L-OspA. To confirm the down-regulation of TLR5 expression on exposure to BL observed in both oligoarray analysis and RT-PCR, we measured TLR5 protein levels by Western blot in monocytes incubated with BL, L-OspA, and Pam_3CSK_4 (figure 3). The TLR5 protein level was down-regulated in a dose-dependent manner in cells stimulated with 0.1–10 μg/mL BL and 0.01–1 μg/mL L-OspA. Specifically, 1 μg/mL L-OspA resulted in a 68% reduction in optical density from control values. Although increasing concentrations of Pam_3CSK_4 (0.01–1 μg/mL) produced a dose-dependent curve, 1 μg/mL led to a 39% reduction in levels of TLR5 protein. In contrast, RT-PCR results showed a more pronounced decrease in TLR5 mRNA with 0.01 μg/mL Pam_3CSK_4 than with 0.1 μg/mL L-OspA and 1 μg/mL BL. Blocking TLR2 ligation with anti-TLR2 antibody reversed the down-regulation of TLR5 (figure 3*B*). These results confirm that lipoproteins in BL, L-OspA, and Pam_3CSK_4 are able to suppress TLR5 expression and that this effect occurs via TLR2 stimulation.

Induction of TLR5 protein expression by* B. burgdorferi *p37 and fliC. To test whether the down-regulation of TLR5 was a general phenomenon of TLR stimulation, we incubated monocytes with various concentrations of fliC or p37 and measured the TLR5 protein levels by Western blot. Both TLR5 ligands increased TLR5 protein levels in a dose-dependent manner (figure 4). At the highest concentration (0.1 μg/mL), fliC and p37 induced a 4.55- and 4.05-fold increase, respectively, in TLR5

