Differentiated THP-1 Cells Exposed to Pathogenic and Nonpathogenic *Borrelia* Species Demonstrate Minimal Differences in Production of Four Inflammatory Cytokines

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Abstract

Tick-borne borreliae include Lyme disease and relapsing fever agents, and they are transmitted primarily by ixodid (hard) and argasid (soft) tick vectors, respectively. Tick–host interactions during feeding are complex, with host immune responses influenced by biological differences in tick feeding and individual differences within and between host species. One of the first encounters for spirochetes entering vertebrate host skin is with local antigen-presenting cells, regardless of whether the tick-associated *Borrelia* sp. is pathogenic. In this study, we performed a basic comparison of cytokine responses in THP-1-derived macrophages after exposure to selected borreliae, including a nonpathogen. By using THP-1 cells, differentiated to macrophages, we eliminated variations in host response and reduced the system to an in vitro model to evaluate the extent to which the *Borrelia* spp. influence cytokine production. Differentiated THP-1 cells were exposed to four *Borrelia* spp., *Borrelia hermsii* (DAH), *Borrelia burgdorferi* (B31), *B. burgdorferi* (NC-2), or *Borrelia lonestari* (LS-1), or lipopolysaccharides (LPS) (activated) or media (no treatment) controls. Intracellular and secreted interferon (IFN)- γ , interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α were measured using flow cytometric and Luminex-based assays, respectively, at 6, 24, and 48 h postexposure time points. Using a general linear model ANOVA for each cytokine, treatment (all *Borrelia* spp. and LPS compared to no treatment) had a significant effect on secreted TNF- α only. Time point had a significant effect on intracellular IFN- γ , TNF- α and IL-6. However, we did not see significant differences in selected cytokines among *Borrelia* spp. treatments. Thus, in this model, we were unable to distinguish pathogenic from nonpathogenic borreliae using the limited array of selected cytokines. While unique immune profiles may be detectable in an in vitro model and may reveal predictors for pathogenicity in borreliae of unknown pathogenicity, a larger panel of cytokines would be desirable to test.

Keywords: *Borrelia*, immunology, flow cytometry

Introduction

BORRELIA SPIROCHETES are responsible for diseases including Lyme disease and relapsing fever. Hosts become exposed primarily via the bite of ixodid or argasid tick vectors, when spirochetes are introduced with saliva. Immature and adult ixodid ticks (vectors of Lyme disease spirochetes) may remain attached for days while nymphal and adult argasid ticks (vectors of relapsing fever spirochetes) typically attach and feed for 15–30 min. Thus, host innate and acquired immune responses to tick feeding, and consequently, timing of spirochete exposure to host defense mechanisms, may differ due to tick biology, *Borrelia* species or strain and in-

dividual differences in immune response within and among host species. While the host response is complex and varies, resident antigen-presenting cells—dendritic cell subsets and sentinel macrophages—are likely to be critical in initial spirochetal encounters (Mason et al. 2014). During feeding, tick salivary proteins that have anti-hemostatic and anti-inflammatory properties, and that directly interfere with dendritic cell function, modulate the final immune response (Cavassani et al. 2005, Skallova et al. 2008, Slamova et al. 2011, Lieskovska et al. 2015). Both resident and recruited macrophages are involved in proinflammatory cytokine secretion. For *Borrelia burgdorferi*, the proinflammatory response includes production of tumor necrosis factor (TNF)- α ,

interferon (IFN)- γ , interleukin (IL)-6, IL-8, and IL-1 β (Salazar et al. 2003, Strle et al. 2009, Buffen et al. 2013). In vitro studies with *B. burgdorferi* use various immune cell types to evaluate cytokine production, including peripheral blood mononuclear cells (PBMCs), dendritic cells derived from monocytes in PBMCs, THP-1 (human monocytic) cells treated with 1 α ,25-dihydroxyvitamin D₃ (Vitamin D₃), and THP-1 cells treated with phorbol 12-myristate 13-acetate (PMA) (Giambartolomei et al. 1998, 2002, Suhonen et al. 2003, Dennis et al. 2009, Strle et al. 2009, Buffen et al. 2013). While *B. burgdorferi* cytokine studies are common, cytokine studies using tick-borne relapsing fever spirochetes are limited. In THP-1 cells differentiated with vitamin D₃, exposure to *B. burgdorferi* (B31) induced expression of myeloid differentiation primary response 88 protein (MYD88), and associated increases in TNF- α , IL-6, and IL-8, by 24 h post-exposure (Dennis et al. 2009). In patients with louse-borne relapsing fever (caused by *Borrelia recurrentis*), with a Jarish-Herxheimer reaction (J-HR), antibiotic administration likely drives elevated TNF and IL-6 serum levels (Cuevas et al. 1995). Increases in anti-inflammatory cytokine, IL-10, are considered protective during tick-borne relapsing fever (Cooper et al. 2000, Gelderblom et al. 2007), and administration of rhIL-10 to decrease acute inflammation associated with the J-HR is also associated with an increase in proinflammatory cytokines, TNF- α , IL-6, and IL-8 (Cooper et al. 2000). Here, we investigated production of selected inflammatory cytokines using differentiated THP-1 cells exposed to *Borrelia*, in the absence of tick saliva. We selected four cytokines, TNF- α , IL-6, IL-1 β , and IFN- γ , which have been associated with infection by at least one of the *Borrelia* species. To our knowledge, IFN- γ has not been measured in THP-1 cells, however, it has been detected in human macrophages in response to *Mycobacterium tuberculosis* and after co-stimulation of monocyte-derived human macrophages with IL-12 and IL-18 (Darwich et al. 2009, Robinson et al. 2010).

