The Journal of Immunology

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J Immunol 1999; 163:2382-2386; ;
http://www.jimmunol.org/content/163/5/2382

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cutting Edge: Inflammatory Signaling by *Borrelia burgdorferi* Lipoproteins Is Mediated by Toll-Like Receptor 2[1]

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The agent of Lyme disease, *Borrelia burgdorferi*, produces membrane lipoproteins possessing potent inflammatory properties linked to disease pathology. The recent association of toll-like receptors (TLR) 2 and 4 with LPS responses prompted the examination of TLR involvement in lipoprotein signaling. The ability of human cell lines to respond to lipoproteins was correlated with the expression of TLR2. Transfection of TLR2 into cell lines conferred responsiveness to lipoproteins, lipopeptides, and sonicated *B. burgdorferi*, as measured by nuclear translocation of NF-κB and cytokine production. The physiological importance of this interaction was demonstrated by the 10-fold greater sensitivity of TLR2-transfected cells to lipoproteins than LPS. Futhermore, TLR2-dependant signaling by lipoproteins was facilitated by CD14. These data indicate that TLR2 facilitates the inflammatory events associated with Lyme arthritis. In addition, the widespread expression of lipoproteins by other bacterial species suggests that this interaction may have broad implications in microbial inflammation and pathogenesis. *The Journal of Immunology*, 1999, 163: 2382–2386.

Lyme disease is caused by infection with the tick-borne spirochete *Borrelia burgdorferi* (1). A subacute inflammatory arthritis develops in 60% of individuals not treated at the time of the tick bite (2), and is associated with invasion of the joint tissue by spirochetes (3, 4). *B. burgdorferi* possess surface-associated proteins with the tripalmitoyl-*S*-glyceryl-cysteine (Pam_3Cys)[3] modification common to many bacterial species (5), and numerous inflammatory activities have been ascribed to the Pam_3Cys modification (6–9). Unique to *B. burgdorferi* is the presence of 130 genes, accounting for >10% of the genome, that possess a sequence encoding the signal peptidase II cleavage site necessary for synthesis of the lipid modification (10). Expression of lipoprotein genes is environmentally regulated (11), with the outer surface protein (Osp)A lipoprotein expressed primarily in the tick mid-gut and late in infection of humans (11–13). Serological evidence indicates that other *B. burgdorferi* lipoproteins are preferentially expressed during infection of mammals (11, 13), and it is likely that they possess similar stimulatory properties (6–9).

The inflammatory activities attributed to *B. burgdorferi* lipoproteins include the ability to directly induce NF-κB nuclear translocation, resulting in cytokine production, adhesion molecule expression, and generation of nitric oxide and superoxide (6–9). Lipoproteins or their derivatives have been shown to activate endothelial cells, neutrophils, macrophages, and B lymphocytes in vitro, and to induce localized inflammatory infiltrate into knee joints and dermal sites in vivo (6–9, 14–16). *B. burgdorferi* does not produce LPS (17), nor does its genome encode the enzymes required for LPS synthesis (10). Thus, lipoproteins provide the major inflammatory stimulus associated with chronic infection. The existence of a specific receptor for the highly inflammatory lipoproteins has been hypothesized, and would provide a mechanism by which the localized bacteria could directly activate inflammation.

Bacterial lipoproteins and LPS share many characteristics, including a biologically active lipid modification, the cell types that are responsive, and the types of responses that are induced (18). These observations argue that a similar molecule may be involved in signaling, and is supported by the finding that the LPS coreceptor CD14 also facilitates lipoprotein signaling in several cell types (19–21). Toll-like receptor (TLR)2 and TLR4 are two candidate proteins that have recently been implicated in LPS signaling (22–27). TLR4 has been identified by positional cloning as the gene responsible for LPS hyporesponsiveness in C3H/HeJ mice (24–26). These mice respond normally to lipoproteins (7, 14), arguing that TLR4 is not required for lipoprotein signaling. Transfection of either TLR2 or TLR4 confers responsiveness to LPS in cell lines

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Received for publication June 9, 1999. Accepted for publication July 8, 1999.

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[1] This work was supported by U.S. Public Health Service Grants AI-32223 and AI-43521 (to J.J.W.), AI-24158 (to J.H.W.), and 5P30-CA-42014 (to the University of Utah). The project described was also supported in part by an award from the American Lung Association (to J.H.W.) and an Arthritis Foundation Postdoctoral Fellowship (to R.M.W.).