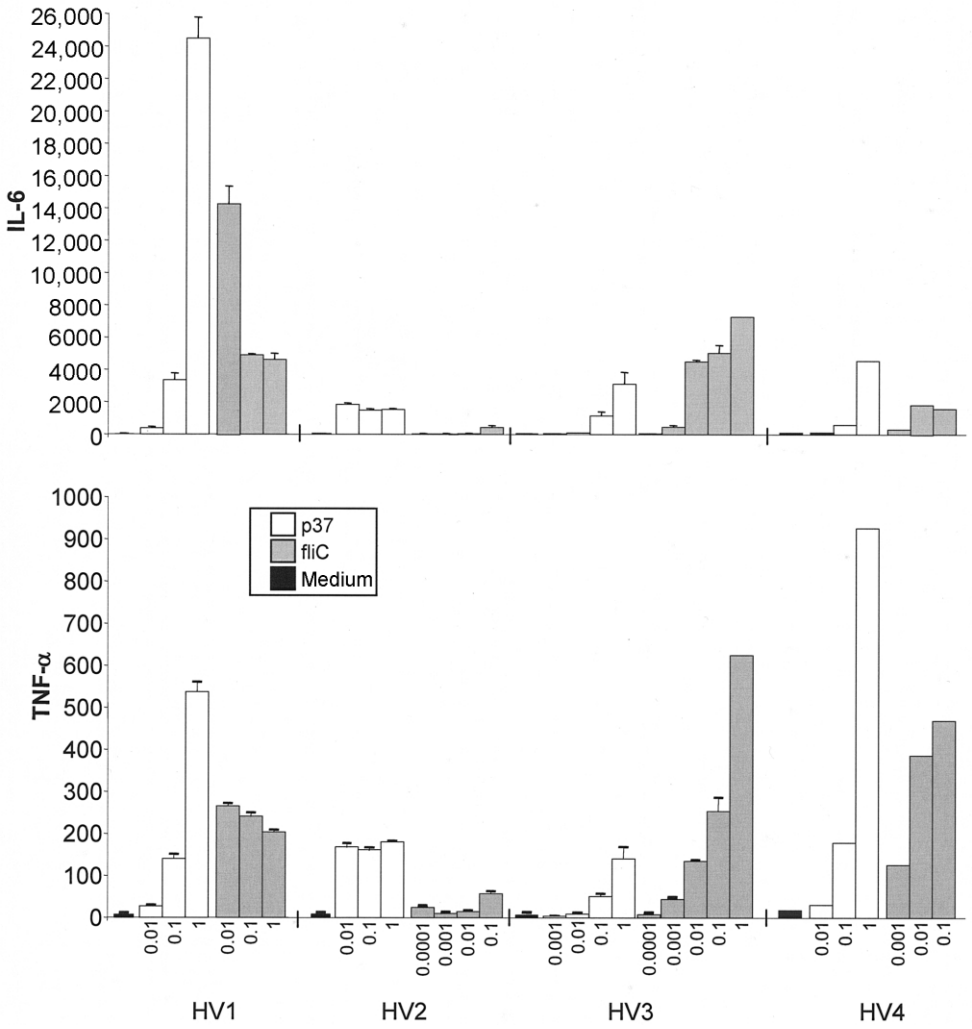

Figure 5. Interleukin (IL)–6 and tumor necrosis factor (TNF)–α protein quantification after stimulation with flagellin antigens p37 and fliC. Shown are the values for IL-6 and TNF-α protein levels after exposure of monocytes from 4 healthy volunteers (HVs) to the bacterial flagellin antigens p37 and fliC at increasing concentrations (in micrograms per milliliter) for 48 h, as assessed by the Lincoplex Multiplex Immunoassay Kit. Data are the mean and SD of triplicate samples from each experiment. Where error bars are not shown, only 1 well was measured. Although there was individual variation in the magnitude of the response, both p37 and fliC stimulated cytokine production.

protein levels. These data indicate not only that the down-regulation of TLR5 was caused by TLR2 stimulation but that TLR5 protein expression is enhanced on TLR5 stimulation. It also shows that p37 can activate human TLR5.

Cytokine profile of monocytes stimulated with p37 and fliC. To assess the functional consequences of TLR5 activation by p37, we incubated monocytes with various concentrations of p37 and fliC and measured IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12 (p70), and TNF-α protein secretion at 48 h. Both TLR5 agonists stimulated monocytes from different individuals to produce cytokines, albeit at different amounts. Increasing concentrations (0.01–1 μg/mL) of p37 resulted in a dose-dependent response for IL-6 and TNF-α levels (figure 5) in samples from 3 of 4 individuals. Both p37 and fliC also stimulated production of IL-8, IL-1β, and IL-10, whereas IFN-γ and IL-12 (p70) were undetectable (data not shown).

Induction of tolerance in human monocytes. Given our finding that TLR2 agonists down-regulate TLR5, we examined whether they are able to induce cross-tolerance to flagellin, possibly by decreasing TLR5 expression. To test this hypothesis, we compared the ability of L-OspA, Pam_3CSK_4 (at a concentration with little effect on TLR5 protein levels), LPS, and fliC to induce tolerance and cross-tolerance. Monocytes were incubated with L-OspA (1 μg/mL), Pam_3CSK_4 (0.01 μg/mL), LPS (100 ng/mL), fliC (0.1 μg/mL), or medium alone for 48 h as a pretreatment. Cells were then washed twice and rested for 2 h in fresh medium before challenge with 0.01 μg/mL Pam_3CSK_4 or 0.1 μg/mL fliC for 24 h. TNF-α and IL-6 protein secretion were measured by