Materials and Methods

Borrelia culture and treatment of THP-1 cells

In this study, we used *Borrelia hermsii* (DAH strain), *B. burgdorferi* (strain B31), and *B. burgdorferi* (strain NC-2) cultivated in BSK-H medium (Sigma-Aldrich, St. Louis, MO) with 6% Gibco[®] rabbit serum (Life Technologies, Grand Island, NY) at 37°C and 5% CO₂ in a humidified incubator until use. *Borrelia lonestari* (strain LS-1), the nonpathogenic species, was grown in ISE6 (*Ixodes scapularis* embryonic) cells as described (Varela et al. 2004). To prepare ISE6-free *B. lonestari*, we passed infected ISE6 cells through a 27-gauge needle, then centrifuged at 80 g for 10 min to recover supernatant with spirochetes. Spirochetes were counted before THP-1 exposure. THP-1 (human monocytic) cells were maintained in tissue culture flasks at 37°C and 5% CO₂ in a humidified incubator before and during infections. Cells were cultivated with RPMI medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 50 μ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and penicillin/streptomycin (Life Technologies, Grand Island, NY) at a concentration of 100 U/mL penicillin, 100 μ g/mL streptomycin until use. We performed each treatment exposure trial (6-, 24-, 48-h time

points) three times. For each time point, 4, 24-well plates with $\sim 2.5 \times 10^5$ differentiated THP-1 cells in each well were used. To prepare cells, $\sim 5 \times 10^5$ THP-1 cells were seeded into wells, with the addition of 100 nM phorbol 12-myristate 13-acetate (PMA; Life Technologies, Grand Island, NY) to differentiate cells. Spent media and any nondifferentiated cells (estimating $\sim 50\%$ differentiation) were removed 48 h later and wells were replaced with fresh THP-1 medium (RPMI with 10% FBS) without PMA or antibiotics for 24 h before exposure to treatments.

For spirochete treatments, four wells in each of two replicate plates were inoculated as follows: *B. hermsii* (DAH), *B. burgdorferi* (B31), *B. burgdorferi* (NC-2), and *B. lonestari* (LS-1) at a multiplicity of infection (MOI) of 10:1. In each of the two other plates, four wells were treated with 1 μ g/mL lipopolysaccharides (LPS) from *Escherichia coli* 026:B6, four were isotype controls, four were no antibody controls for intracellular cytokine staining in flow cytometry analyses, and four were used as antibody controls for intracellular cytokine staining in flow cytometry analyses. All wells received THP-1 medium with addition of 10% BSK-H that included 6% rabbit serum (thus, final concentration of rabbit serum in the media of all treatment and control wells was 0.6%). At each time point and for each of the three experimental replicates, supernatant from individual wells was pooled by control and treatment groups (*B. hermsii*, *B. burgdorferi* B31, *B. burgdorferi* NC-2, *B. lonestari*, LPS, no treatment). Pooled supernatants were centrifuged (400 g for 7 min) to remove cell debris, and aliquots frozen (-20°C) for analysis using a custom Milliplex multiplex assay (EMD Millipore, Billerica, MA) and Luminex[®] 200 system (Luminex Corporation).

Measurement of extracellular and intracellular cytokines

Using two 96-well Milliplex Human Cytokine Panel 1, Magnetic, Custom 4-plex panels (EMD Millipore Corporation, Billerica, MA) we analyzed secreted (extracellular) cytokines, IFN- γ , IL-1 β , IL-6, and TNF- α . Cell culture supernatants from treatment groups, pooled as described above, were assayed in duplicate for each of the three experimental replicates for each time point. The no treatment control and LPS-treated samples were assayed in duplicate for one experimental replicate in each of the time points. Samples were prepared according to manufacturer specifications. We used averages of the duplicates for each experimental replicate in our statistical analyses.

