Distinct roles for MyD88, TLR2, TLR5, and TLR9 in phagocytosis of Borrelia burgdorferi and inflammatory signaling

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Running title: Role of MyD88 in the phagocytosis of Borrelia Burgdorferi

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ABSTRACT

The contribution of toll-like receptors (TLRs) to phagocytosis of *Borrelia burgdorferi* has not been extensively studied. We show that bone marrow derived macrophages (BMDM) from MyD88<sup>−/−</sup> mice or Raw cells transfected with a dominant negative MyD88 were unable to efficiently internalize *B. burgdorferi*. Knockouts of TLR2 and TLR9 or knockdown of TLR5 by siRNA produced no defects in phagocytosis of *B. burgdorferi*. Production of inflammatory cytokines was greatly diminished in MyD88<sup>−/−</sup> BMDM, but only partially affected in TLR2<sup>−/−</sup> BMDM or knockdown of TLR5 and unaffected in TLR9<sup>−/−</sup> BMDM. Cytochalasin D (cytoD) reduced cytokine induction, but not to the level of the MyD88<sup>−/−</sup>. Addition of cytoD to TLR2<sup>−/−</sup> BMDM inhibited inflammatory responses to *B. burgdorferi* to the level of MyD88<sup>−/−</sup> BMDM--consistent with a role for TLR2 in both recognition of extracellular products and lysosomal sampling by TLR2 after processing of the organism. CytoD had no impact on cytokine productions in cells undergoing TLR5 knockdown. These results suggest that MyD88, but not TLR2, TLR5 and TLR9, is important for the uptake of *B. burgdorferi* and MyD88 affects inflammatory responses both through its effects on phagocytosis and its role in transducing signals from TLR2 and TLR5.
INTRODUCTION

Toll-like receptors (TLRs) play an important role in host innate immune responses to microbial pathogens. Engagement of surface-associated TLRs results in the activation of signaling pathways, which release multiple inflammatory mediators that shape the early host response. TLRs have been shown to be important in dendritic cell maturation and antigen processing and thus also have an important role in determining adaptive immune responses (23, 34, 42).

Deficiency of an adapter molecule, myeloid differentiation primary response gene 88 (MyD88), which is a key signaling molecule utilized by most of the TLRs, results in a severe immune impairment of host defenses against many microorganisms (7, 11, 13, 18, 38, 39, 43).

*Borrelia burgdorferi* is the etiologic agent of Lyme disease. The *B. burgdorferi* genome encodes for over 160 lipoproteins that become expressed during different stages of its life cycle. Borrelial lipoproteins are recognized by TLR1/2 heterodimers and have been shown to activate peripheral blood mononuclear cells (PBMC) to produce inflammatory cytokines and chemokines (21, 36, 40). Studies of mice deficient in either TLR2 or MyD88 have found that loss of either of these proteins results in a severely impaired ability to clear spirochetes from infected mice (5, 9, 28, 49, 51). This impairment does not appear to be due to the effects of TLR signaling on the appropriate development of the adaptive immune response as mice deficient in MyD88 develop an antibody response that is essentially indistinguishable from that of wild type mice (28).

One possible reason for the inability of TLR2−/− or MyD88−/− mice to appropriately control infection with *B. burgdorferi* is that TLR signaling may be important for the early killing of the organism by phagocytes such as macrophages. The role of TLR signaling in the phagocytosis of...
bacteria has varied depending upon the organisms studied. For many bacteria, the major phagocytic defect in MyD88−/− cells is in the killing of the organism after it reaches the phagosome. This may be due to a reduction in phagosome maturation or in oxidative killing (8, 26). Blander and Medzhitov have shown that phagocytosis and killing of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* is greatly decreased in the absence of TLR2 (for *S. aureus*), TLR4 (for *E. coli* and *S. typhimurium*) or MyD88 (all three) (8). This defect appears to be due to a loss of activation of p38 mitogen-activated protein kinase (MAPK), which plays an important role in both phagosome maturation and oxidative killing (8, 26). However, there remains some controversy about this mechanism as Yates and Russell, using defined microparticles, have suggested that phagosome maturation proceeds independently of TLR2 and TLR4 signaling (52). In their studies, while MyD88−/− cells did exhibit a defect in phagolysosome maturation, it appeared that this defect was not due to the loss of direct activation of TLR signaling pathways by the TLR ligands, but rather attributed to baseline differences between the cells. These defects may arise secondary to the role of TLRs in cellular development and/or the impact of low-level stimulation on the cytokine “milieu” and activation state of the cells.

TLR activation also appears to play a role in the internalization of some, but not all, bacteria by macrophages. Internalization of *S. aureus*, *E. coli* and *S. typhimurium* is decreased in the absence of TLR activation, while loss of TLR activation has no effect on the internalization of either group B streptococcus or *L. monocytogenes* (20, 50).
TLR2 has been known to be a major signaling receptor for *B. burgdorferi* lipoprotein, outer surface protein (OspA) (21, 51). While blocking TLR2 results in great reduction of inflammatory signaling in response to purified lipoproteins, antibody blocking of surface bound TLR2 does not block inflammatory signaling in response to whole organisms (6). There are several possible explanations for this. First, receptors other than TLR2 may recognize products of *B. burgdorferi* and contribute the inflammatory response. Second, as it has been shown that TLR2 may be recruited to phagosomes, it is possible that the majority of TLR2 signaling does not occur through ligation of extracellular lipoproteins (14, 24, 33).

Liu *et al* have previously reported that MyD88−/− macrophages are able to bring *spirochetes* into phagosomes but show defective killing of the organism in phagosomes (28). The contribution of individual TLRs to this process for *B. burgdorferi* has not been reported. In this report, we will detail our studies on the role of MyD88 in phagocytosis of *B. burgdorferi*. Surprisingly, our data show that the major defect in phagocytosis in the absence of MyD88 is not in phagolysosomal killing but in uptake of the organism. We will also report our studies on the contribution of three TLRs, which were thought most likely to recognize *B. burgdorferi* products, TLR2, TLR5 and TLR9, to phagocytosis of *B. burgdorferi* as well as their effects on inflammatory signaling.