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[3] Abbreviations used in this paper: Pam_3Cys, tripalmitoyl-*S*-glyceryl-cysteine; TLR, toll-like receptor; RSV, Rous sarcoma virus; Osp, outer surface protein; ELAM-1, endothelial cell-leukocyte adhesion molecule (E-selectin); HPRT, hypoxanthine phosphoribosyl transferase.

0022-1767/99/$02.00

(22, 23, 27). In this study, the possibility that members of the toll-receptor-like family are involved in signaling by bacterial lipoproteins was investigated.

Materials and Methods

Cell lines and reagents

U373 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). 293 cells were a subclone from Tularik (South San Francisco, CA). The TLR, endothelial cell-leukocyte adhesion molecule (ELAM-1) luciferase, and Rous sarcoma virus (RSV)-β-galactosidase DNA constructs are described by Kirschning et al. (22). Polymyxin B, LPS from *Salmonella typhimurium*, and human serum were obtained from Sigma (St. Louis, MO). Pam_3Cys-Ser-Lys_4-OH was obtained from Boehringer Mannheim (Indianapolis, IN). Sonicated *B. burgdorferi* and OspA purified from *B. burgdorferi* were isolated as described (14). Recombinant OspA, OspB, and unlipidated OspA were provided by John Dunn (Brookhaven National Laboratories, Upton, NY) (28). The recombinant and native OspA and sonicated *B. burgdorferi* contained <0.3 endotoxin units (EU) per 500 ng, as determined by *Limulus* amebocyte assay (Associates of Cape Cod, Woods Hole, MA). The 60bca cell line was obtained from ATCC, and mopc 21 Ab was from Sigma.

Quantification of TLR cDNA

mRNA and cDNA were prepared (29) from U373 cells cultured in DMEM with serum replacement Nutridoma-HU (Boehringer Mannheim). Transcript levels were quantified using the Light Cycler (Idaho Technology, Idaho Falls, ID) and normalized to hypoxanthine phosphoribosyl transferase (HPRT). This technique continuously monitors the accumulation of fluorescently labeled product to calculate starting transcript numbers and is described in detail by Morrison et al. (29). The oligonucleotide primers used to quantify HPRT were CTGGCGTCGTGATTAGTG and CCAA CACTTCGTGGGGTC; TLR1, CTATACACCAAGTTGTCAGC and GTCTCCAACTCAGTAAGGTG; TLR2, TCACCTACATTAGCAACAG and GATCTGAAGCATCAATCTC; TLR3, GATCTGTCTCATAATG GCTTG and GACAGATTCCGAATGCTTGTG; TLR4, GCTTACTT TCACTTCCAAC and GCCATTGAAGATGCCATTG; and TLR5, CTAGCTCCTAATCCTGATG and CCATGTGAAGTCTTTGCTGC.

Transfections

U373 cells were transfected using pFx-2 (Invitrogen, Carlsbad, CA) with 4 μg of either pFLAG alone or pFLAG containing a TLR construct. Cells were grown for 24 h in DMEM with Nutridoma-HU, followed by stimulation for an additional 24 h with agonist. 293 cells were cotransfected using a calcium phosphate kit (Life Technologies, Gaithersburg, MD) at a ratio of 3:1:2 μg for the pFLAG plasmid alone or pFLAG containing a TLR construct: the ELAM-1 luciferase reporter construct: and an RSV β-galactosidase construct to normalize for transfection efficiency. Cells were grown for 36 h and stimulated with the indicated agonist for an additional 6 h.

Luciferase and cytokine assays

IL-6 (U373) and IL-8 (293) production was measured by ELISA using matched-pair Abs (Endogen, Woburn, MA). 293 cells were then lysed using reporter lysis buffer (Promega, Madison, WI), and 20 μl of lysate was assayed for luciferase and β-galactosidase activity using a Dynatec MLX luminometer after incubation in luciferase assay reagent (Promega) or Galacto-Light with light emission accelerator (Tropix, Bedford, MA), respectively.