To evaluate intracellular cytokines, we then prepared cells from these wells for analysis by flow cytometry. Wells were first incubated with 1 μ g/mL brefeldin A (BD GolgiPlug protein transport inhibitor; BD Biosciences, San Jose, CA) in complete medium for 5 h. Media was then removed and cells incubated with StemPro[®] Accutase[®] (Life Technologies, Grand Island, NY) for 15 min at 37°C. Cells were resuspended and washed in phosphate-buffered saline (PBS) twice before fixation/permeabilization (BD Cytofix/Cytoperm[™] Plus, BD Biosciences). After fixation/permeabilization, cells were washed with cold permeabilization buffer (BD Perm/Wash; BD Biosciences) and incubated 10 min in the dark at RT, centrifuged (400 g for 7 min), and resuspended in 100 μ L BD Perm/Wash. We blocked Fc receptors by incubating cells 10 min at RT with Human TruStain FcX (BioLegend[®], San Diego, CA) before adding each cytokine antibody. For each

cytokine analyzed, IFN- γ , IL-1 β , IL-6, and TNF- α , cell aliquots from each treatment were separately stained with each cytokine antibody directly conjugated with allophycocyanin (APC) in the dark for 30 min at RT, with matched isotype controls included on all time points and replicates. We used cytokine antibodies as follows: anti-human IFN- γ clone 25723 (R&D Systems, Inc., Minneapolis, MN), anti-human TNF- α clone Mab11 (eBioscience, San Diego, CA), anti-human IL-1 β clone 8516 (R&D Systems, Inc., Minneapolis, MN), and anti-human IL-6 clone MQ2-13A5 (eBioscience, San Diego, CA). After staining, cells were washed in permeabilization/wash buffer, centrifuged at 400 g for 7 min and the pellet resuspended in 400 μ L PBS with 0.2% BSA by vortexing, followed by flow cytometry. Stained THP-1 cells were acquired using a FACSCalibur (BD Biosciences) and gated based on expected scatter properties for live cells. For data analysis, we used CellQuest Pro software (BD Biosciences, San Jose, CA) to analyze a total of 10,000 events through the live gate. Geometric mean fluorescence intensity from live cells (generally 90% of cells, based on scatter properties) within these events was used for analyses. A

general linear model (GLM)-ANOVA was used for each cytokine to evaluate effects of time point, treatment, and treatment $\times$ time interaction on cytokine production. Non-normally distributed data were square-root transformed. When appropriate, a Tukey post hoc test was used. Alpha level was set at $p < 0.05$ for all analyses.

Results

In our analysis of secreted cytokines using the Luminex assay, GLM-ANOVA demonstrated that co-incubation with *Borrelia* spp. or LPS had a significant effect only on secreted TNF- α ($p = 0.00405$, $F_{2,5} = 5.068$), as all treatments were significantly different from the no treatment control (Fig. 1A). There was no effect of these treatments on other secreted cytokines (IFN- γ , IL-6, and IL-1 β). However, as we did not include the addition of ATP to stimulate secretion of IL-1 β , we may have underestimated secreted levels of that cytokine. Regarding intracellular cytokines, as measured by flow cytometry, GLM-ANOVA analysis of geometric mean fluorescence intensity demonstrated that neither the *Borrelia*

FIG. 1. Mean concentration (pg/mL) of TNF- α secreted (measured by Luminex assay) when cells were co-incubated with borreliae (BH: *B. hermsii*, BL: *Borrelia lonestari* LS-1, Bb-B31: *B. burgdorferi* B31, Bb-NC2: *B. burgdorferi* NC-2) or LPS (LPS from *Escherichia coli*) was significantly higher than the NT control; error bars are the standard error of the mean, calculated for data collected from the three replicate experiments (A). Geometric MFI of intracellular cytokines (measured by flow cytometry using FACSCalibur) in THP-1 cells exposed to all *Borrelia* species differed at three different time points (6, 24, and 48 h), as detected by flow cytometry. Only cytokines where time point had a significant effect are shown: IFN- γ (—); IL-6 (---); TNF- α (...); IL-1 β is not included due to lack of significant effect. Error bars are the standard error of the mean, calculated for data collected from the three replicate experiments (B). LPS, lipopolysaccharides; MFI, mean fluorescence intensity; NT, no treatment.

spp. treatments nor LPS co-incubation showed a significant effect on measured cytokine levels (data not shown). Thus, the *Borrelia* spp. treatments did not differ from LPS control. Only time point (exposure for 6-, 24-, or 48-h) was found to have a significant effect on intracellular levels of three cytokines, IFN- γ ($p=0.00323$, $F_{2,5}=6.984$), IL-6 ($p=0.000777$, $F_{2,5}=9.177$), and TNF- α ($p=0.0132$, $F_{2,5}=5.043$), when all *Borrelia* treatments were analyzed together, demonstrating higher levels for these three cytokines at 48 h. There was no significant effect of time on IL-1 β , LPS, or the no treatment control (data not shown). Using Tukey's post hoc test, we found significantly more intracellular IFN- γ and IL-6 at the 48 h time point exposure than at 6 and 24 h ($p<0.05$), and at 24 h, there was significantly less intracellular TNF- α (Fig. 1B). The treatment $\times$ group interaction had no effect on any of the cytokines measured.