**Materials and Methods**

*Mice, cells and bacteria*

MyD88−/− mice were maintained as heterozygous breeding pairs at the sixth-generation backcross on the C57BL/6 background. MyD88−/−, MyD88+/+, and MyD88+/− littermates were genotyped as described previously (27). TLR2−/− and TLR9−/− mice on a C57BL/6 background were generated as
described in (19, 44). Age and sex-matched C57BL/6 control mice were obtained from the
Jackson Laboratory and used as controls for either TLR2−/− or TLR9−/− mice. The procedures used
for our animal studies were reviewed and approved by Tufts University Institutional Animal
Care and Use committee.

Mouse bone marrow-derived macrophages (BMDM) were recovered from mouse femurs and
differentiated as described in (41). In brief, bone marrow cells were flushed from mouse femurs
with sterile RPMI media and cultured on plastic petri dishes for 5-7 days in medium containing
RPMI supplemented with 30% L929 cell conditioned media, 20% fetal calf serum (FBS) and 1%
penicillin-streptomycin. BMDM were harvested from 100 x 15mm petri dishes and plated at
0.5x10⁶ macrophages/well in 24 well tissue culture plates. The murine macrophage cell line Raw
264.7 cells (ATCC, Manassas, VA) were grown in Dulbecco Modified Eagle Medium (DMEM)
with L-glutamine, supplemented with 10% FBS and 1% penicillin-streptomycin.

Clonal isolates of infectious low passage B. burgdorferi sensu stricto strain N40 (clone D10E9)
were used for all the experiments. B. burgdorferi was cultured in Barbour-Stoenner-Kelly
(BSK-H) medium (Sigma, St. Louis, MO) at 35°C as previously described (2, 22).

Phagocytosis assay

In a 24-well plate, coverslips were coated with 1% rat collagen in 60% ethanol solution and dried
overnight. Fully differentiated bone marrow-derived macrophages (BMDM) were plated in
RPMI supplemented with 30% L-cell conditioned media, 20% fetal calf serum (FBS) and 1%
penicillin-streptomycin. Cells were maintained in this media for 24 hours and then placed into
serum-free RPMI for 12-16 hours prior to use in assays. *B. burgdorferi* were added to the cultures at a multiplicity of infection (MOI) of 10. Plates were centrifuged at 1200 rpm at 4°C for 5 min to bring *B. burgdorferi* in contact with the cells. The plates were then moved to room temperature (time zero). For phagocytosis to occur, the plates were moved to 37°C for 5, 20, and 60 minutes. Coverslips were removed at various timepoints after the addition of *B. burgdorferi* and washed with cold PBS three times to remove unbound *B. burgdorferi*. Cells were fixed in 3.7% paraformaldehyde with 5% sucrose in PBS for 20 min at 25°C. Coverslips were washed three times in phosphate buffered saline (PBS) and stored at 4°C until use.

For inhibitor treatments, inhibitors were added to the cells 1 h prior to addition of *B. burgdorferi*. SB203580 (p38 MAPK inhibitor) were purchased from Calbiochem (San Diego, CA). The activity of p38 MAPK inhibitor at the concentrations used was confirmed by measuring the effects on mRNA expression of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) from lipopolysaccharide (LPS)-stimulated BMDM by qRT-PCR.

For experiments inhibiting phagocytosis, cytochalasin D was purchased from Tocris bioscience (Ellisville, Missouri). The concentration of cytochalasin D was determined based on the previous studies (24, 33). Cells were treated with 1µM cytochalasin D 1 hour before stimulation with *B. burgdorferi*, and the phagocytosis assay was performed as above. Cytochalasin D treatment had no cytotoxic effect on BMDM or Raw 264.7 cells, as determined by trypan blue exclusion assay.
**Immunofluorescence (IF) microscopy**

For immunofluorescence studies, the coverslips were incubated three times for 5 min in blocking buffer (PBS containing 2% goat serum) at room temperature. All antibody incubations were continued for 1 h at 37°C in a humidified incubator. After blocking, the coverslips were incubated for 1 h at 37°C with an anti-*B. burgdorferi* polyclonal rabbit antibody (a generous gift of Dr. Jenifer Coburn) diluted 1:10,000 in blocking buffer. Coverslips were then washed three times with blocking buffer and incubated with a Cascade blue-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) diluted in 1:500 in blocking buffer. Samples were again washed three times in phosphate-buffered saline (PBS) for 5 min and then permeabilized with chilled methanol for 10 sec. After incubating three times for 5 min in blocking buffer, the coverslips were again incubated with the anti-*B. burgdorferi* rabbit antibody diluted 1:10,000 in blocking buffer. Following three 5-min washes with blocking buffer, the coverslips were incubated with an anti-lysosomal associated membrane protein (LAMP-1) rat monoclonal antibody (IB4, from the Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Md., and the Department of Biology, University of Iowa, Iowa City) diluted 1:100 in blocking buffer. After washing three times for 5 min in blocking buffer, samples were incubated simultaneously with a Texas Red conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) and a fluorescein isothiocyanate-conjugated goat anti-rat IgG antibody (Zymed Laboratories, South San Francisco, CA). After washing, coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.) and examined by differential interference contrast (DIC) and fluorescence microscopy by using a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany). Images were captured with a digital CCD.
camera (Hamamatsu, Hamamatsu-City, Japan). Analysis of co-localization of the fluorescent labels was performed using OpenLab software (Improvision Inc., Lexington, MA).

For quantitative analyses, the percentage of cells with one or more internalized *B. burgdorferi* particles were counted by examining sequential fields from minimum three independent experiments (minimum 3 sets of 100-200 cells/set). Cells containing any internalized *B. burgdorferi* particles or cells containing internalized/intact *B. burgdorferi* were counted and expressed as a percent of the total number cells examined. The mean percent of minimum three independent experiments were plotted over time and the statistical significance between groups was analyzed using the nonparametric Mann-Whitney U test.

