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Phagosomal signaling by *Borrelia burgdorferi* in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN- β

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Phagocytosed *Borrelia burgdorferi* (Bb) induces inflammatory signals that differ both quantitatively and qualitatively from those generated by spirochetal lipoproteins interacting with Toll-like receptor (TLR) 1/2 on the surface of human monocytes. Of particular significance, and in contrast to lipoproteins, internalized spirochetes induce transcription of IFN- β . Using inhibitory immunoregulatory DNA sequences (IRSs) specific to TLR7, TLR8, and TLR9, we show that the TLR8 inhibitor IRS957 significantly diminishes production of TNF- α , IL-6, and IL-10 and completely abrogates transcription of IFN- β in Bb-stimulated monocytes. We demonstrate that live Bb induces transcription of TLR2 and TLR8, whereas IRS957 interferes with their transcriptional regulation. Using confocal and epifluorescence microscopy, we show that baseline TLR expression in unstimulated monocytes is greater for TLR2 than for TLR8, whereas expression of both TLRs increases significantly upon stimulation with live spirochetes. By confocal microscopy, we show that TLR2 colocalization with Bb coincides with binding, uptake, and formation of the phagosomal vacuole, whereas recruitment of both TLR2 and TLR8 overlaps with degradation of the spirochete. We provide evidence that IFN regulatory factor (IRF) 7 is translocated into the nucleus of Bb-infected monocytes, suggesting its activation through phosphorylation. Taken together, these findings indicate that the phagosome is an efficient platform for the recognition of diverse ligands; in the case of Bb, phagosomal signaling involves a cooperative interaction between TLR2 and TLR8 in pro- and antiinflammatory cytokine responses, whereas TLR8 is solely responsible for IRF7-mediated induction of IFN- β .

Lyme disease | endosomal receptors | type I interferons | phagocytosis

Lyme disease (LD), the most commonly reported vector-borne illness in the United States (1), is a multisystem infectious disorder caused by the spirochetal bacterium *Borrelia burgdorferi* (Bb). Monocytes and macrophages are considered to be critical cellular elements of the innate immune response to Bb (2–5). Recognition of the spirochete was previously thought to result primarily from the interactions of the bacterium's abundant outer membrane-associated lipoproteins with Toll-like receptor (TLR) 1/2 on the surface of innate immune cells (6). More recently, we and others have provided evidence that phagocytosed live Bb induces inflammatory signals that differ both quantitatively and qualitatively from those generated by lipoproteins (2, 4, 7–9). In addition to enhanced cytokine production, phagocytosed live Bb induced transcription of IFN- β and several IFN-stimulated genes (ISGs) in isolated human monocytes, whereas spirochetal lipoproteins were unable to do so (7).

Although production of type I IFNs was previously ascribed solely to antiviral immune responses (10), it now is well established that both intracellular (11–14) and extracellular bacteria (11, 15–17) also induce transcription of these cytokines. Bacteria can elicit type I IFNs either by activating TLRs (11, 12) or through TLR-independent recognition of bacterial pathogen-

associated molecular patterns (PAMPs) within the host cell cytosol (11, 18). We and others have begun to demarcate the pathways by which live Bb induces type I IFNs in both mouse and human cells (7, 19–22). Miller et al. (20) demonstrated that live Bb induces transcription of several ISGs in bone marrow-derived murine macrophages (BMDMs) independent of both MyD88 (20) and TRIF (21) yet requiring the transcription factor IFN regulatory factor (IRF) 3. Using Bb-infected human peripheral blood mononuclear cells (PBMCs), Petzke et al. (22) provided evidence that human plasmacytoid dendritic cells (pDCs) are a principal source of IFN- α in response to phagocytosed live spirochetes and demonstrated that transcription and secretion of this type I IFN was inhibited by blocking TLR7 and TLR9. These two TLRs, together with TLR8, make up a family of endosomal pattern recognition receptors that are capable of generating MyD88-dependent type I IFNs by sensing pathogen-derived nucleic acids (23, 24). In contrast to pDCs, human monocytes constitutively express TLR8, and although they also express TLR7, they do not express TLR9 (23, 25–27). The role of TLR8 in modulating innate immune responses to Bb, including its involvement in the production of type I IFNs, has not been previously examined.

In this study, we used highly purified human monocytes to characterize more precisely the mechanisms whereby internalization of the LD spirochete induces production of pro- and antiinflammatory cytokines, including type I IFNs. We provide evidence that phagosomal signaling in Bb-infected monocytes involves a sequential and cooperative interaction between TLR2 and TLR8, which occurs exclusively by activation of TLR8 in the case of IFN- β . Our combined observations take us well beyond the simplistic notion that innate immune cell activation by Bb occurs only at the plasma membrane through TLR1/2 and CD14 signaling and, instead, reveal the importance of the phagosome as an efficient platform for the recognition of diverse bacterial PAMPs.