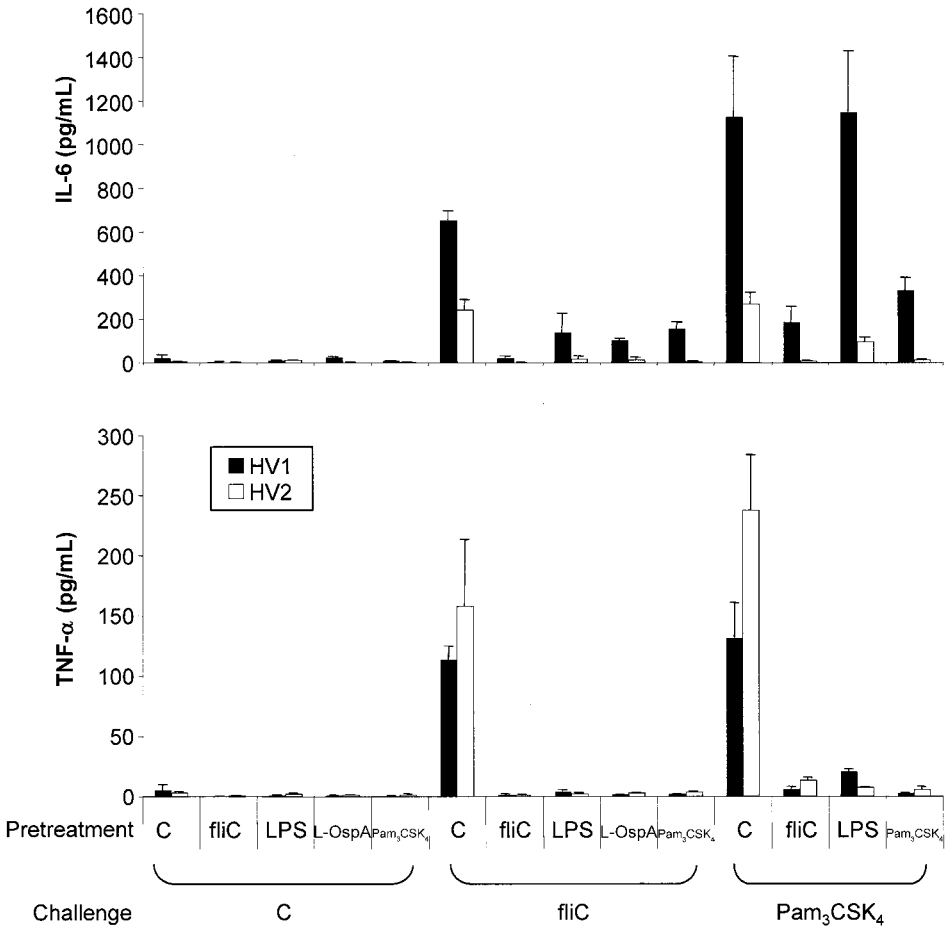

Figure 6. Comparison of tumor necrosis factor (TNF)–α and interleukin (IL)–6 production induced by the bacterial flagellin antigen fliC in nontolerized and lipidated outer surface protein A (L-OspA), $Pam_3CysSerLys_4$ (Pam_3CSK_4), fliC, and lipopolysaccharide (LPS) tolerized human monocytes. Human monocytes were pretreated with medium only (C), L-OspA (1 μg/mL), Pam_3CSK_4 (0.01 μg/mL), LPS (100 ng/mL), or fliC (0.1 μl/mL) for 48 h. Cells were then washed twice, rested for 2 h at 37°C, and restimulated with fliC (0.1 μg/mL) and Pam_3CSK_4 (0.01 μg/mL) for 24 h. Levels of TNF-α and IL-6 were assessed with the Lincoplex Multiplex Immunoassay Kit. Data are the mean and SD of experiments performed in triplicate for 2 individuals. TLR2, TLR4, and TLR5 stimulation caused hyporesponsiveness on secondary fliC and Pam_3CSK_4 stimulation. HV, healthy volunteer.

ELISA. Pretreatment with L-OspA, Pam_3CSK_4, LPS, and fliC caused decreased TNF-α and IL-6 secretion on stimulation with fliC and Pam_3CSK_4 (figure 6). These data demonstrate that TLR2, TLR4, and TLR5 ligands render monocytes hyporesponsive to a second TLR5 and TLR2 stimulation and that, therefore, tolerance and cross-tolerance occur independently of TLR modulation.

DISCUSSION

The expression of different human TLRs may act to either enhance or inhibit innate immune system recognition of particular pathogen-associated molecular patterns, which suggests that cellular responses to these patterns are dependent on the total repertoire of TLRs present on a cell, necessary cofactors, and the extent to which levels of each protein are expressed [14–16].