Results

The human glioma cell line U373 responds vigorously to LPS, but not to OspA, as assayed by IL-6 production (Fig. 1*B*). In addition, U373 cells produce detectable levels of transcript for TLR1, TLR3, TLR4, and TLR5 (Fig. 1*A*). No transcript was detected for TLR2 in U373 cells (Fig. 1*A*). Interestingly, the human monocytic line THP-1 was OspA-responsive and possessed very high levels of TLR2 transcript (data not shown), while the nonresponsive U373 cells produced none. These findings suggest a correlation between expression of TLR2 and the ability of cell lines to respond to the *B. burgdorferi* lipoprotein OspA.

To test whether the lipoprotein unresponsive phenotype could be corrected in U373 cells, expression constructs encoding four of the TLR proteins were transfected individually into this cell line, and the ability to respond to lipoproteins was measured by IL-6 production. Transfection of TLR2 conferred responsiveness to OspA in U373 cells (Fig. 1*B*), whereas transfection of vector alone, TLR1, TLR3, or TLR4 did not. TLR4 did significantly increase IL-6 production of U373 cells when stimulated with LPS (Fig. 1*B*), a result that agrees with previous reports suggesting that TLR4 is involved in LPS signaling (24–27).

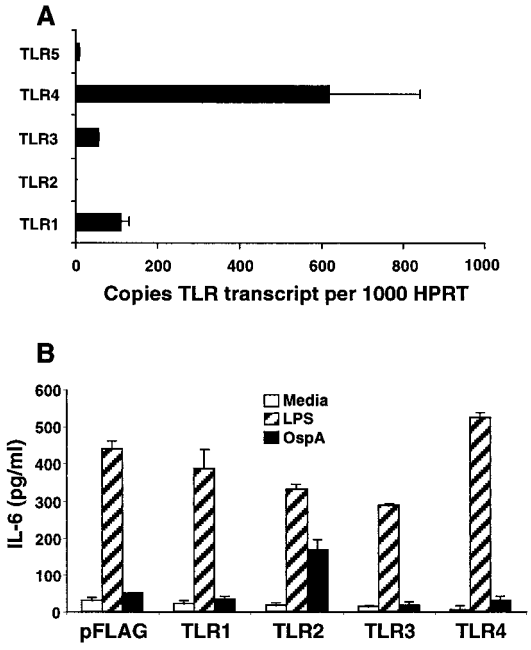

FIGURE 1. Lipoprotein responsiveness correlates to TLR2 expression in U373 cells. *A*, RT-PCR detection of TLR expression. mRNA expression for TLR1-TLR5 was quantified in U373 cells and normalized to expression of HPRT. *B*, Transfection of TLR2 corrects lipoprotein-unresponsiveness in U373 cells. U373 cells were transiently transfected with expression vector alone (pFLAG) or expression vector with specific TLR construct. Cells were treated with: media with 5 μg/ml polymyxin B, LPS at 100 ng/ml with 2% human serum, recombinant OspA at 500 ng/ml with 5 μg/ml polymyxin B. Supernatants were collected after 24 h and were assayed for IL-6 production by ELISA.

To test this effect in a second TLR2-negative cell line (22), expression constructs encoding the four TLR proteins were transiently transfected into the human embryonic kidney line 293. The TLR expression plasmids were cotransfected with a NF-κB-dependent luciferase reporter plasmid that contained the E-selectin (ELAM-1) promoter. To normalize for transfection efficiency, an RSV-β-galactocidase control plasmid was also cotransfected. Only transfection of the TLR2 expression construct conferred responsiveness to OspA in 293 cells (Fig. 2, *A* and *B*) and was reflected in both luciferase and endogenous IL-8 production. As reported previously, TLR2 also conferred responsiveness to LPS (22, 23). Because TLR2 conferred responsiveness to both lipoproteins and LPS, polymyxin B was added to all lipoprotein samples to eliminate stimulation due to possible LPS contamination during experimental manipulation. Transfection of TLR4 resulted in constitutive activation, as reported by others in this cell type (22), and was not up-regulated by either LPS or OspA. None of the other constructs, pFLAG vector alone, TLR1, or TLR3, conferred responsiveness to either agonist (Fig. 2, *A* and *B*).

The experiments in Figs. 1, 2*A*, and 2*B* used recombinant lipidated OspA, which was previously shown to possess the same stimulatory properties as OspA purified from *B. burgdorferi* (30).