**Quantitative *B. burgdorferi* growth assay**

For quantitative analysis of *B. burgdorferi* survival in the BMDM from wild-type littermates and *MyD88*−/− mice, *B. burgdorferi* were incubated with BMDM as described for the phagocytosis assays. After 1 hour post *B. burgdorferi* infection, cells were washed with cold PBS three times to remove unbound/uninternalized *B. burgdorferi*. 10⁶ cells were resuspended in BSK liquid media and serially-diluted 1:10 of columns in 96 well plates. The plates were incubated at 37°C and monitored for color change that would indicate the growth of bacteria. Samples from the wells were checked with color change or every three days (which ever came first). The well with the highest dilution that was positive for *B. burgdorferi* growth was assumed to have a single bacteria at the time of initial dilution and the number of *B. burgdorferi* per 10⁶ cells was calculated by multiplying the dilution required to reach a well with no *B. burgdorferi*. 
**Transient transfection of MyD88 DN plasmid**

Raw 264.7 cells were transiently transfected with a dominant-negative (DN) mutant of MyD88 (15) or pCDNA3-GFP plasmid (Invitrogen, Carlsbad, CA), using a 4:1 lipid/DNA ratio of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol (21). The transfection mix was added to cells in DMEM serum-free media and incubated at 37°C. After 6 hours, the media was replaced with 10% FBS added DMEM, and 24 hours later, we performed phagocytosis assay as described. To briefly describe how we estimated transfection efficiency of Raw 264.7 cells, we randomly chose 10 fields from each well of 24 well plate and counted both total cells and cells expressing GFP after transient transfection of cells with pCDNA3-GFP plasmid. We then divided the number of GFP expressing cells by the total number of cells for all 10 fields. Estimated transfection efficiency for all experiments was approximately 70-80%.

**Quantitative reverse transcriptase PCR (qRT-PCR)**

RNA from BMDM or Raw 264.7 cells was extracted using Trizol as per the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Total RNA was DNase (Ambion, Austin, TX) treated, following the manufacturer’s protocol. First-strand synthesis of cDNA from total RNA was performed using Improm II reverse transcriptase (Promega, Madison, WI) as per the manufacturer’s instructions. Quantification of cDNA was performed by quantitative PCR (iCycler, Biorad) using Sybrgreen dye (Quantitect SYBR green PCR mix; Qiagen, Valencia, CA). Cycling parameters were 55°C for 5 min and 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 1min. The primers used were as previously described (5). The specificity of each reaction was checked by melt curve analysis and electrophoresis of PCR.
products on agarose gels. Expression of target genes was normalized to that of β-actin. Calculations of expression were normalized using the \( \Delta\Delta C_t \) method where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by \( 2^{-\Delta\Delta C_t} \), where \( C_t \) is the cycle number of the detection threshold.

Small interfering RNA (siRNA) transfection

Raw 264.7 cells were transiently transfected with a 100nM control siRNA or custom-designed TLR5 siRNA (Sigma, St. Louis, MO) using a 2:1 lipid/siRNA ratio of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol (21). The transfection mix was added to the cells in DMEM serum free media. At least two different working siRNA constructs for each target were identified by their ability to knock-down targeted gene expression compared to control siRNA to ensure that the effects were due to siRNA targeting and not non-specific effects. 24 hours later, cells were infected with \( B. burgdorferi \) and RNA was harvested after 24 hours post infection. Quantitative RT-PCR was performed to determine knockdown effect of TLR5 and expression of TLR5 was normalized to that of β-actin. Transfection of siRNA had no cytotoxic effect on Raw 264.7 cells, as determined by trypan blue exclusion assay. Primers for detecting TLR5 mRNA expression were as followed. F: ATGGCATGTCAACTTGACTT, R: GATCCTAAGATTGGGCAGGT.

Statistical analysis

Experiments were repeated three to six times as indicated. The statistical significance between groups was analyzed using the nonparametric Mann-Whitney U test. Differences were considered statistically significant when the \( p \) values were equal to or less than 0.05.
RESULTS

**MyD88 is important for uptake of B. burgdorferi by primary bone marrow derived macrophages**

It has previously been shown that *B. burgdorferi* are efficiently phagocytosed by macrophages (28, 30-32). In order to determine whether MyD88 plays a role in the phagocytosis of *B. burgdorferi*, we used immunofluorescence imaging to examine the phagocytosis of *B. burgdorferi* by BMDM from MyD88^{−/−} mice and their wild type littermates. Using different fluorescent markers to label *B. burgdorferi* before and after cell permeabilization allowed us to distinguish between internalized and extracellular *B. burgdorferi*. At 5 min, most *B. burgdorferi* have not been phagocytosed by either wild-type or MyD88^{−/−} BMDM (Fig. 1A). However, by 20 min, a clear separation occurs between phagocytosis in wild-type and MyD88^{−/−} mice. The majority of organisms incubated with the wild-type BMDM have been phagocytosed and degraded into small particles by 20 min and almost no intact spirochetes can be seen by 60 min. In contrast, the majority of *B. burgdorferi* are still intact in MyD88^{−/−} BMDM at 20 min and even at 60 min. Almost all of these spirochetes are visualized with incubation with the *B. burgdorferi* antibody prior to permeabilization, suggesting a defect in the uptake into BMDM, rather than a defect in degradation in the phago-lysosome. At 60 minutes, 80.5±18.7 % of wild-type BMDM contain internalized spirochetes while only 25.7±12.9% of MyD88^{−/−} BMDM contain any spirochetes (*p*≤0.05) (Fig. 1B). Intact spirochetes were only rarely seen within either wild-type or MyD88^{−/−} BMDM (Fig. 1C), suggesting that the difference in phagocytosis was due to uptake of the organism and not degradation of the organism after uptake.
To ensure that the defect in uptake of *B. burgdorferi* by MyD88−/− BMDM was not due to a global defect in uptake, we compared uptake of *E. coli* in MyD88−/− and wild-type BMDM. Anti-OmpA antibody was used to detect *E. coli* (25). *E. coli* were very rapidly ingested by BMDM and no defects or delays were seen in uptake of *E. coli* at 5, 20 and 60 min by MyD88−/− BMDM (data not shown). The rapid uptake of *E. coli* by the MyD88−/− cells suggests that the defect in uptake of *B. burgdorferi* is not due to an intrinsic inability of the cells to take up other organisms.