In the present study, we have demonstrated the differential expression of human TLR1, TLR2, and TLR5 in cells stimulated with *B. burgdorferi*. In agreement with previous data that showed cooperation between TLR1 and TLR2 for the recognition of triacylated lipoproteins [6, 7, 15, 16], expression of both receptors was induced on stimulation with BL, L-OspA, and Pam_3CSK_4. These findings were confirmed at the cellular phenotypic level. Although the induction of TLR2 by TLR2 ligands in human cells, including monocytes, has been described elsewhere [17–20], to our knowledge, the parallel induction of expression of both receptors in human PBMCs and monocytes has not yet been reported. TLR2 ligands have also been shown to up-regulate TLR2 on murine cells [17, 21, 22].

Blocking the ligation of TLR2 on monocytes with neutralizing antibody reduced the TLR1 and TLR2 phenotypic expression induced by TLR2 ligands, thereby demonstrating that these changes are TLR2 dependent. The up-regulation of TLR1 and TLR2 appears to be a specific response to TLR2 stimulation,

given that both LPS and flagellin, which stimulate TLR4 and TLR5, respectively, did not affect human TLR1 and TLR2 expression. The lack of effect of LPS on TLR2 expression and the up-regulation of TLR4 by LPS are in accord with previous results in human monocytes [23]. These results differ from findings in murine cells, in which LPS causes the up-regulation of TLR2 in macrophages [21, 24–26]. In contrast, TLR4 expression remained unchanged or decreased in murine macrophages stimulated with LPS [25, 27], whereas LPS increased TLR4 mRNA in rat cardiomyocytes [28]. The reasons for such differences in TLR expression between human and mouse cells remain unclear. They may represent species-specific as well as cell-specific responses. For example, although LPS does not change TLR2 expression on human monocytes, it does up-regulate the receptor in human polymorphonuclear cells [23]. Also, although immature dendritic cells down-regulate TLR1, TLR2, TLR4, and TLR5 in response to LPS, this phenomenon did not occur in monocytes [29].

Interestingly, although we found TLR1 and TLR2 mRNA and protein levels to be significantly up-regulated in response to TLR2 agonists, we also observed a down-regulation of TLR5. TLR5 recognizes flagellin [8], the structural protein subunit of the flagellum. TLR5 protein levels were reduced in a dose-dependent manner after monocytes were incubated with BL and L-OspA. This down-regulation was less pronounced in cells stimulated with Pam_3CSK_4 and seems to have been specific to TLR2 stimulation, given that LPS did not induce the down-regulation of TLR5. Moreover, TLR1 and TLR2 were unchanged and TLR5 was up-regulated in cells stimulated with TLR5 ligands. The up-regulation of TLR5 on stimulation with its ligand has been reported in SV40 immortalized human airway epithelial cells and SV40 transformed human bronchial epithelial cells [28]. To our knowledge, this is the first report of the up-regulation of TLR5 by TLR5 ligands in human monocytes and that flaA, the outer layer protein that composes the *B. burgdorferi* flagella [30], can enhance TLR5 protein expression.

Of particular interest is the observation that TLR2 stimulation resulted in the down-regulation of TLR5. Because flagella play an essential role in motility and cell morphology [31] and are important virulence factors [32–34] of *B. burgdorferi,* we hypothesized that the down-regulation of TLR5 could be useful to the organism by decreasing the cell response to flagellin. This may represent an interesting mechanism of bacterial subversion of the host response and could be useful for spirochete persistence. Because flagella are indispensable for the organism, they cannot be down-regulated—a mechanism of immune evasion that has been demonstrated for other surface antigens [35]. This is an attractive hypothesis; however, *B. burgdorferi* flagella are not exposed at the cell surface. The flagella of *B. burgdorferi* are contained within the periplasm, a space between the protoplasmic cell cylinder and the outer membrane sheath. The possibility remains that *Borrelia* flagella could be exposed, perhaps via transient gaps, as has been implied for *Treponema pallidum* [36]. Finally, other organisms recognized by TLR2 and TLR5 may explore this mechanism. Alternatively, the down-regulation of TLR5 could also be useful for the host. It is possible that such down-regulation could be relevant in an environment where strong and persistent innate immune responses are not desirable—for example, the central nervous system or intestinal mucosa [37].

