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Borrelia burgdorferi clinical isolates induce human innate immune responses that are not dependent on genotype

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ABSTRACT

Borrelia burgdorferi can be categorized based on restriction fragment length polymorphism analysis into ribosomal spacer type (RST) 1, 2 and 3. A correlation between RST type and invasiveness of *Borrelia* isolates has been demonstrated in clinical studies and experimental models, and RST 1 isolates are more likely to cause disseminated disease than RST 3 isolates. We hypothesized that this could partially be due to increased susceptibility of RST 3 isolates to killing by the innate immune system early in infection. Thus, we investigated the interaction of five RST 1 and five RST 3 isolates with various components of the human innate immune system *in vitro*. RST 3 isolates induced significantly greater upregulation of activation markers in monocyte-derived dendritic cells compared to RST 1 isolates at a low multiplicity of infection. However, RST 1 isolates stimulated greater interleukin-6 production. At a high multiplicity of infection no differences in dendritic cell activation or cytokine production were observed. In addition, we observed no differences in the ability of RST 1 and RST 3 isolates to activate monocytes or neutrophils and all strains were phagocytosed at a comparable rate. Finally, all isolates tested were equally resistant to complement-mediated killing, as determined by dark-field microscopy and a growth inhibition assay. In conclusion, we demonstrate that the RST 1 and 3 isolates showed no distinction in their susceptibility to the various components of the human immune system studied here, suggesting that other factors are responsible for their differential invasiveness.

1. Introduction

Lyme borreliosis is caused by the spirochaete *Borrelia burgdorferi* sensu lato and transmitted through the bite of infected *Ixodes* ticks into the host's skin. From the skin, invasive strains of *Borrelia* disseminate haematogenously to cause symptoms in the joints, heart, nervous system or distant skin regions (Steere, 2001). Local infection and dissemination of *Borrelia* is combatted by the innate immune system. Dendritic cells (DCs) residing in the skin engulf *Borrelia*, whereupon they become activated and migrate to lymph nodes to present *Borrelia* antigen to naïve T cells, inducing an adaptive immune response against *Borrelia* (Mason et al., 2013). Monocytes and neutrophils are recruited to the tick bite site where they are able to phagocytose *Borrelia*, clearing it from the area, become activated and produce pro-inflammatory cytokines, which augment the immune response (Menten-Dedoyart et al., 2012; Radolf et al., 2012; Salazar et al., 2003). Apoptosis is induced in monocytes with ingested *Borrelia*, which may act as an additional immune defence tactic (Cruz et al., 2008). In the blood, proteins of the complement system bind to *Borrelia* and form membrane attack complexes which induce cell lysis and lead to opsonisation (de Taeye et al., 2013).

Abbreviations: RST, ribosomal spacer type; DC, dendritic cell; moDC, monocyte-derived dendritic cell; EM, erythema migrans; PBMC, peripheral blood mononuclear cell; MOI, multiplicity of infection; MFI, mean fluorescence intensity; LPS, lipopolysaccharide; HLA-DR, human leukocyte antigen-DR; CD, cluster of differentiation; IL, interleukin; TNF, tumour necrosis factor; CFSE, carboxyfluorescein siacetate succinimidyl ester; NHS, normal human serum; HIS, heat inactivated serum; CBA, cytokine bead array; MAC, membrane attack complex; OspC, outer surface protein C; IFN, interferon; Lp, linear plasmid.

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Borrelia can be categorised into distinct ribosomal spacer types (RST) 1, 2 and 3, based on restriction fragment length polymorphism (RFLP) sequencing of a non-coding region of ribosomal RNA operon (Liveris et al., 1996). A substantial body of clinical and experimental evidence reveals a correlation between RST type and the invasiveness of *Borrelia* isolates. In a clinical study in which the RST type of *Borrelia* isolates recovered from erythema migrans (EM) lesions of 104 untreated patients was analysed, patients with spirochaetaemia and/or multiple EM, markers of invasive disease, were found to be five times more likely to be infected with RST 1 than RST 3 isolates, with RST 2 isolates displaying an intermediate phenotype (Wormser et al., 1999). Subsequent studies corroborated this observation, finding a significant association between RST type and frequency of disseminated infection (Jones et al., 2006; Wormser et al., 2008). In addition, RST 1 isolates more frequently caused antibiotic-refractory lyme arthritis (Jones et al., 2009). Furthermore, in murine models of lyme borreliosis, needle inoculated RST 1 isolates persisted longer and gave rise to greater spirochaete loads than RST 3 isolates (Wang et al., 2001, 2002).

It has been demonstrated that *Borrelia* possess genes which may be differentially expressed to bypass or modulate host immunity (Hovius et al., 2007). Genetic differences or differential gene expression between isolates might determine the interaction of the spirochetes with the host immune system. We hypothesised that RST 3 isolates are more susceptible to killing by the innate immune system than RST 1 isolates, which would explain why RST 1 strains are more invasive than RST 3 isolates. Therefore, we tested the susceptibility of five isolates of each RST type to various elements of the innate immune system: complement-mediated killing, phagocytosis by and activation of neutrophils and monocytes, apoptosis of monocytes and activation of DCs. Although, we saw significant differences in the DC response to RST 1 and RST 3 isolates, we found no differences in the susceptibility of isolates to the other arms of the innate immunity.