In order to further confirm immunofluorescence data, we performed quantitative cultures for *B. burgdorferi* after 60 min incubations with wild-type and MyD88−/− BMDM. Unbound *B. burgdorferi* were washed away prior to culture, so this assay measures the numbers of live spirochetes that are bound to the surface of the cells or are internalized. We recovered 2 logs higher numbers of spirochetes from cultures where *B. burgdorferi* were incubated with MyD88−/− BMDM, compared with wild type BMDM (10,000 organisms/10⁶ cells vs 100 organisms/10⁶ cells; Fig.1D).

*Transient transfection of MyD88 dominant negative (DN) plasmid in Raw 264.7 cells reduces the uptake of B. burgdorferi.*

To confirm the effects of MyD88 deficiency on the phagocytosis of *B. burgdorferi* and eliminate the possibility of unrecognized or unintended effects related to the knockout, we wished to test the effects of blocking MyD88 signaling through a different mechanism. As one issue that has been previously raised in studies of phagocytosis of other organisms with MyD88-deficient cells has been the effect of the absence of TLR signaling during cellular maturation (52), we chose to transf ect mature mouse macrophage cells (Raw 264.7) with a MyD88 DN plasmid. This has the
advantage that the cells have matured normally in the presence of TLR signaling and that MyD88 is not eliminated by the presence of the dominant negative. Only the effects of MyD88 engagement through signaling of the TLR ligands are reduced due to competitive binding to the dominant negative protein.

We transiently transfected a MyD88 DN plasmid or an empty vector control into a mouse macrophage cell line, Raw 264.7. The effect of abrogating MyD88 function in these cells was confirmed by impaired TNF-α and IL-6 mRNA production in response to B. burgdorferi (data not shown). Cells transiently transfected with an empty vector control behaved similarly to untransfected Raw 264.7 cells; B. burgdorferi were ingested by the cells and, by 60 min, were found completely degraded and associated with the phagolysosomes. However, transfection with dominant negative MyD88 resulted in a reduction in phagocytosis that was similar to that seen in the MyD88−/− BMDM (77.1±9.5% control transfected cells vs. 29.6±25.7% MyD88 DN transfected cells (p<0.05) Fig. 2). These results confirm the results with our MyD88−/− BMDM and strongly suggest that MyD88-mediated phagocytosis could be due to a requirement for engagement of upstream receptors that signal through MyD88.

**TLR2, TLR5 and TLR9 are not required for the uptake of B. burgdorferi.**

TLR2 has been shown to recognize borrelial lipoproteins (21) and studies of TLR2 or MyD88 deficient mice indicated that both are important in control of B. burgdorferi infection, as bacterial burdens were greatly increased in the absence of either TLR2 or MyD88 (5, 9, 28, 49, 51). Therefore, we sought to examine whether TLR2 is also important for uptake of B. burgdorferi. We performed phagocytosis assays using BMDM from TLR2−/− mice as before.
Again, wild type control BMDM took up and degraded *B. burgdorferi* within phago-lysosomes of macrophages by 20 min with almost no *B. burgdorferi* seen extracellularly in association with cells. In marked distinction to MyD88\(^{-/-}\) BMDM, the absence of TLR2 did not affect phagocytosis of *B. burgdorferi* and at 20 min and 60min, almost all the organisms were degraded with the same percentage of cells containing degraded *B. burgdorferi* as control BMDM (82.2±23.2% wild type control BMDM vs. 88.0±13.4% TLR2\(^{-/-}\) BMDM at 60min post infection; Fig. 3A).

Very little is known about the interaction of TLR5 and TLR9 with *B. burgdorferi* products. TLR5 recognizes protein flagellin that is required for bacterial motility (16) and TLR9 recognizes CpG motifs in bacterial DNA (3, 19). *B. burgdorferi* does contain flagellin, which could be recognized by TLR5, although this has not been previously documented. TLR5 has not been previously associated with phagocytosis of any organism. To determine whether the effects of MyD88 on phagocytosis of *B. burgdorferi* by macrophages may be mediated by TLR5, we transfected Raw 264.7 cells with different siRNAs directed against TLR5. Unfortunately, we were unable to find an antibody suitable for determining knockdown of TLR5 at the protein level; however, we identified 2 separate siRNA constructs that were able to reduce mRNA expression of TLR5 by 60% after 24-48 hour post-transfection, confirmed by qRT-PCR (data not shown). In addition, these effects were specific for TLR5 ligands as transfection with TLR5 siRNA reduced the induction of TNF-\(\alpha\) mRNA in response to *S. typhimurium* flagellin and did not affect cellular responses to CpG DNA (data not shown). Phagocytosis assays were performed as shown in figure 1. No defect in the phagocytosis of *B. burgdorferi* was seen with transfection of TLR5 siRNA compared with control siRNA (57.0±19.5% control siRNA-
transfected macrophages vs. 68.8±27.8% TLR5 siRNA-transfected macrophages contained *B. burgdorferi* particles at 60 min post infection (Fig. 3B). This suggests either that TLR5 is not involved in phagocytosis or that small amounts of TLR5 are sufficient to result in efficient phagocytosis.

TLR9 is an intracellularly located receptor that would not be predicted to have an effect on phagocytosis. However, we confirmed this utilizing TLR9−/− BMDM. As expected, no effects on phagocytosis were seen in the TLR9−/− BMDM (81.4±7.1% of wild type control BMDM vs. 67.1±20.3% of TLR9−/− BMDM containing degraded *B. burgdorferi* particles at 60 min post infection; Fig. 3C).

*p38 MAPK does not play a role in phagocytosis of B. burgdorferi.*

Signaling through TLRs can activate many different signaling pathways. The majority of studies of TLR involvement in phagocytosis have focused on the role of p38 MAPK (8, 12, 26). In order to determine the effect of p38 MAPK in the phagocytosis of *B. burgdorferi*, we performed phagocytosis assays in the presence of a p38 MAPK inhibitor. BMDM from wild-type mice were pre-incubated with 10µM p38 MAPK inhibitor SB203580 for 1 hour prior to the addition of *B. burgdorferi*. Doses of the inhibitor was chosen based on previously published studies (8, 17) and the activity of the inhibitor at these doses for suppression of downstream gene activation were confirmed by qRT-PCR in separate experiments (data not shown).