Discussion

Associating measurable changes in selected cytokines with pathogenicity may allow for predicting the pathogenic potential of uncharacterized bacteria from hematophagous arthropod vectors. In this study, we used the *B. hermsii* DAH strain, two *B. burgdorferi* sensu stricto strains, and *B. lonestari* LS-1 strain that is considered nonpathogenic to humans. We found that by analyzing all exposure time points together, secreted TNF- α was higher in differentiated THP-1 cells exposed to *Borrelia* species or LPS compared to no treatment. However, there was no significant difference among *Borrelia* species. In addition, cells secreted progressively more IFN- γ and IL-6, with significantly higher levels at 48 h of exposure. TNF- α , on the other hand, was secreted significantly less at the 24 h time point than at 6 or 48 h. Our experiments did not use dendritic cells, a more relevant cell type for exposure to bacteria introduced into the skin. The cytokine response of THP-1 cells to *Borrelia* spp. may reflect specific pathogen-associated molecular pattern (PAMPs) expressed by spirochetes in vitro, and not necessarily reflect unique responses due to spirochete pathogenicity in vivo. Further, we were unable to examine THP-1 cells by microscopy for intracellular borreliae using this approach. Unlike PBMCs, THP-1 cells do not readily internalize *B. burgdorferi*, or other spirochetes, and the cytokine production we measured here may reflect minimal activation of cells as seen by Moore et al. (2007). Buffen et al. (2013) demonstrated enhanced IL-1 β production by human PBMCs in the presence of *B. burgdorferi* after inhibition of autophagy. Although PMA-differentiated THP-1 cells can initiate autophagy, and we visualized motile (viable) spirochetes during bacterial enumeration, we do not know whether THP-1 cells here internalized spirochetes or whether autophagy occurred. Borreliae have been shown to lose viability in RPMI in comparison to BSK and RPMI with 25% BSK (Lazarus et al. 2008). *Borrelia* viability was not assessed; however, we used 10% BSK in our complete medium to more closely follow the proportion in tick cell media used for *B. lonestari* propagation (Varela et al. 2004).

In general, our data support previous data showing elevations in IFN- γ , IL-6, and TNF- α over time after exposure to *B. burgdorferi*, and reveal that these changes are consistent with exposure to other *Borrelia* species. While IFN- γ production by THP-1 cells has not been evaluated previously, our detection of intracellular IFN- γ from viable stimulated THP-1

cells, which increased 48 h after co-incubation with borreliae or LPS, supports observations that stimulated macrophages produce this cytokine (Darwich et al. 2009, Robinson et al. 2010). Interestingly, we found a relative decrease in intracellular TNF- α at the 24 h time point and then increase at 48 h, and increase in IL-6 over the three time points. A previous study using THP-1 cells to evaluate the mechanism for production of proinflammatory mediators after *B. burgdorferi* stimulation, found increases in extracellular cytokines, TNF- α , IL-8, and IL-6 over a 24 h period that correlated with expression of MYD88; a slight decrease in extracellular TNF- α could be seen at the 24 h time point compared to the 2, 4, and 6 h time points but a 48 h time point was not evaluated (Dennis et al. 2009). Considering the additional time point in our study, the increase in TNF- α observed here may also reflect spirochete death by that time, and the additional response of THP-1 cells to released PAMPs, which could be evaluated in future studies. While we did not see significant differences in extracellular (secreted) TNF- α over time, intracellular TNF- α was elevated in all treatment groups compared to no treatment.

Conclusions

Certainly, the complexity of immune responses initiated during tick feeding are influenced by tick biology and behavior, including tick saliva, and the individual host immune response, components not investigated in this study. However, removing these factors allowed us to study the immune response in a fundamentally important, though basic approach. In exploring future utility of cytokine profiles for evaluating or potentially predicting pathogenicity of uncharacterized bacteria species, testing a larger panel of relevant cytokines and other immune markers, and including several MOI for *Borrelia* spp., may help identify whether specific profiles occur consistently.

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Author Disclosure Statement

No competing financial interests exist.

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