Results

Phagocytosis of Bb Is Required for Transcription of IFN- β in Bb-Infected Human Monocytes. We previously showed that inhibition of phagocytosis diminished NF- κ B-dependent cytokine production in Bb-stimulated PBMCs (2). More recently, we demonstrated that the monocyte is a major source of these

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cytokines and solely responsible for the production of IFN- β (7). We now provide evidence that internalization of the bacterium is essential for the induction of IFN- β and for optimal production of TNF- α in isolated human monocytes. When the actin filament disrupter Cytochalasin-D is used to block phagocytosis, transcription of *ifnb* is abrogated and secretion of TNF- α is significantly diminished (Fig. S1). Thus, if sensing of the spirochete is restricted to cell surface TLR2, induction of type I IFNs does not occur and production of NF- κ B-mediated cytokines is significantly impaired.

Bb-Mediated Induction of IFN- β Cannot Be Explained by TLR2 Signaling. TLR2 signals were previously believed to be incapable of inducing type I IFNs (28). Two recent studies have challenged this notion by demonstrating that activation of TLR2, by either viral (29) or bacterial (30) ligands, can induce transcription of IFN- β in inflammatory human monocytes and murine macrophages, respectively. In a previous study, we provided evidence that lipoprotein-rich borrelial lysates do not induce transcription of IFN- β by human monocytes (7). In the same study, we showed that production of IFN- β in Bb-infected murine BMDMs occurs independent of TLR2. Nonetheless, in light of the recent finding that a bacterial TLR2 ligand (Pam₃CSK4) induces production of IFN- β in murine macrophages (30), we have reexamined herein the contribution of TLR2 activation in the production of this cytokine by human monocytes. We first established that transcription of IFN- β in Bb-stimulated monocytes does not begin in earnest until 2 h of incubation and increases noticeably after 4 h of stimulation (Fig. S2). In contrast to the robust type I IFN responses elicited by either LPS or live Bb, even high concentrations of Pam₃CSSNA failed to induce transcription of IFN- β in monocytes (Fig. S3). These combined observations are indicative that TLR2 signaling does not play a significant role in the induction of type I IFNs in Bb-infected monocytes.

TLR8 Is Required for Transcription of IFN- β and Cooperates with TLR2 in Generating Pro- and Antiinflammatory Cytokine Production in Human Monocytes. Having established that TLR2 signaling alone cannot explain the type I IFN responses to live Bb, we hypothesized that activation of an endosomal TLR could be responsible for the induction of IFN- β in the monocyte. To explore this possibility, we took advantage of the availability of specific inhibitory DNA-oligonucleotide sequences (IRSs) that have the ability to block ligation of TLR7, TLR8, or TLR9 (31, 32). These inhibitors were recently used by Petzke et al. (22) to demonstrate that induction of IFN- α in Bb-infected pDCs is dependent on TLR7 and TLR9. For these experiments, we first corroborated that healthy volunteers' monocytes expressed TLR7 and TLR8 and very little TLR9 (27) (Table S1). As depicted in Fig. 1A, we then showed that inhibition of TLR8 by IRS957 eliminated transcription of IFN- β , whereas induction of this cytokine by the spirochete was unaffected by blocking TLR7 with IRS661 ($P = 0.17$ vs. no inhibitor) or TLR9 with IRS869 ($P = 0.27$ vs. no inhibitor). The specificity of IRS957 for TLR8 in human monocytes has been previously confirmed by exclusion of reactivity to TLR7 and TLR9 (32). Herein, we confirmed the specific inhibitory effect of IRS957 by demonstrating that production of IFN- β was markedly decreased in response to the TLR7/8 agonist R848 (Fig. S4) and the TLR8-specific ligand 3M-002 (Fig. S5), whereas TLR7 inhibition had no effect on 3M-002-mediated transcription of IFN- β . Moreover, the response to LPS, which induces IFN- β through TLR4 (28), was unaffected by the TLR8 inhibitor (Fig. 1A). In concert with the role of TLR8 in the production of NF- κ B-dependent cytokines (33–35), IRS957 also caused a significant decrease in secretion of TNF- α , IL-6, and IL-10 by Bb-infected monocytes (Fig. 1B). In parallel experiments, we verified using confocal microscopy that internalization of the spirochete was not significantly affected by blocking TLR8 (no in-