In the vehicle (DMSO) treated controls, *B. burgdorferi* were found to be degraded and associated with phagolysosomes of wild-type BMDM by 60 min with almost no *B. burgdorferi* seen
extracellularly in association with cells. Although it affected cytokine induction, the p38 MAPK inhibitor, SB203580, did not affect phagocytosis of *B. burgdorferi* and by 60 min, almost all the organisms were degraded and the same percentage of cells contained degraded *B. burgdorferi* as vehicle treated (84.54±26.8% vehicle treated cells vs. 84.19±8.0% SB203580 treated cells at 60 min post infection; Fig. 4).

**Role of MyD88, TLR2, TLR5 and TLR9 in inflammatory signaling**

Given the important role of TLRs and MyD88 in inflammatory signaling, we next wished to examine the impact of loss of these receptors/adaptors on release of cytokines and chemokines in *B. burgdorferi* infected BMDM. BMDM from either MyD88−/− or TLR2−/− mice were stimulated with whole *B. burgdorferi* to analyze the contribution of these molecules to the cellular response in terms of cytokine and chemokine production. Cells were stimulated with *B. burgdorferi* and 24 hours later, RNA was harvested. The transcriptional levels of cytokines and chemokines were examined by qRT-PCR.

As has been well established in previous studies, the addition of *B. burgdorferi* to BMDM results in induction of cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1) and chemokine (C-X-C motif) ligand 2 (CXCL-2) (4-6, 9, 21, 53). However, MyD88−/− BMDM showed little to no induction of these cytokines and chemokines following *B. burgdorferi* infection (Fig. 5A).

In contrast, the absence of TLR2 only partially affected induction of cytokines and chemokines by *B. burgdorferi*. The effects did show some variation depending upon the specific cytokine or
chemokine. Compared to wild type control, mRNA transcript expression of TNF-α, IL-6, IL-1β, MCP-1, and CXCL-2 from TLR2<sup>−/−</sup> BMDM was decreased by 41.7%, 61.9%, 88.9%, 37.9% and 73.5% (p < 0.05 for all) (Fig. 5B).

Although TLR5 did not appear to have a role in phagocytosis of <i>B. burgdorferi</i>, it does not preclude a role in recognition of <i>B. burgdorferi</i> products and induction of inflammatory signaling. Recognition of <i>B. burgdorferi</i> products by TLR5 has not previously been reported. We performed siRNA knockdown of TLR5 and stimulated cells with live <i>B. burgdorferi</i>. Raw 264.7 cells were transfected with either control or TLR5 siRNAs and stimulated with <i>B. burgdorferi</i>. Cells were harvested 24 h post-infection, and total RNA was analyzed by qRT-PCR. While a non-targeting small interfering RNA (siRNA control) had no effect on TLR5 mRNA levels, transfection with siRNA targeted at TLR5 decreased TLR5 mRNA levels by 60% after 24-48 hours transfection (data not shown). We found that 60% reduction of TLR5 mRNA by siRNA resulted in 43%, 57%, and 65% reductions (p<0.05 for all) in the expression of TNF-α, IL-6, and IL-1β in response to <i>B. burgdorferi</i> stimulation (Fig. 6A). However, knockdown of TLR5 expression did not have significant effects on the transcription of chemokines such as MCP-1 and CXCL-2.

The role of TLR9 signaling in the recognition of <i>B. burgdorferi</i> has also not previously been reported. <i>B. burgdorferi</i> is known to release DNA in the culture and could provide a ligand for TLR9. Phagocytosis of <i>B. burgdorferi</i> may result in the release of CpG DNA that is sampled by TLR9 inside the cell. We stimulated wild-type and TLR9<sup>−/−</sup> BMDM with <i>B. burgdorferi</i> and examined induction of mRNA for different cytokines by q-RT-PCR. However, TLR9 deficiency...
had no effect on cytokine secretion induced by *B. burgdorferi*. Stimulation with either purified genomic DNA or whole *B. burgdorferi* resulted in the production of similar levels of TNF-α by BMDM from wild-type and TLR9\(^{-/-}\) mice (Fig. 6B). Similar results were shown in the production of IL-6 and IL-1β (data not shown). Thus, TLR5 but not TLR9, is important for induction of inflammatory responses to *B. burgdorferi* that we observed.

**TLR2 samples *B. burgdorferi* lipoproteins both extracellular and intracellularly, whereas TLR5 recognizes predominantly extracellular *B. burgdorferi* products.**

Phagocytosis of *B. burgdorferi* has been reported to be required for induction of inflammatory signaling and production of cytokines (33). To determine whether the different effects of *B. burgdorferi* on TLR2\(^{-/-}\) and MyD88\(^{-/-}\) BMDM is due to the difference in phagocytosis and internalization between the cells, we examined the effect of a phagocytosis inhibitor, cytochalasin D, on signaling in wild type and TLR2\(^{+/+}\) BMDM. The cytochalasins have been used extensively to inhibit phagocytosis of many different types of bacteria (10, 33, 52). First, we confirmed that cytochalasin D blocked the majority (>95%) of phagocytosis of *B. burgdorferi* by immunofluorescence imaging (data not shown).

To confirm that blocking phagocytosis of *B. burgdorferi* by cytochalasin D treatment leads to decreased levels of cytokines and chemokines, we examined expression of TNF-α, IL-6, IL-1β, MCP-1, and CXCL-2 in cytochalasin D-treated wild type BMDM. Cells were harvested 24 hours post infection and cytokine and chemokine expression was examined by qRT-PCR. The presence of cytochalasin D reduced expression of TNF-α, IL-6, IL-1β, MCP-1, and CXCL-2 mRNA by 74%, 68%, 77%, 81%, and 72% respectively (\(p \leq 0.05\) for all compared with sham treated...
controls, Fig. 7A). Of note, this level of reduction is less than was seen with the MyD88<sup>−/−</sup> BMDM.