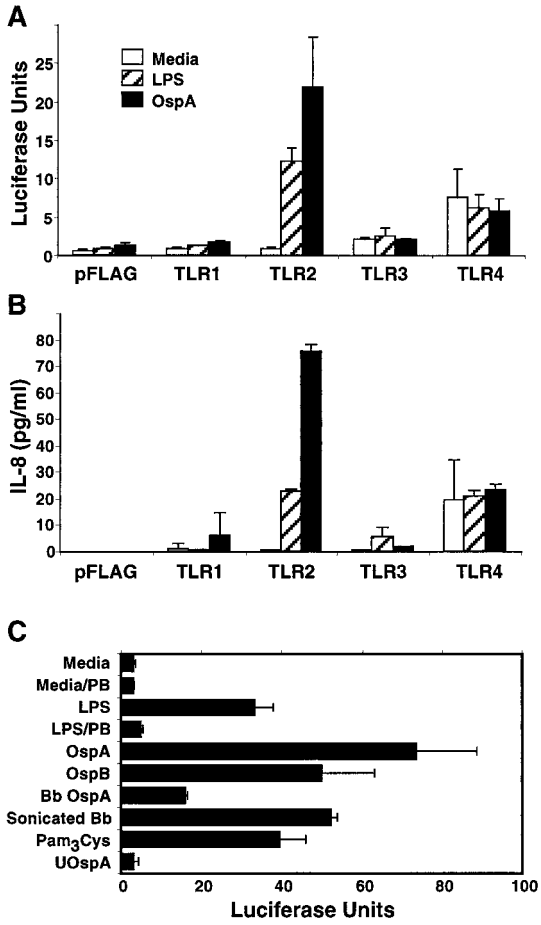

FIGURE 2. Transfection of TLR2 confers responsiveness to lipoproteins in 293 cells. *A*, NF-κB nuclear translocation in Toll-like receptor-expressing cells. 293 cells were transiently transfected with expression vector alone (pFLAG) or expression vector with specific TLR construct, plus the ELAM-1 luciferase reporter construct. Cells were stimulated for 6 h with the indicated agonist. NF-κB nuclear translocation is indicated by luciferase units. Media with 5 μg/ml polymyxin B, LPS at 100 ng/ml, recombinant OspA at 500 ng/ml plus 5 μg/ml polymyxin B. *B*, Induction of IL-8 secretion in Toll-like receptor-transfected cells. Supernatants from the samples shown in *A* were assayed for IL-8 production by ELISA. *C*, Specificity of TLR2-induced responsiveness to lipoproteins. 293 cells were transiently cotransfected with the TLR2 expression plasmid and the ELAM-1-luciferase construct. Cells were stimulated for 6 h and NF-κB nuclear translocation is indicated by luciferase units. Media is media alone; Media/PB has 5 μg/ml of polymyxin B; LPS is 100 ng/ml LPS; LPS/PB is 100 ng/ml LPS plus 5 μg/ml polymyxin B. The remaining samples all contained 5 μg/ml polymyxin B and are as follows: OspA, recombinant OspA at 500 ng/ml; Bb OspA, OspA purified from strain N40 of *B. burgdorferi* at 100 ng/ml; OspB, recombinant OspB at 500 ng/ml; Sonicated Bb, 1 μg/ml sonicated strain N40 of *B. burgdorferi*; Pam3Cys, Pam_3Cys-Ser-Lys_4-OH at 200 ng/ml; and UOspA, unlipidated recombinant OspA at 500 ng/ml.