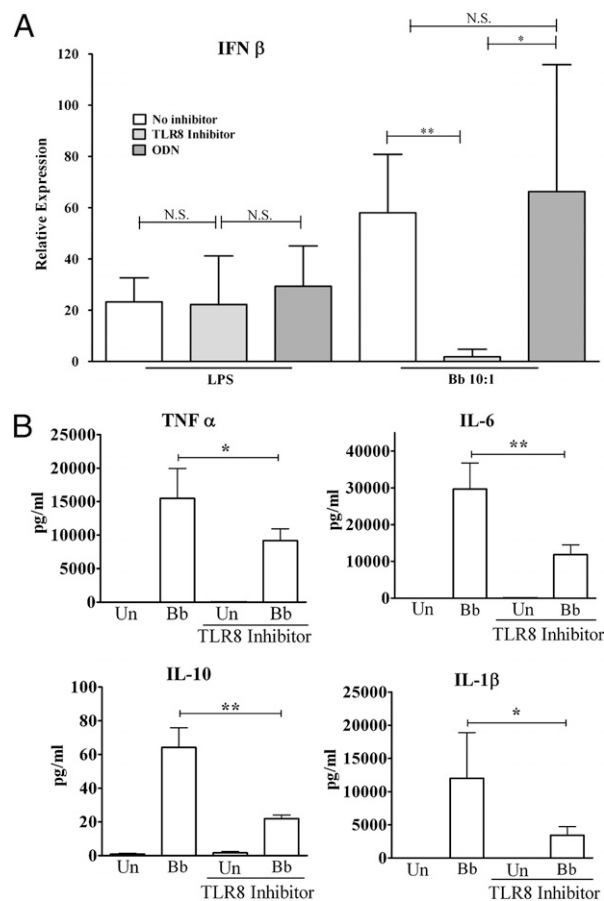


Fig. 1. Transcription of IFN- β is abrogated after TLR8 inhibition in human monocytes. (A) TLR8 inhibition by IRS-957 eliminated transcription of IFN- β in Bb-infected monocytes (10:1 multiplicity of infection), whereas a control ODN sequence did not. Transcription of IFN- β in response to LPS was not affected by either treatment. Relative expression refers to *ifnb* gene expression normalized to *gapdh*. N.S., not significant; ODN, oligodeoxynucleotide. (B) Secretion of NF- κ B-dependent cytokines (TNF- α , IL-6, IL-10, and IL-1 β) was diminished on inhibition of TLR8 in human monocytes. Un, uninfected. P values correspond to paired analysis [Mann-Whitney U test (A) and paired t test (B)] from a minimum of four independent experiments. * $P < 0.05$; ** $P < 0.01$.

hibition, $19.2\% \pm 4.1$ vs. TLR8 inhibited, $14.2\% \pm 1.2$; $P = 0.2$). Taken as a whole, we interpreted these results as evidence that in Bb-infected monocytes, TLR8 cooperates with TLR2 in the production of NF- κ B-mediated cytokines, whereas TLR8 alone is responsible for transcription of IFN- β .

We next used RT-PCR to examine the expression profile of TLR2 and TLR8 in Bb-infected monocytes and compared these responses with those elicited by synthetic TLR2 and TLR8 ligands. As shown in Table 1, live Bb differentially induced transcription of both TLR2 and TLR8, although it had no effect on TLR7 or TLR9. In parallel experiments, we demonstrated that up-regulation of both TLR2 and TLR8 was greatly diminished by blocking TLR8 (Table 1). The inhibitory effect of IRS957 on TLR8 protein levels was confirmed by Western blot analysis (Fig. S6). The regulatory effect of TLR8 stimulation on TLR2 is not at all unanticipated, given that IFN- β has been shown to up-regulate expression of TLR2 (36). In support of this idea, R848 induced transcription of both TLR8 and TLR2 (Table 1), whereas Pam₃CSSNA only affected TLR2. The inhibitory effect of IRS957 on TLR8 transcription is indicative that TLR8 is self-regulated on engagement of its cognate spirochetal PAMP. These findings

Table 1. TLR relative expression in stimulated human monocytes

n = 6	Bb, 10:1 MOI			
	No inhibitor	TLR8-inhibited	Pam3CS5NA, 10 μ g	R848, 1 μ g
TLR2	3.1 \pm 0.5	1.9 \pm 0.2*	2.8 \pm 1.7	2.2 \pm 0.4
TLR8	5.8 \pm 0.7	-1.5 \pm 0.6*	-0.4 \pm 1.9	14.2 \pm 4.3
TLR7	1.4 \pm 0.1	-0.4 \pm 1.2*	-1.3 \pm 0.2	3.6 \pm 2.7
TLR9	-3.6 \pm 0.9	-2.5 \pm 0.7	-1.5 \pm 0.5	-3.1 \pm 1.3

Values represent relative changes in gene expression compared with GAPDH. MOI, multiplicity of infection.