TLR2 is usually located on cell surface but upon microbial recognition, it can be recruited to phagosomes (48). In order to determine whether activation of TLR2 following *B. burgdorferi* infection occurs in phagosomes or through recognition of borrelial products in the extracellular space, we incubated TLR2<sup>−/−</sup> BMDM with cytochalasin D for 1 hour before stimulating cells with *B. burgdorferi*. The combination of cytochalasin D to TLR2<sup>−/−</sup> cells would be expected to abrogate all signaling requiring TLR2 as well as any non-TLR2 mediated signaling requiring phagocytosis. The addition of cytochalasin D to TLR2<sup>−/−</sup> BMDM resulted in additional reduction of induction of inflammatory cytokines beyond that seen with either cytochalasin D or TLR2<sup>−/−</sup> BMDM alone (Fig. 7A). The decrease in induction of inflammatory cytokines approached that seen with MyD88<sup>−/−</sup> BMDM (which would lack both phagocytosis and TLR signaling) for many molecules. This suggests that: 1) Based on the increased reduction of induction of inflammatory mediators with the addition of cytochalasin D to TLR2<sup>−/−</sup> BMDM, that there are TLR2 independent pathways that are activated after phagocytosis of the organism; and 2) Based on increased reduction of cytokines and chemokines in TLR2<sup>−/−</sup> cells compared with wild-type BMDM treated with cytochalasin D, that TLR2 signaling can be activated both through recognition of external *B. burgdorferi* and through recognition of products after phagocytosis. The relative contribution of recognition of external products versus phagosome sampling by TLR2 varied depending upon the cytokine involved. A rough estimation of the contribution of recognition of extracellular *B. burgdorferi* products by TLR2 to total induction for a specific cytokine ranged from 22-37% (formula= % wild-type BMDM treated with cytochalasin D – %
TLR2\(^{+/−}\) BMDM treated with cytochalasin D). The contribution of recognition of intracellular *B. burgdorferi* products by TLR2 was estimated to range from 6-56% of the total induction of a specific cytokine (formula = \[100 − \% TLR2\(^{−/−}\) BMDM\] – \% TLR2 external signaling).

We also studied the effects of addition of cytochalasin D to cells treated with TLR5 siRNA. In contrast to our findings for TLR2, the addition of cytochalasin D showed no significant effects on TLR5 signaling, suggesting that the majority of TLR5 signaling is through recognition of extracellular *B. burgdorferi* products by surface bound receptor (Fig. 7B).

DISCUSSION

Phagocytosis involves a series of coordinated events beginning with recognition of a foreign pathogen and proceeding through activation of receptors and complex signaling networks, leading to internalization, killing, and processing of the pathogen (47). In this study, we provide evidence that MyD88 plays an important role in the internalization of *B. burgdorferi* into mouse macrophages. BMDM from MyD88\(^{−/−}\) mice were unable to efficiently ingest *B. burgdorferi*. In addition, reduced phagocytosis in a MyD88 DN plasmid-transfected macrophages indicates that even short-term inhibition of MyD88 signaling decreases phagocytosis.

Previous studies have suggested that although there may be some deficiency in the ability of MyD88\(^{+/−}\) BMDM to ingest certain bacteria, the primary defect in their ability to kill bacteria secondary to deficiencies in phagosome maturation or oxidative killing (8, 26). Of note, we did not observe any defect in uptake of *E. coli* by BMDM from MyD88\(^{−/−}\) mice in our hands. It is possible that *E. coli* may be recognized by other receptors that are important in activating
phagocytosis and replacing the signals that are lost with the deletion of MyD88. For example, *E. coli* LPS is recognized by TLR4 (35). TLR4 can signal both through MyD88-dependent and -independent pathways. There is an overlap in the downstream signaling pathway activated by both MyD88-dependent and -independent pathways so that recognition of *E. coli* LPS could bypass MyD88 and still provide the required downstream signal for phagocytosis.

For *B. burgdorferi*, the defect in phagocytosis appears to be primarily in internalization of the organism. We did not see elongated, intact *B. burgdorferi* within BMDM from MyD88−/− mice at 60 min suggesting that once spirochetes were taken up by the macrophages, degradation occurred normally. Interestingly, loss of p38 signaling, which is important for phagosome maturation and is thought to be the defect in the lack of killing of other organisms in MyD88−/− macrophages, did not affect phagocytosis of *B. burgdorferi*—although it did reduce inflammatory signaling. Previous reports have suggested that phagocytosis of *B. burgdorferi* may occur in part through coiling phagocytosis and extra-phagosomal killing (37), which would not require p38 signaling, however, our experiments do not allow us to distinguish this.

Our results differ significantly from previous reports from Liu *et al* that suggested normal uptake of *B. burgdorferi*, but impaired killing by macrophages (28). There are a number of differences in the methodologies that may account for different results, including differences in the source and background of the mice, the use of peritoneal macrophages versus BMDM, and differences in the strain of *B. burgdorferi*. Most significantly, their experiments relied on H$_2$O washes of cells incubated with *B. burgdorferi* to osmotically lyse non-internalized organisms without further confirmation of whether all externally bound bacteria were lysed. In our hands, while
H₂O washing did lyse the majority of extracellular bacteria, our staining revealed the continued presence of intact bacteria bound externally to the cells. We hardly saw intact *B. burgdorferi* internalized within cells. It is also possible that the osmotic shock may causes changes to cellular membranes or function that could affect phagocytosis.

Although MyD88 seems to be required for efficient internalization of *B. burgdorferi*, TLR2, TLR5 and TLR9 do not appear to play any role in the phagocytosis of *B. burgdorferi*. This is particularly surprising for TLR2, which is the primary host receptor for *B. burgdorferi* lipoproteins. It is certainly possible that the phagocytic effects involving MyD88 are mediated through an as yet unrecognized TLR, although none of the other TLRs have been confirmed to recognize a *B. burgdorferi* product. Another possibility is that the effects of MyD88 on phagocytosis are due to activation through non-TLR pathways, such as IL-1 or IL-18, which can also utilize MyD88 as an adaptor molecule (1, 29).

There have been several potential mechanisms proposed by which MyD88 may affect internalization of organisms. One possible mechanism is that it may affect the expression of scavenger receptors on the surface of macrophages that are responsible for binding and internalizing bacteria (46). Some scavenger receptors have been shown to be up-regulated by TLR signaling and participate in TLR-mediated phagocytosis of other organisms (12). Another possible mechanism is that MyD88 signaling may be important in regulation of actin polymerization and membrane extension for internalization of *B. burgdorferi*.