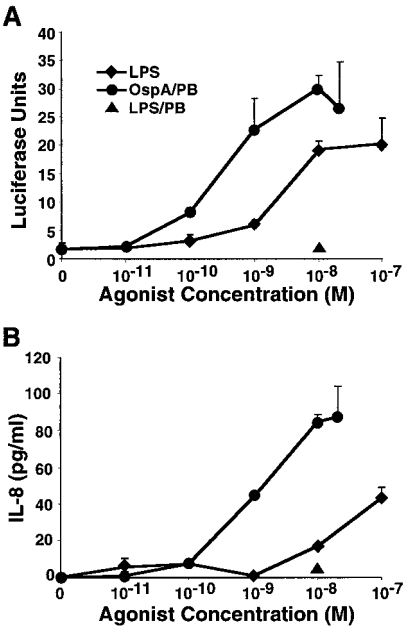

FIGURE 3. Comparison of the sensitivity of TLR2-dependent responses to OspA and LPS. *A*, NF-κB nuclear translocation in response to increasing doses of OspA or LPS. 293 cells transiently cotransfected with the TLR2 expression plasmid and ELAM-1 luciferase construct were incubated with increasing concentrations of LPS, OspA plus 5 μg/ml polymyxin B, or LPS plus 5 μg/ml polymyxin B for 6 h. Samples were assayed for NF-κB translocation by luciferase. *B*, IL-8 production in response to increasing doses of OspA or LPS. Supernatants from the samples shown in *A* were assayed for IL-8 production by ELISA.

This was confirmed in Fig. 2*C*, where OspA purified from *B. burgdorferi* was found to activate NF-κB in 293 cells transfected with TLR2. Sonicated *B. burgdorferi*, an abundant source of lipoproteins, was also a strong stimulant in TLR2-expressing cells. A second recombinant lipoprotein, OspB, which possesses the same lipid modification on a distinct polypeptide, also strongly activated TLR2-expressing cells. The fact that the synthetic lipopeptide Pam_3Cys-Ser-Lys_4-OH also possesses this activity further supports the requirement for the lipid modification. These findings indicate that several distinct Pam_3Cys-containing molecules induce inflammatory signaling through TLR2, and suggest that the interaction is dependent on the common lipid modification. This specificity was confirmed with unlipidated OspA, a recombinant protein that possesses the same amino acid sequence as lipidated OspA, but lacks the amino-terminal cysteine required for the Pam_3Cys modification (31). The unlipidated OspA did not activate either NF-κB translocation (Fig. 2*C*) or IL-8 production (data not shown) in TLR2-expressing 293 cells. These results indicate that the lipid modification is required for cellular activation via TLR2, and that several lipoproteins and a synthetic lipopeptide possess this activity.

Further evidence that lipoproteins are a biologically important ligand for TLR2 was sought by comparing the dose required for NF-κB translocation and IL-8 production by LPS and OspA. Human neutrophils have been found to respond to 10-fold lower molar concentrations of LPS than OspA when adequate amounts of LPS binding protein and CD14 are present (9). In contrast, TLR2-transfected 293 cells responded to at least 10-fold lower concentrations of OspA than LPS (Fig. 3, *A* and *B*). The greater sensitivity of TLR2-expressing cells to OspA than LPS strongly argues that TLR2 is important in mediating inflammatory signaling by lipoproteins in humans.

The finding that TLR2-negative U373 cells are responsive to LPS but not lipoproteins suggests that endogenously expressed TLR2 mediates inflammatory signaling by bacterial lipoproteins. The extremely high expression of TLR2 obtained in transiently transfected 293 cells may promote responsiveness to LPS that is not observed under physiological conditions. When the amount of TLR2 construct transfected into 293 cells was reduced, responsiveness to both LPS and OspA were conferred by transfection with as little as 0.03 μg of TLR2-expressing plasmid (Fig. 4). Very low concentrations of plasmid conferred maximal responsiveness

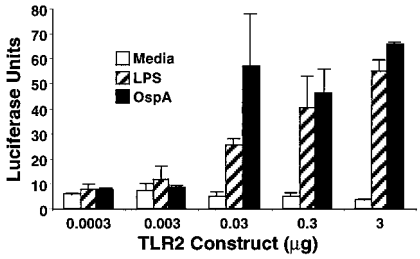

FIGURE 4. Dose dependence of TLR2 conferred responsiveness to OspA. 293 cells were transiently cotransfected with the indicated amount of TLR2 expression plasmid plus the ELAM-1 luciferase construct. Luciferase activity was measured in cells collected 6 h following stimulation. Media, media with 5 μg/ml polymyxin B; LPS, 100 ng/ml LPS; OspA, recombinant OspA at 500 ng/ml plus 5 μg/ml polymyxin B.

in 293 cells, and luciferase units remained high at the highest concentrations of plasmid, arguing that the difference in dose response seen between LPS and OspA did not reflect differential saturation of cofactors by either agonist. This finding further suggests that the dependence of LPS responsiveness on TLR2 expression in transfected 293 cells and its independence of TLR2 in U373 cells may reflect the influence of other essential components of the signaling pathway present in these different cell types.