* $P < 0.05$ (Mann-Whitney U test). Comparison analysis between TLR8-inhibited and -uninhibited Bb-stimulated monocytes.

provide additional evidence that innate immune responses to Bb in human monocytes result from a cooperative interaction between TLR2 and TLR8.

TLR2 and TLR8 Colocalize with GFP-Bb to the Phagosomal Vacuole of the Monocyte. Having established that phagocytosis is an essential element of the innate immune response to the spirochete and that TLR2 and TLR8 cooperate in generating phagosomal signals, we then used confocal microscopy to characterize the spatial-temporal distributions between phagocytosed Bb and TLR2 and TLR8 more precisely. We first showed that the baseline expression of TLR2 in unstimulated monocytes was greater than that of TLR8 (Fig. 2 and Fig. S7). TLR2 was visualized on the monocyte's surface as well as on the cytoplasm, whereas TLR8 was seen exclusively inside the cell. Within 30 min of coincubation with Bb, expression of both TLR2 and TLR8 increased in Bb-infected monocytes and to a lesser extent in bystander uninfected cells (Fig. 2 and Fig. S7). To capture spirochete-TLR localization at both early and later stages of phagocytosis, we analyzed multiple confocal images obtained at several times of incubation, beginning with the 30-min stimulation time point. TLR2 colocalized at the cell surface with spirochetes that were attached and/or partially embedded in the monocyte's membrane (Fig. 3A and Movie S1). TLR2 also colocalized to the phagosomal vacuole, with recently internalized spirochetes shown as tightly wound bacterial coils (Fig. 3B and Movie S2). TLR8 colocalized to phagosomal vacuoles containing fully degraded spirochetes in the shape of bright fluorescent blebs (Fig. 3C and Movie S3). Concurrent staining with TLR2 and TLR8 confirmed that both TLRs colocalize simultaneously to phagosomal vacuoles containing degraded spirochetes (Movie S4). Of particular significance, TLR2 colocalized with TLR8 in the cytoplasm of Bb-infected monocytes (Fig. 2, *Inset* and Movie S4), which suggests that TLR2 and TLR8 traffic to the phagosome from similar endosomal compartments. Taken together, these images provide visual evidence that Bb is initially sensed by TLR2 on the monocyte cell surface during binding and uptake, whereas partially and fully degraded spirochetes interact with both TLR2 and TLR8 in the phagosomal vacuole.

Live Bb Induces Transcription and Nuclear Translocation of IRF7. We next sought to elucidate which transcription factor is associated with TLR8-mediated induction of IFN- β . We focused on IRF3, IRF5, and IRF7, all of which are associated with the induction of genes that encode type I IFNs (37–41). We have previously demonstrated that IRF7 is the only IRF that is significantly up-regulated in PBMCs stimulated with live Bb (7). Herein, we show by RT-PCR that IRF7 (but not IRF3 or IRF5) is also induced in Bb-infected isolated human monocytes (Table 2). We also demonstrate that the transcriptional regulation of IRF7 is eliminated by blocking TLR8. Using epifluorescence microscopy, we then confirmed that IRF7 is translocated into the nucleus of Bb-

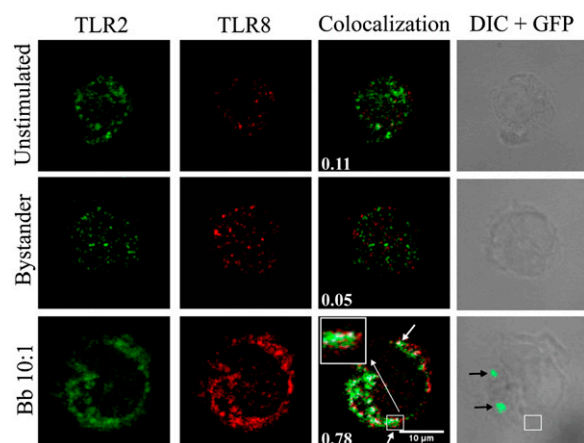


Fig. 2. TLR2 and TLR8 cellular expression. Costaining for TLR2 (green) and TLR8 (red) is shown in unstimulated monocytes and in both infected and uninfected cells (bystander) within 30 min of incubation with Bb (10:1 multiplicity of infection). TLR2 fluorescent signals are visualized in unstimulated and bystander cells, greatly increasing in Bb-infected monocytes. TLR8 signals are of very low intensity in uninfected monocytes and, like TLR2, increase in Bb-infected monocytes. Black arrows point to degraded GFP-Bb. TLR2 colocalizes with TLR8 even at locations that do not coincide with internalized GFP-Bb (white arrows and region of interest). Numerical values shown in white correspond to Mander's colocalization coefficient (M2), indicative of the proportion of the green signal (TLR2) overlapping with the signal in the red channel (TLR8). DIC, differential interference contrast.