To investigate this hypothesis, we studied whether stimulation with L-OspA and Pam_3CSK_4 resulted in tolerance to TLR5 and TLR2 stimulation in human monocytes and compared it with stimulation with LPS and fliC. Tolerance, a phenomenon usually studied with LPS but also seen with the engagement of other TLR/IL-1 receptors, is defined as a reduced capacity to respond to activation after the first exposure to a stimulus. It can be divided into tolerance or cross-tolerance, depending on whether the tolerizing and challenge stimuli use the same or different TLRs [38]. The mechanisms of tolerance are not fully resolved, with one of the possible mechanisms being the down-regulation of receptors [39, 40]. Our results demonstrate that monocytes stimulated with TLR2 ligands become tolerant to flagellin and to Pam_3CSK_4. The same occurred with monocytes stimulated with LPS and fliC. Therefore, TLR modulation does not seem to be involved in the development of tolerance. It is likely that multiple factors contribute to this phenomenon, because numerous signaling proteins are altered during tolerance. These include a decreased association of TLR4 with MyD88 [41], increased IL-1R–associated kinase (IRAK) degradation and decreased association of IRAK with MyD88 [42, 43], the expression of MyD88 short [44], the induction of IRAK-M [45–47], the induction of suppressor of cytokine signaling–1 [48, 49], increased IκB inhibitory proteins and NF-κB p50 homodimer [38, 50], and disruption of the NF-κB p65 transactivating stage and alteration of chromatin remodeling at the IL-1β promoter [51]. IL-10 and transforming growth factor–β also participate in the mechanism of tolerance [20, 52]. A combination of these mechanisms is likely to be involved in monocyte hyporesponsiveness observed after TLR2, TLR4, and TLR5 stimulation.

In conclusion, stimulation with TLR2 ligands induces the up-regulation of TLR2 and TLR1 and down-regulates TLR5 in human monocytes. It is intriguing to consider that TLR expression patterns may change in response to diverse environments and the surrounding conditions and that they may be regulated differently at the inflammatory site. These changes may be useful for either the pathogen or the host or for both.

References

1. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. J Clin Invest **2004**; 113:1093–101.
2. Radolf JD, Arndt LL, Akins DR, et al. *Treponema pallidum* and *Borrelia*