OspA activation of human endothelial cells, neutrophils, monocytes, and mouse monocytes has been shown to be facilitated by the LPS coreceptor CD14 (20, 21). Although 293 cells do not express CD14 on their surface (22), human serum present in the culture medium provides soluble CD14. OspA activation of 293 cells transfected with the TLR2 expression plasmid was diminished in the presence of the CD14-blocking mAb, 60bca, when compared with an isotype control (Fig. 5). The diminished activity in the presence of blocking Ab is consistent with the 10- to 20-fold inhibition previously reported in endothelial cells, monoctyes, and neutrophils (20, 21). These findings indicate that TLR2-mediated lipoprotein signaling can be enhanced by soluble CD14 in a manner consistent with that exhibited by cell types naturally responsive to bacterial lipoproteins.

Discussion

Innate recognition of microbial products forms the initial defense against pathogen infection. In *Drosophila*, activation of Toll controls the induction of the antifungal peptide drosomycin in response to fungal infection (32), while two of the mammalian homologues, TLR2 and TLR4, have been associated with LPS responses. Our results demonstrate that TLR2 is required for bacterial lipoprotein signaling in two different TLR2-negative cell lines, U373 and 293, and that signaling requires the lipid modification. Although both LPS and lipoproteins can induce signaling through TLR2, transfected 293 cells responded to at least 10-fold lower concentrations of lipoprotein than LPS, supporting the physiological relevance of the lipoprotein interaction. Additionally, the defect in TLR4 that has been associated with the inability to C3H/HeJ mice to respond to LPS while responding normally to *B. burgdorferi* lipoproteins suggests that additional regulatory pathways are present in mammalian cells.

Other properties have been described for the Pam_3Cys modification of bacterial lipoproteins, including that of immunological adjuvant (31). Animal studies of the OspA-based Lyme vaccine have demonstrated that its immungenicity is dependent on this lipid modification (31). The identification of TLR2 as a critical component of Pam_3Cys signaling may provide a mechanism by which lipoproteins stimulate humoral immune responses without additional adjuvant.

Lyme arthritis, the most common manifestation of late Lyme disease, is associated with the presence of *B. burgdorferi* in the joint (3, 4). The abundantly expressed lipoproteins are intricately involved in the pathology of Lyme disease. These lipoproteins possess potent stimulatory properties for inflammatory cells that are associated with affected joints: namely neutrophils, mononuclear cells, and endothelial cells (6–9, 15, 16). Thus, understanding the molecular basis of the signaling events caused by lipoproteins will lead to a greater understanding of the inflammatory events associated with Lyme arthritis in general. In addition, lipoproteins are broadly expressed by numerous pathogen species, many of which have been associated with inflammatory pathology (33–35). Thus, an understanding of lipoprotein-induced signaling events will have widespread implications in the understanding of the diverse pathologies caused by these microbial species.

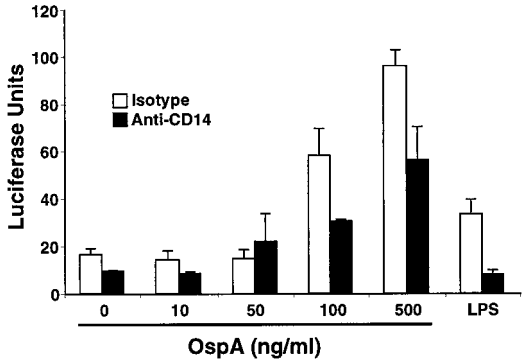

FIGURE 5. CD14 facilitates inflammatory activation by OspA. 293 cells were transfected with 0.03 μg of the TLR2 expression plasmid in the presence of the ELAM-1 luciferase construct. Transfected cells were then cultured in DMEM with 2% human serum for 36 h followed by the addition of increasing concentrations of OspA in the presence of 5 μg/ml of polymyxin B and 5 μg/ml of either a CD14 blocking Ab (60bca) or its isotype control (mopc 21). LPS, 100 ng/ml of LPS. NF-κB translocation to the nucleus was measured by luciferase.

Acknowledgments

We thank John Dunn for providing the recombinant OspA, OspB, and unlipidated OspA, and both Andy Hines and the members of the Weis labs for helpful discussions.

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