Phosphatidylinositol-3 kinase (PI3K) signaling is involved in membrane extension and fusion behind bound particles, and may play a role in insertion of new membrane at the site of particle
internalization (47). We have found that *B. burgdorferi*-induced activation of PI3K is dependent upon MyD88 signaling (unpublished data), but it is unclear whether this is directly responsible for the defects in phagocytosis. Third, it is possible that MyD88-mediated phagocytosis may be through regulation of lysosomal recruitment, which is necessary for opening the membrane for the organisms to enter. This mechanism has been proposed as the entry mechanism for other organisms, such as *T. cruzi* (45).

Processing of microbial invaders by phagocytes plays an important role in inflammation and host defense for many different organisms (14, 24, 33). However, for *B. burgdorferi*, it has long been assumed that activation of TLR2 occurs through the recognition of *B. burgdorferi* lipoproteins by membrane-bound TLR2 prior to phagocytic processing. Recent studies have cast doubt upon the importance of recognition of *B. burgdorferi* lipoproteins in the extracellular space. Behera *et al* have shown that antibodies to TLR2 are unable to block induction of inflammatory signaling by live *B. burgdorferi*—although they efficiently block cytokine induction by purified *B. burgdorferi* lipoproteins (6). Moore *et al.* have shown that treatment of human monocytes with cytochalasin D results in a reduction of TNF-α and IFN-γ induction in response to live *B. burgdorferi* (33). Our results confirm the importance of phagocytosis and bacterial processing for induction of inflammatory cytokines, but also suggest that there is still a more minor component of recognition of *B. burgdorferi* products by membrane-associated TLR2. The lack of an effect on phagocytosis by the loss of TLR2 compared with MyD88 has allowed us to distinguish the contributions of phagocytosis and external TLR2 signaling to overall release of inflammatory products. It appears that activation of TLR2 occurs through both binding of
borrelial lipoproteins to TLR on the surface of cells, and sampling of lysosomes containing
degraded bacterial products.

The role of TLR5 in response to *B. burgdorferi* has not been previously reported. Our data
support that TLR5 signaling may be important for induction of cytokines in response to *B.
burgdorferi*. Interestingly, this effect was restricted to specific cytokines and the transcription of
chemokines, such as MCP-1 and CXCL-2, were not affected by the loss of TLR5 signaling.
Although our data suggest that both TLR2 and TLR5 signaling contribute to the inflammatory
response to *B. burgdorferi*, it remains possible (likely) that other receptors may contribute to the
inflammatory response. Of note, our TLR5 studies utilized Raw 264.7 cells rather than primary
BMDM since primary murine BMDM express minimal to no TLR5; thus the relative
contribution of TLR5 signaling is also likely to be cell-type specific. An interesting aspect to our
data is that *B. burgdorferi* flagellin is encased within the outer surface membrane and would not
be predicted to interact directly with TLR5. It is possible that some flagellin is released from the
within the bacteria through bacterial death or blebbing of the membrane and is accessible to
TLR5. However, we cannot rule out the possibility that a *B. burgdorferi* product other than
flagellin is being recognized by TLR5.

In summary, we have found that MyD88, but not TLR2, TLR5 or TLR9, plays an important role
in phagocytosis of *B. burgdorferi*. The contribution of MyD88 to inflammatory signaling in *B.
burgdorferi* infection is in large part due to its role in phagocytosis, but also in its transduction of
TLR signals. TLR2 recognizes both extracellular and internalized products of *B. burgdorferi,*
both of which contribute to overall inflammatory signaling. TLR5, which has not been shown to
be activated by *B. burgdorferi*, appears to respond primarily to extracellular products. A better understanding of complex mechanisms involved in the phagocytic responses to specific organisms and resulting effects on the inflammasome may lead to new insights into the contributions of specific pathways to inflammation and mechanisms by which the host response can differentiate between microbial invaders.

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FIGURE LEGENDS

Figure 1. MyD88^{-/} BMDM show deficient uptake of B. burgdorferi.

(A) BMDM from wild-type littermate and MyD88^{-/} mice were isolated. B. burgdorferi were added to BMDM at a MOI of 10. Cells were incubated with B. burgdorferi at 5, 20, or 60 min. Cells were fixed at each timepoint and then probed with various antibodies. DIC: differential interference contrast, LAMP-1: anti-LAMP-1 (lysosomal marker) antibody (Green); Bb: rabbit anti-B. burgdorferi antibody prior to permeabilization of the cells to identify extracellular organisms (Blue); Bb(permeabilized): rabbit anti-B. burgdorferi antibody after permeabilization of the cells to identify both internalized and extracellular B. burgdorferi epitopes (Red). Merge shows a merged image of LAMP-1, Bb, and Bb (permeabilized). Experiments were repeated five times with cells from 2 different mice per experiment and representative images are shown. Scale bar, 10µM.

(B) Cells containing internalized B. burgdorferi particles were counted and expressed as a percent of the total number cells examined. (C) Cells containing spirochetes that appeared intact and not degraded (i.e. maintained their spirochetal architecture) were counted and expressed as a percent of total cells. Each experiment was repeated five times using cells from two different mice each time. Error bars represent standard deviations. *=p<0.05, compared with wild type cells at matched post infection timepoints.

(D) For quantitative analysis of B. burgdorferi survival in the BMDM from wild-type littermates and MyD88^{-/} mice, B. burgdorferi were incubated with macrophages as before. After 1 hour of incubation, cells were washed three times with cold PBS to eliminate unbound B. burgdorferi. Cells (10^6) were resuspended in BSK liquid media and serial-diluted in 96 well plates. Wells were monitored for growth of B. burgdorferi. Experiments were performed in duplicate and
repeated three times with cells from 2 different mice per experiment. Each bar represents one independent experiment.

Figure 2. Transient transfection of a MyD88 DN plasmid in Raw 264.7 cells reduces the uptake of *B. burgdorferi.*

Raw 264.7 cells were transiently transfected with pCDNA3-GFP control or MyD88 DN plasmid. After 24 hours, cells were incubated with *B. burgdorferi* for 60 min. Fixed cells were incubated with different fluorescently labeled antibodies as described in Fig. 1 and then visualized by fluorescent microscopy. Cells containing internalized *B. burgdorferi* particles at 1 hour post infection were counted and expressed as a percent of the total number cells examined. Data are representative of three independent experiments. Error bars represent standard deviations. * = p < 0.05, compared with control-transfected cells.