burgdorferi lipoproteins and synthetic lipopeptides activate monocytes/macrophages. J Immunol **1995**; 154:2866–77.
3. Ebnet K, Brown KD, Siebenlist UK, Simon MM, Shaw S. *Borrelia burgdorferi* activates nuclear factor-κ B and is a potent inducer of chemokine and adhesion molecule gene expression in endothelial cells and fibroblasts. J Immunol **1997**; 158:3285–92.
4. Giambartolomei GH, Dennis VA, Lasater BL, Philipp MT. Induction of pro- and anti-inflammatory cytokines by *Borrelia burgdorferi* lipoproteins in monocytes is mediated by CD14. Infect Immun **1999**; 67: 140–7.
5. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. Science **2003**; 300:1524–5.
6. Takeuchi O, Sato S, Horiuchi T, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol **2002**; 169:10–4.
7. Alexopoulou L, Thomas V, Schnare M, et al. Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. Nat Med **2002**; 8:878–84.
8. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **2001**; 410: 1099–103.
9. Abrahamsen TG, Carter CS, Read EJ, et al. Stimulatory effect of counterflow centrifugal elutriation in large-scale separation of peripheral blood monocytes can be reversed by storing the cells at 37 degrees C. J Clin Apheresis **1991**; 6:48–53.
10. Munson PJ. A consistency test for determining the significance of gene expression changes on replicate samples and two convenient variance-stabilizing transformations. In: Program and abstracts of the GeneLogic Workshop of Low Level Analysis of Affymetrix GeneChip Data (Bethesda, MD). Available at: http://stat-www.berkeley.edu/users/terry/zarray/Affy/GL_Workshop/genelogic2001.html. Accessed 7 February 2006.
11. Durbin B, Hardin J, Hawkins D, Rocke D. A variance-stabilizing transformation for gene-expression microarray data. Bioinformatics **2002**; 18(Suppl 1):S105–10.
12. Huber W, Von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics **2002**; 18(Suppl 1):S96–104.
13. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc B **1995**; 57:289–300.
14. Hajjar AM, O'Mahony DS, Ozinsky A, et al. Cutting edge: functional interactions between Toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J Immunol **2001**; 166:15–9.
15. Wyllie DH, Kiss-Toth E, Visintin A, et al. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J Immunol **2000**; 165:7125–32.
16. Ozinsky A, Underhill DM, Fontenot JD, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc Natl Acad Sci USA **2000**; 97:13766–71.
17. Liu Y, Wang Y, Yamakuchi M, et al. Upregulation of Toll-like receptor 2 gene expression in macrophage response to peptidoglycan and high concentration of lipopolysaccharide is involved in NF-κ B activation. Infect Immun **2001**; 69:2788–96.
18. Hajishengallis G, Martin M, Schifferle RE, Genco RJ. Counteracting interactions between lipopolysaccharide molecules with differential activation of Toll-like receptors. Infect Immun **2002**; 70:6658–64.
19. Seibl R, Birchler T, Loeliger S, et al. Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium. Am J Pathol **2003**; 162:1221–7.
20. Diterich I, Rauter C, Kirschning CJ, Hartung T. *Borrelia burgdorferi*-induced tolerance as a model of persistence via immunosuppression. Infect Immun **2003**; 71:3979–87.
21. Nilsen NJ, Nonstad U, Khan N, et al. Lipopolysaccharide and double-stranded RNA up-regulate toll-like receptor 2 independently of myeloid differentiation factor 88. J Biol Chem **2004**; 279:39727–35.
22. Ray A, Chatterjee NS, Bhattacharya SK, Biswas T. Porin of *Shigella dysenteriae* enhances mRNA levels for Toll-like receptor 2 and MyD88, up-regulates CD80 of murine macrophage, and induces the release of interleukin-12. FEMS Immunol Med Microbiol **2003**; 39:213–9.
23. Muzio M, Bosisio D, Polentarutti N, et al. Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol **2000**; 164:5998–6004.
24. Oshikawa K, Sugiyama Y. Regulation of Toll-like receptor 2 and 4 gene expression in murine alveolar macrophages. Exp Lung Res **2003**; 29: 401–12.
25. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and Toll-like receptor 2 and 4 gene expression. J Immunol **2000**; 164:5564–74.
26. Laflamme N, Echchannaoui H, Landmann R, Rivest S. Cooperation between Toll-like receptor 2 and 4 in the brain of mice challenged with cell wall components derived from gram-negative and gram-positive bacteria. Eur J Immunol **2003**; 33:1127–38.
27. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. Science **1998**; 282:2085–8.
28. Adamo R, Sokol S, Soong G, Gomez MI, Prince A. *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and Toll-like receptor 2 as well as Toll-like receptor 5. Am J Respir Cell Mol Biol **2004**; 30:627–34.
29. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol **2001**; 166:249–55.
30. Ge Y, Li C, Corum L, Slaughter CA, Charon NW. Structure and expression of the FlaA periplasmic flagellar protein of *Borrelia burgdorferi*. J Bacteriol **1998**; 180:2418–25.
31. Motaleb MA, Corum L, Bono JL, et al. *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci USA **2000**; 97:10899–904.
32. Zambrano MC, Beklemisheva AA, Bryksin AV, Newman SA, Cabello FC. *Borrelia burgdorferi* binds to, invades, and colonizes native type I collagen lattices. Infect Immun **2004**; 72:3138–46.
33. Sellek RE, Escudero R, Gil H, et al. In vitro culture of *Borrelia garinii* results in loss of flagella and decreased invasiveness. Infect Immun **2002**; 70:4851–8.
34. Sadziene A, Thomas DD, Bundoc VG, Holt SC, Barbour AG. A flagellaless mutant of *Borrelia burgdorferi:* structural, molecular, and in vitro functional characterization. J Clin Invest **1991**; 88:82–92.
35. Liang FT, Jacobs MB, Bowers LC, Philipp MT. An immune evasion mechanism for spirochetal persistence in Lyme borreliosis. J Exp Med **2002**; 195:415–22.
36. Blanco DR, Radolf JD, Lovett MA, Miller JN. The antigenic interrelationship between the endoflagella of *Treponema phagedenis* biotype Reiter and *Treponema pallidum* Nichols strain. I. Treponemicidal activity of cross-reactive endoflagellar antibodies against *T. pallidum.* J Immunol **1986**; 137:2973–9.
37. Hausmann M, Kiessling S, Mestermann S, et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology **2002**; 122:1987–2000.
38. Dobrovolskaia MA, Medvedev AE, Thomas KE, et al. Induction of in vitro reprogramming by Toll-like receptor (TLR)2 and TLR4 agonists in murine macrophages: effects of TLR "homotolerance" versus "heterotolerance" on NF-κ B signaling pathway components. J Immunol **2003**; 170:508–19.
39. Wang JH, Doyle M, Manning BJ, Di Wu Q, Blankson S, Redmond HP. Induction of bacterial lipoprotein tolerance is associated with suppression of Toll-like receptor 2 expression. J Biol Chem **2002**; 277: 36068–75.
40. Nomura F, Akashi S, Sakao Y, et al. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. J Immunol **2000**; 164:3476–9.
41. Medvedev AE, Lentschat A, Wahl LM, Golenbock DT, Vogel SN. Dys-