infected monocytes and R848-stimulated cells (Fig. 4), a finding that is indicative of IRF7 activation through phosphorylation (42). A similar process did not occur in unstimulated monocytes or bystander uninfected cells. A two-step process involving IRF3 activation before activation of IRF7 does not seem plausible, because we did not observe any increase in IRF3 signal by mi-

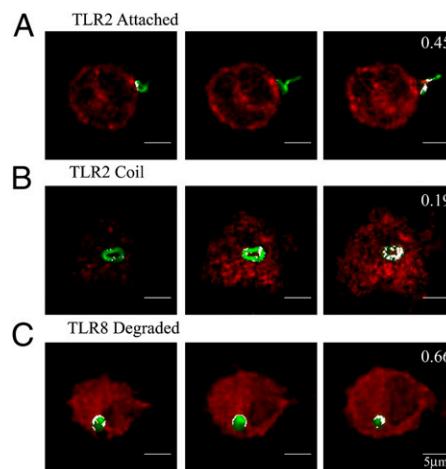


Fig. 3. Phagocytosed Bb colocalizes with TLR2 and TLR8 in human monocytes. A composite of representative confocal micrographs shows three consecutive Z-stack planes of GFP-Bb-stimulated human monocytes. Extracellular Bb interacts with TLR2 at the plasma membrane of the monocyte (A), whereas recently internalized (bacterial coil) and fully degraded (fluorescent bleb) Bb colocalizes with TLR2 (B) and TLR8 (C). Mander's colocalization coefficient values (M2), shown in the upper right corner of each figure, are indicative of the proportion of the green signal overlapping with the signal in the red channel. Red depicts TLR2 signals in A and B and TLR8 in C, green depicts GFP-Bb, and white represents red and green pixels that colocalize. (Scale bar, 5 μ m.) Colocalization through the Z-stack images illustrated in A and B (TLR2) and in C (TLR8) is shown in Movie S1, Movie S2, and Movie S3.

Table 2. IRF Expression in Bb-stimulated human monocytes

n = 6	No inhibitor	TLR8-inhibited
IRF 3	-1.1 ± 0.5*	-2.1 ± 0.1
IRF 5	0.03 ± 0.6*	-2.2 ± 0.3
IRF 7	7.2 ± 1.3*	1.6 ± 0.1

Values represent relative changes in gene expression compared with GAPDH.

* $P < 0.05$ (Mann-Whitney U test). Comparison analysis between inhibited and uninhibited Bb-stimulated human monocytes.

scopy or translocation of IRF3 into the nucleus (Fig. S8). Moreover, using Bb-infected monocytes in a time-chase experiment, we confirmed that relative increases in IRF7 sequentially preceded relative increases in IFN- β (Fig. S2). Blocking IRF7 phosphorylation using the kinase inhibitor staurosporine (43) completely abrogated IFN- β induction (Fig. S9A) and greatly impaired up-regulation of IRF7 (Fig. S9B). The amount of staurosporine used was enough to block nuclear translocation of IRF7 in the absence of apoptosis (Fig. S9C). Taken as a whole, these results provide evidence that IRF7 induces transcription of IFN- β in Bb-infected monocytes.

Discussion

The experimental evidence presented herein indicates that the LD spirochete induces multifaceted innate inflammatory signals in human monocytes that can only be fully coordinated during internalization of the bacterium and formation of the phagosome. As maturation of the phagosome progresses, this unique vacuolar structure very quickly gains capabilities for the recognition of diverse spirochetal PAMPs and becomes an increasingly more efficient platform for signal generation, leading to enhanced production of NF- κ B-mediated cytokines and MyD88-dependent induction of IFN- β . Our findings are unique in that they suggest that internalization and degradation of the spirochete by the monocyte

facilitates sampling of liberated PAMPs through a sequential activation of cell surface TLR2, followed by signaling through TLR2 and TLR8 cooperatively at the phagosomal membrane.