Figure 3. TLR2, TLR5 and TLR9 do not participate in the phagocytosis of *B. burgdorferi.*

Wild type control or TLR2−/− BMDM cells (A) were plated in 24 well plates and infected with *B. burgdorferi* for 5 min, 20 min, and 60 min. Raw 264.7 cells were transfected with either control or TLR5 siRNAs (B) and after 24 hours, cells were infected with *B. burgdorferi* for 5 min, 20 min, and 60 min. Wild type control or TLR9−/− BMDM cells (C) were plated in 24 well plates and infected with *B. burgdorferi* for 5 min, 20 min, and 60 min. The phagocytosis assay and staining was performed as described in Fig 1. Cells containing internalized *B. burgdorferi* particles were counted and expressed as a percent of the total number cells examined. Data are representative of three to four independent experiments. Error bars represent standard deviations.
Figure 4. p38 MAPK is not involved in the phagocytosis of *B. burgdorferi*.

Wild-type BMDM were pre-incubated with either a vehicle control or 10µM p38 MAPK inhibitor SB203580 for 1 hour prior to the addition of *B. burgdorferi* (MOI 10). The phagocytosis assay and immunofluorescence staining was performed as described in Fig 1. Cells containing internalized *B. burgdorferi* particles at 1 hour post *B. burgdorferi* infection were counted and expressed as a percent of the total number cells examined. Data are representative of three independent experiments. Error bars represent standard deviations.

Figure 5. Both MyD88 and TLR2 regulate *B. burgdorferi*-induced expression of inflammatory molecules from BMDM.

Wild type, MyD88−/−, and TLR2−/− BMDM were infected with *B. burgdorferi* at a MOI 10 for 24 hours, and transcriptional expression of TNF-α, IL-6, IL-1β, MCP-1 and CXCL-2 was measured by qRT-PCR, as described in *Materials and Methods*. MyD88−/− and littermate wild-type control macrophages are shown in (A), and TLR2−/− and wild type control BMDM are shown in (B). Expression of target genes was normalized to that of β-actin. The results shown are from three independent experiments performed in duplicate. Expression from wild type cells infected with *B. burgdorferi* was arbitrarily set to 1 for all the experiments and other values shown relative to that expression level. Error bars represent standard deviations. *=p<0.05, compared to wild type cells infected with *B. burgdorferi*.

Figure 6. TLR5, but not TLR9, is involved in inflammatory signaling in response to *B. burgdorferi*. 
(A) Raw 264.7 cells were transfected with either control or TLR5-specific siRNAs. 24 hours later, cells were stimulated with either media or *B. burgdorferi* for 24 hours. Transcriptional expression of TNF-α, IL-6, IL-1β, MCP-1 and CXCL-2 was measured by qRT-PCR, as described in *materials and methods*. Expression with cells transfected with control siRNA and infected with *B. burgdorferi* was arbitrarily set to 1 for all the experiments and other values shown relative to that expression level. (B) Wild type and TLR9⁻/⁻ BMDM were collected and plated in 6 well plates. BMDM were stimulated with either 200nM CpG DNA or 1µg purified *B. burgdorferi* DNA or whole *B. burgdorferi* for 4 hours and cells were harvested for RNA isolation. Transcriptional expression of TNF-α is shown. The expression of cytokines in wild-type BMDM infected with *B. burgdorferi* was arbitrarily set to 1. The real-time PCR experiments were performed in duplicate and repeated three times and average of all experiments is shown. Error bars represent standard deviations. *=p<0.05 compared to control siRNA stimulated with *B. burgdorferi*.

**Figure 7. Effects of inhibition of phagocytosis by cytochalasin D on inflammatory signaling via TLR2 and TLR5.**

(A) BMDM from either wild-type control or TLR2⁻/⁻ mice were plated in 6 well plates. Cytochalasin D (1µM) was added to macrophages 1 hour before incubation with *B. burgdorferi* to block uptake of the bacteria into BMDM. BMDM were stimulated with *B. burgdorferi* for 24 hours and cells were harvested for RNA isolation. Transcriptional expression of TNF-α, IL-6, IL-1β, MCP-1, and CXCL-2 was measured by qRT-PCR, as described in *materials and methods*. Expression of target genes was normalized to that of β-actin. Expression with wild type cells infected with *B. burgdorferi* was arbitrarily set to 1 for all the experiments and other values
shown relative to that expression level. Experiments were performed three times in duplicate and
the averages of the experiments are shown. Error bars represent standard deviations. The \( p \)
values for all tested cytokines and chemokines, comparing \( B.\ burgdorferi \)-infected wild type and
TLR2\(^{+/−}\) BMDM were \( \leq 0.05 \); Comparisons between \( B.\ burgdorferi \)-infected wild type cells
treated with or without cytochalasin D and between \( B.\ burgdorferi \)-infected wild type cells and
TLR2\(^{+/−}\) cells treated with cytochalasin D were also all statistically significant with \( p \leq 0.05 \).

(B) Raw 264.7 cells were transfected with either control or TLR5 siRNAs. After 24 hours later,
cytochalasin D (1\( \mu \)M) was added to macrophages 1 hour before incubation with \( B.\ burgdorferi \)
to block uptake of the bacteria into Raw 264.7 cells. Cells were stimulated with \( B.\ burgdorferi \)
for 24 hours and harvested for RNA isolation. Transcriptional expression of TNF-\( \alpha \), IL-6, IL-
1\( \beta \), MCP-1, and CXCL-2 was measured by qRT-PCR, as described in materials and methods.
Expression of target genes was normalized to that of \( \beta \)-actin. Expression with control siRNA
transfected cells infected with \( B.\ burgdorferi \) was arbitrarily set to 1 for all the experiments and
other values shown relative to that expression level. Experiments were performed three times in
duplicate and the averages of the experiments are shown. Error bars represent standard
deviations.
REFERENCES


Figure 1

A. DIC                LAMP-1            Bb          Bb(permeabilized)      Merge

B. % cells containing internalized Bb

C. % cells with internalized, intact Bb

D. Live Bb/10^6 cells

*
Figure 2

% cells with internalized Bb

Control    MyD88 DN

*
Figure 4

% cells with internalized Bb

Control                         SB203580
Figure 6

A

B
Figure 7

A.

B.