2. Materials and methods

2.1. Borrelia burgdorferi *culture*

B. burgdorferi sensu stricto RST 1 isolates B515, BL203, BL206, B479, B491, RST 3 isolates B331, B348, B356, B418 and B485 and *B. garinii* isolate A87S were cultured in modified Barbour–Stoenner–Kelly (BSK)-2 medium (AMC, Amsterdam, The Netherlands) plus sodium bicarbonate supplemented with 6% rabbit serum (Sigma–Aldrich, Zwijndrecht, The Netherlands) at 33 °C until cultures reached the mid to late log growth phrase. Spirochaetes were assessed for motility, quantified by dark-field microscopy and recovered by centrifugation at 4000 × *g* for 8 min and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, UK) supplemented with 10% Fetal Calf Serum (FCS) (Lonza, Verviers, Belgium) and L-glutamine (Gibco) for stimulation. Only low passage (<10) isolates were included in experiments.

2.2. Monocyte derived dendritic cell (moDC) generation and stimulation

MoDCs were generated as previously described (Hovius et al., 2008a,b). Briefly, peripheral blood mononuclear cells (PBMCs) from fresh heparinised blood donated by consenting, healthy volunteers were isolated by density gradient using Ficoll paque (GE-healthcare, Uppsala, Sweden) and monocytes were identified as adherent cells at 37 °C after isolation using Percoll (GE-healthcare). Monocytes were cultured at a concentration of 4×10^5 cells/ml in RPMI with 10% FCS and L-glutamine supplemented with 1000 U/ml interleukin (IL)-4 (Prospec, East Brunswick, NJ, USA) and 50 ng/ml Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (Gibco) at 37 °C with 5% CO_2. After one week, 1×10^5 moDCs were stimulated by each of the ten isolates for 48 h at different multiplicities of infection (MOIs). Lipopolysaccharide (LPS, 10 ng/ml) of *Escherichia coli* (Invitrogen, Paisley, UK) was used as a positive control for cell stimulation. Cells were stained with fluorophore-conjugated monoclonal antibodies anti-human leukocyte antigen-DR (HLA-DR)-PE-Cy7 and anti-cluster of differentiation (CD) 86-PE (BD Pharming, San Diego, CA) and the geometric mean fluorescence intensity (MFI) was measured using FACSCanto II (BD Biosciences). Analysis was performed using FlowJo (Treestar, Ashland, OR).

2.3. Whole blood stimulation

A 100 μl heparinised human whole blood (approximately 8×10^5 leukocytes) was stimulated with each isolate for 16 h at 37 °C with 5% CO_2 at different MOIs. MOI was calculated for leukocytes present in whole blood. Lipopolysaccharide (LPS, 10 ng/ml) of *E. coli* (Invitrogen, Paisley, UK) was used as a positive control for cell stimulation. Cells were stained with fluorophore-conjugated monoclonal antibodies anti-HLA-DR-PE-Cy7, anti-CD11b-APC, anti-CD66-FITC and anti-CD14-PE (BD Pharming) and analysed by flow cytometry as described above.

2.4. Cytokine measurement

The concentration of cytokines IL-6, IL-8, IL-1β, IL-10, tumour necrosis factor (TNF)-α and IL-12p70 produced by stimulated cells was measured as previously described (Hovius et al., 2008a,b) in cell supernatant using a human inflammatory cytokine bead array (CBA) kit (BD Biosciences) according to the manufacturer's instructions.

2.5. Whole blood phagocytosis assay

Phagocytosis assays were performed in essence as previously described (Hovius et al., 2009). Briefly, *Borrelia* isolates were centrifuged, pelleted and washed three times with phosphate buffered saline (PBS) (Fresenius Kabi, Graz, Austria), resuspended in 5 μM carboxyfluorescein siacetate succinimidyl ester (CFSE) (Invitrogen) in PBS and agitated for 30 min at 37 °C in the dark. The isolates were washed twice and resuspended at a concentration of 4×10^6 spirochaetes per ml. Heparinised human whole blood was incubated with each CFSE-labelled isolate separately at an MOI of 10 and agitated at 37 °C (or 4 °C as a binding control). At various time intervals 20 ul of blood was removed and phagocytosis was stopped by adding ice cold FACS Buffer (0.5% bovine serum albumin (BSA), 0.01% NaN_3 and 0,35 mM EDTA in PBS). The cells were washed twice in FACS buffer and erythrocytes were lysed using erythrocyte lysis buffer before performing flow cytometry as described above. Cell populations were distinguished based on forward and side scatter characteristics.

2.6. Monocyte apoptosis (supplemental data)

Two representative RST 1 (B515, BL206) (Wang et al., 2001, 2002; Bockenstedt et al., 2006; Wang et al., 2004a,b) and two representative RST 3 (B331, B356) (Wang et al., 2001, 2002; Bockenstedt et al., 2006; Wang et al., 2004a,b) clinical isolates were incubated with human PBMCs at an MOI of 10 for 6 h at 37 °C. A 0.1 μM staurosporine was used as a positive control for the induction of apoptosis. Cells were harvested and stained with anti-CD14-APC (Miltenyi Biotec, Auburn, CA). Apoptosis was measured using the Apo-BrdU-Red *in situ* DNA Fragmentation Assay Kit (BioVision,