Mammalian TLR8 is thought to signal exclusively through MyD88 after recognition of single-stranded RNA ligands (44). Human TLR8 can be activated by nucleic acids derived from a variety of RNA viruses (45–48) as well as *Escherichia coli* RNA in genetically complemented HEK293 cells (49). To date, TLR8 has been linked to the recognition of two different intracellular bacteria, *Mycobacterium bovis* bacille Calmette–Guérin (bacillus Calmette–Guérin) (35) and *Helicobacter pylori* (50). In the case of bacillus Calmette–Guérin, TLR8 protein expression levels markedly increased in infected macrophages derived from a human acute monocytic leukemia cell line (THP-1 cells). Interestingly, a significant boost in TLR8 expression levels was also seen in the blood of patients with untreated *Mycobacterium tuberculosis* infection (50). Using activated THP-1 cells, Gantier et al. (35) demonstrated that expression of the two main human TLR8 variant mRNAs significantly increased following *H. pylori* phagocytosis. In this study, we reveal a previously unappreciated direct role for human TLR8 in transcription of IFN- β in Bb-infected monocytes and cooperativity with TLR2 in the production of several NF- κ B-dependent cytokines. We hypothesize that endosomal TLR8 is activated in response to spirochetal mRNA released upon degradation of the bacterium into the phagosomal cargo. In contrast to bacillus Calmette–Guérin and *H. pylori*, Bb is an extracellular pathogen that is rapidly degraded once phagocytosed; thus, it is not surprising that engagement of TLR8 in the monocytes occurred soon after internalization of the bacterium. Given that induction of type I IFNs in Bb-infected pDCs is partially dependent on TLR9 (22), it is conceivable that borrelial DNA also may play a role in activating TLR8 in human monocytes. This premise is supported by the recent finding that murine TLR8, which is functional in mice in the context of a viral infection (51), can be activated by vaccinia virus (VV) DNA through recognition of poly(A)T-rich motifs (52). Like VV DNA, borrelial DNA also contains poly(A)T-rich motifs (53).

TLR2–spirochetal PAMP interactions were originally studied as cell surface phenomena (54). Several studies have now demonstrated that TLR2 is recruited to the phagosome, where it becomes available for signaling (14, 55–57). Structurally, this model is not at all unanticipated, given that the phagosomal membrane is initially derived from the plasma membrane (58, 59). Consistent with this concept, Underhill et al. (55) found that in a mouse leukemic monocyte macrophage cell line (RAW-cells), cell surface TLR2 was recruited to phagosomes and colocalized with zymosan particles within minutes of their internalization, suggesting that TLR2-mediated signals occur within a circumscribed window of time during the initial phase of phagosome maturation. Studies conducted in Stephanie Vogel's laboratory (60), however, have revealed that prolonged TLR2-dependent signaling from the phagosomal vacuole takes place in murine macrophages infected with a mutant strain of *Francisella tularensis* (Ft), which is unable to escape the phagosome. The same group demonstrated that TLR2 and MyD88 colocalize to the phagosomal vacuole of RAW macrophages infected with a live vaccine Ft strain (14). TLR2 also can become available for signaling from within endosomal vacuoles in human monocytes stimulated with internalized staphylococcal lipoteichoic acid (56). O'Connell et al. (57) demonstrated that intracellular TLR2 was responsible for the initiation of signal transduction events by *Chlamydia trachomatis*. Herein, we validate that TLR2 senses live spirochetes at the plasma membrane during binding and uptake and provide previously undescribed evidence that TLR2 is also available for signaling at the phagosomal membrane of human monocytes. The finding that TLR2 colocalizes with TLR8 intracellularly in areas other than the phagosome (Fig. 4, region of interest) suggests that TLR2 traffics to the phagosomal vacuole

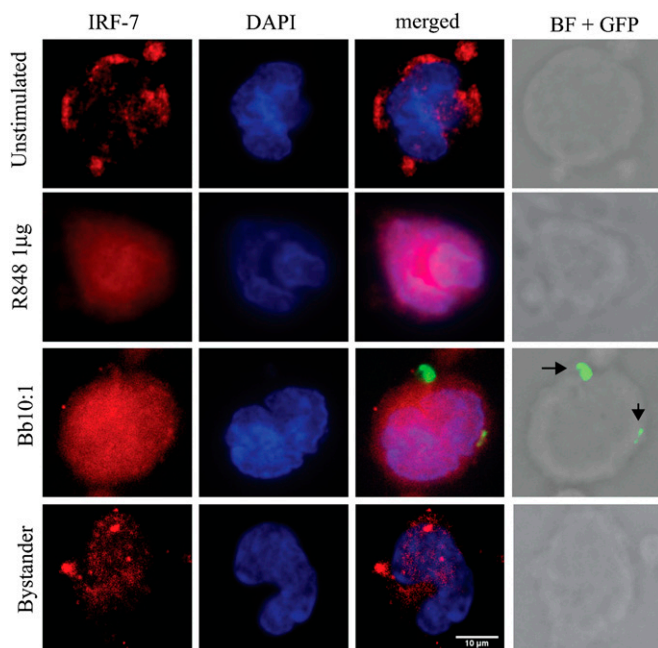


Fig. 4. Live Bb induces nuclear translocation of IRF7. IRF7 (red) is expressed in the cytoplasm of uninfected monocytes and translocates to the nucleus (DAPI, blue) in GFP-Bb-infected monocytes (indicated by black arrows) and in R848-stimulated controls. Translocation of IRF7 into the nucleus is shown in pink. BF, bright field.