regulation of LPS-induced Toll-like receptor 4-MyD88 complex formation and IL-1 receptor-associated kinase 1 activation in endotoxin-tolerant cells. J Immunol **2002**; 169:5209–16.
42. Siedlar M, Frankenberger M, Benkhart E, et al. Tolerance induced by the lipopeptide Pam3Cys is due to ablation of IL-1R-associated kinase-1. J Immunol **2004**; 173:2736–45.
43. Li L, Cousart S, Hu J, McCall CE. Characterization of interleukin-1 receptor-associated kinase in normal and endotoxin-tolerant cells. J Biol Chem **2000**; 275:23340–5.
44. Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J Exp Med **2003**; 197:263–8.
45. Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell **2002**; 110:191–202.
46. Nakayama K, Okugawa S, Yanagimoto S, et al. Involvement of IRAK-M in peptidoglycan-induced tolerance in macrophages. J Biol Chem **2004**; 279:6629–34.
47. Escoll P, del Fresno C, Garcia L, et al. Rapid up-regulation of IRAK-M expression following a second endotoxin challenge in human monocytes and in monocytes isolated from septic patients. Biochem Biophys Res Commun **2003**; 311:465–72.
48. Kinjyo I, Hanada T, Inagaki-Ohara K, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity **2002**; 17: 583–91.
49. Nakagawa R, Naka T, Tsutsui H, et al. SOCS-1 participates in negative regulation of LPS responses. Immunity **2002**; 17:677–87.
50. Bohuslav J, Kravchenko VV, Parry GC, et al. Regulation of an essential innate immune response by the p50 subunit of NF-κB. J Clin Invest **1998**; 102:1645–52.
51. Chan C, Li L, McCall CE, Yoza BK. Endotoxin tolerance disrupts chromatin remodeling and NF-κB transactivation at the IL-1β promoter. J Immunol **2005**; 175:461–8.
52. Schroder M, Meisel C, Buhl K, et al. Different modes of IL-10 and TGF-β to inhibit cytokine-dependent IFN-γ production: consequences for reversal of lipopolysaccharide desensitization. J Immunol **2003**; 170:5260–7.