from two different locations: most certainly from the plasma membrane but also from an as yet undefined cytosolic location/structure, similar to the intracellular trafficking of TLR8. Taken as a whole, we envision a model in which a superior TLR2-receptor density would become available for signaling in phagosomal vacuoles than can occur at the plasma membrane. The presence of specific “docking” molecules within the budding phagosomal vacuole would thus provide an effective scaffold for inserting spirochetal lipoproteins proximal to their cognate receptors. A higher receptor density, in combination with a large concentration of exposed and liberated lipoproteins, would thus greatly improve TLR2 signaling efficiency.

Although cross-talk between TLR2 and TLR8 occurs following stimulation of PBMCs by their individual TLR ligands (61), the transcriptional and translational responses shown here distinctively demonstrate that coordination between TLR2 and TLR8 does occur in response to a live bacterium. How these integrated responses facilitate control of the LD spirochete and whether or not they are beneficial or detrimental to individuals with LD have only begun to be uncovered. Evidence from recently published studies suggests that endosomal TLR activation, including TLR8, can have an impact on the ability of the host cell to respond to stimulation of a different TLR (62, 63), including TLR2 (62). Given that type I IFNs can shape a variety of downstream inflammatory responses through positive and/or negative regulation of the expression of hundreds of additional genes involved in secondary host defenses, TLR8 activation is likely to play a critical role in clearance of the spirochete and, most importantly, disease control. Type I IFNs also can promote inflammatory cell death of host cells (50, 64), a phenomenon that we previously described in Bb-infected monocytes (4), and thus may constitute an additional mechanism for controlling spirochetal replication. Whether or not the presence of *tlr8* SNPs in patients with LD affects spirochetal clearance and/or dissemination, and whether these SNPs are linked to disease progression and/or the severity of the initial clinical presentation, needs to be studied.

Materials and Methods

Human Monocyte Isolation and Stimulation Conditions. All procedures involving human subjects were approved by the Institutional Review Board at the University of Connecticut Health Center. After obtaining written informed consent, monocytes were isolated from LD-seronegative volunteers as previously described (7). Except where noted, cells were incubated for 4 h at 37 °C/5% (vol/vol) CO₂ with live, low-passage, temperature-shifted GFP-Bb 1479 at various multiplicities of infection. Appropriate controls, including a synthetic TLR2 ligand (Pam₃CSNA), were used in the *ex vivo* stimulation model as previously described (7). All culture media and reagents were confirmed to be essentially free of LPS contamination (<10 pg/mL) by Limulus amoebocyte lysate assay (Cambrex). In selected experiments, Cytochalasin-D was used as previously described (2).

Endosomal TLR Blocking. Specific immunoregulatory DNA sequences (IRSs; Integrated DNA Technologies) (31) were used to block TLR7 signaling (IRS661: 5'-TGCTTGCAAGCTTGCAAGCA-3'), TLR7 and TLR8 (IRS957: 5'-TGCTTG-AC-ATCCTGGAGGGTGTGT-3'), and TLR9 (IRS869: 5'-TCCTGGAGGGTGTGT-3') as described by Petzke et al. (22). A non-TLR-specific oligodeoxyribonucleotide sequence was used as a control, in the same concentration as the TLR inhibitor. R848 (Resiquimod; InvivoGen), a known synthetic ligand for human TLR7 and TLR8 (32) and a potent inducer of IFN- β (65), was used to study the response in monocytes. The specificity of the TLR7 and TLR8 inhibitors was assessed with the TLR8-specific ligand 3M-002 (InvivoGen) (66).

Assessment of Monocyte Activation by Quantitative RT-PCR and Cytokine Secretion. At the conclusion of the incubation period, cells were harvested and RNA was extracted using a total RNA isolation kit (Macherey-Nagel). cDNA was prepared from extracted RNA using a high-capacity cDNA RT kit (Applied Biosystems). RT-PCR conditions and reactions have been described previously (7). Commercially available primers (Applied Biosystems) used for amplification included the following: *ifnb* (Hs00277188_s1), *tlr2* (Hs00610101_m1),

tlr7 (Hs00152971_m1), *tlr8* (Hs00152972_m1), *tlr9* (Hs00152973_m1), *irf3* (Hs01547283_m1), *irf5* (Hs00158114_m1), *irf7* (Hs00185375_m1), and *gapdh* (Hs99999905_m1). Expression levels of all transcripts studied were normalized to the GAPDH level, and the relative changes in gene expression generated were calculated using the 2^{- $\Delta\Delta$ CT} method. A cytokine bead array system (Human inflammation kit; Becton Dickinson) was used to assay cytokines secreted by TNF- α , IL-10, IL-6, and IL-1 β in culture supernatants. Secretion of IFN- β was determined using a commercially available ELISA method (VeriKine-HS; PBL Biomedical Laboratories).

Western Blot Analysis of TLR8 Protein Expression. TLR8 expression was measured by Western blot analysis. Briefly, 40 μ g of protein was loaded in each correspondent lane, and protein was transferred to a PVDF membrane. After blocking, the membrane was probed with TLR8 antibody (catalog no. H00051311-MO1; Abnova). Membranes were washed, and secondary antibodies were added. After 1 h, membranes were washed with PBS-Tween and developed. The same membrane was stripped, reblocked, reprobed, and developed for human GAPDH (Santa Cruz Biotechnology, Inc.) Densitometric analysis of obtained bands was done using a Flurchem 8900 Chemiluminescence and Gel Imager (Alpha Innotech).

Cellular Localization of IRF7/IRF3, TLR8/TLR2, and GFP-Bb. To determine the cellular localization of TLR2 and TLR8 in Bb-infected monocytes, we used a modified version of the method described by Ma et al. (67). Briefly, monocytes were plated in poly-D-lysine-treated culture slides (BioCoat; BD Biosciences). Following different time points of stimulation (0 min, 30 min, 60 min, 90 min, 3 h, and 4 h), slides were fixed with 4% (vol/vol) paraformaldehyde for 10 min at room temperature (RT); permeabilized with 0.2% saponin for 10 min; blocked with Connaught Medical Research Laboratories (CMRL) 10% FCS for 2 h at RT; incubated overnight at 4 °C with a rabbit anti-human IRF7 polyclonal or mouse anti-human IRF3 monoclonal antibody (Santa Cruz Biotechnology, Inc.), a rabbit anti-TLR8 polyclonal antibody (IMGEX), or a rabbit anti-TLR2 polyclonal antibody (Rockland Immunochemicals) (1:100 dilution for all); and subsequently incubated with Texas Red^R-X-conjugated goat anti-rabbit antibody (1:200 dilution; Invitrogen) for 1 h at RT or goat anti-mouse DyLight 594 (Thermo Scientific). For TLR2 and TLR8 immunofluorescent costaining, we used the following antibodies: a primary monoclonal mouse antibody to human TLR8 (IMGEX) followed by goat anti-mouse AlexaFluor 546 (Invitrogen) and a primary polyclonal rabbit anti-TLR2 antibody (Rockland Immunochemicals) followed by goat anti-rabbit AlexaFluor 635 (Invitrogen). Slides were washed and mounted in Vectashield antifade reagent (Invitrogen) containing DAPI. Images were acquired on an LSM-510 confocal microscope using a 63 \times (1.4 N.A.) oil immersion objective at a pixel resolution of 512 \times 512. Image analysis was performed using ImageJ (National Institutes of Health); pixel colocalization was displayed using the colocalization plug-in. Mander's overlap colocalization coefficients (M1 and M2) were determined using the JACoP plug-in (68). Movies were generated using ImageJ and Quicktime Pro software (Apple, Inc.). To measure TLR2 and TLR8 cellular expression, ~100 images of human monocytes, stained for either TLR2 or TLR8, were acquired at a magnification of 100 \times in an Olympus BX-41 epifluorescence microscope equipped with a digital camera (Q Imaging), using equal exposure time and gain parameters. Regions of interest corresponding to individual cellular contours were drawn using the Freehand selection tool of the ImageJ software, and mean fluorescence intensity and cellular area were calculated.

Statistical Methods. General statistical analysis was performed using GraphPad Prism 4.0. The fold increase or decrease for each specific gene transcript assayed by quantitative RT-PCR and cytokine concentrations was compared among the different stimuli using either a paired or unpaired Student's *t* test or the equivalent nonparametric method (i.e., Mann-Whitney *U* test). To analyze changes over time, we used a nonparametric statistical test for trend analysis (Kruskal-Wallis test), because a Gaussian distribution could not be assumed. A *P* value <0.05 was considered significant.

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