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Borrelia-induced cytokine production is mediated by spleen tyrosine kinase (Syk) but is Dectin-1 and Dectin-2 independent

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ABSTRACT

Although it is known that *Borrelia* species express sugar-like structures on their outer surface, not much is known about the role of these structures in immune recognition by host cells. Fungi, like *Candida albicans*, are mainly recognized by C-type lectin receptors, in specific Dectin-1 and Dectin-2. In this study we assessed the role of Dectin-1 and Dectin-2 in the recognition process of *Borrelia* spirochetes. Using specific inhibitors against these receptors on human cells did not influenced cytokine production. Individuals carrying a SNP leading to an early stop codon in the *DECTIN-1* gene also did not lead to differential induction of *Borrelia*-dependent cytokines. After injection of live *Borrelia* into knee joints of Dectin-2 deficient mice a trend towards lower inflammation was observed. Inhibition of Syk in human cells resulted in lower cytokine production after *Borrelia* stimulation. In conclusion, Dectin-1 and Dectin-2 seem not to play a major role in *Borrelia* recognition or *Borrelia*-induced inflammation. However, Syk seems to be involved in *Borrelia*-induced cytokine production.

1. Introduction

C-type lectin receptors (CLRs) belong to the family of Pattern Recognition Receptors (PRRs) and recognize mainly sugar and sugar-like structures [12,17]. This family was first described to consist out of calcium-dependent receptors, but in the past years calcium independent members were also added to this group [32]. CLRs consist out of a broad range of members, including Dectin-1, Dectin-2, and Mannose receptor. Mannose receptor (MR) appears on macrophages and monocytes with a large extracellular domain, which mediates binding to mannan, a structure that is mainly present on fungi, including *Candida albicans* [11,20]. However, MR is also capable of binding to bacteria and to influence pro-inflammatory cytokine production, including *Mycobacterium tuberculosis*, and *Borrelia burgdorferi*, the causative agent of Lyme disease [4,35].

Dectin-1 is a nonclassical C-type lectin receptor which displays an extracellular domain that modulates calcium independent binding to β1,3-glucans, and is mainly expressed by dendritic cells, neutrophils, and macrophages [2,27]. The mechanism of ligand recognition by this receptor is still unknown, but Dectin-1 is involved in the production of pro-inflammatory cytokines such as interleukin-(IL)-1β, IL-6, and Tumor Necrosis Factor (TNF)-α, but also anti-inflammatory cytokines such as IL-10 [6]. Dectin-1 signaling is mediated through the spleen tyrosine kinase (Syk)-CARD9 pathway, which leads to the activation of the canonical NFκB subunits, c-Rel and p65, and, subsequently, the production of many cytokines [13]. Dectin-1 was described to play an important role in *C. albicans*-induced signaling [7,12,26]. Individuals bearing a single nucleotide polymorphism (SNP) resulting in an early stop codon (Tyr238X) in the *Dectin-1* gene displayed impaired capacity to produce pro-inflammatory cytokines IL-6 and TNF-α upon *Candida* stimulation, and suffered from severe inflammation on mucosal surfaces. Next to IL-6 and TNF-α production, IL-1β production was also significantly decreased in individuals bearing the SNP, indicating that Dectin-1 can also influence inflammasome-dependent signaling. Earlier, it was shown that Dectin-1 amplifies immune responses which are induced by other PRRs, namely Toll-like receptor (TLR)-4 and TLR-2 [5,8,9].

Dectin-2, a classical CLR member, was first demonstrated in 2000 being expressed on a dendritic cell line [1], but later it could

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also be found on other cell types including macrophages and monocytes [31]. Despite only 20–25% homology between dectin-2 and dectin-1, this receptor recognizes many fungi that are also recognized by dectin-1, and induces subsequent cytokine production by immune cells [1]. After activation, gene expression is upregulated, intracellular signaling pathways are activated, which eventually leads to production of TNF-α [10,29]. At this moment it is unknown whether Dectin-2 is also able to amplify TLR-dependent immune responses.*Borrelia* recognition and induction of inflammation is largely accomplished by Toll-like receptor (TLR)-2 [23,24]. After neutralization of TLR2 with specific antibodies or in individuals bearing SNPs in TLR1/TLR2 heterodimers, *Borrelia*-induced cytokine responses were significantly downregulated [22,24]. Next to that, *Borrelia*-induced IL-1β production was shown to be highly dependent on inflammasome activation [23,25]. Although Dectin-1 and Dectin-2 are both described in fungal-derived cytokine production and inflammasome activation, and been described to amplify the TLR-mediated responses, the role of these CLRs in *Borrelia* responses were not described before. In this study we investigated the role of Dectin-1/Dectin-2 pathway in *Borrelia*-induced cytokine responses.

2. Materials and methods

2.1. Borrelia burgdorferi cultures

B. burgdorferi, ATCC strain 35210, was cultured at 33 °C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma–Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late-logarithmic phase and examined for motility by dark-field microscopy. Organisms were counted using a Petroff-Hauser counting chamber. Bacteria were harvested by centrifugation of the culture at 3000×*g* for 30 min., washed twice with sterile PBS (pH 7.4), and diluted in the specified medium to required concentrations of 1×10^6 spirochetes per mL.

2.2. Animals

Dectin-2-/- mice were bred and maintained in the RadboudUMC, Nijmegen, The Netherlands. Generation of the mice was performed by Ozgene Australia [14]. Dectin-2 deficient mice were backcrossed ⩾10 generations into C57Bl/6J background. Mice (8–14 weeks old, females) were housed in a pathogen-free facility and the animal studies were conducted under EU Directive 2010/63/EU and protocols approved by the Ethics Committee on Animal Experiments of the RadboudUMC. Dectin-1-deficient mice were kindly provided by Prof. Dr. Gordon Brown, Institute of Medical Sciences, University of Aberdeen, United Kingdom.

Female wild-type (C57Bl/6J), Dectin-1 and Dectin-2 knock-out mice between 8 and 14 weeks of age were used. The mice were fed sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*.

2.3. In vitro cytokine production

Murine peritoneal macrophages were isolated after injecting 5 mL of ice cold sterile PBS (pH 7.4) in the peritoneal cavity. After centrifugation and washing, cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 containing 1 mM pyruvate, 2 mM L-glutamine and 50 μg/L gentamicin (culture medium). Cells were counted using a Z1 Coulter Particle Counter Beckman Coulter, Woerden, The Netherlands) and adjusted to 1×10^6 cells/mL. Red blood cells were lysed before counting using Zap-Oglobin reagent (Beckman-Coulter,Woerden, The Netherlands). Cells were cultured in 96-well round-bottom microtiter plates (Costar, Corning, The Netherlands) at 1×10^5 cells/well, in a final volume of 200 μL. After 24 h of incubation of cells with different stimuli at 37 °C in air and 5% CO_2, the plates were centrifuged at 1400×*g* for 8 min, and the supernatant was collected and stored at −20 °C until cytokine assays were performed.

Human venous blood was drawn from the cubital vein of foresters into 10 mL ethylenediaminetetraacetic acid (EDTA) tubes (Monoject). Peripheral blood mononuclear cells (PBMCs) were isolated according to standard protocols, with minor modifications. The PBMC fraction obtained by density centrifugation of blood diluted 1:1 in phosphate-buffered saline (PBS)-buffer over Ficoll-Paque (Pharmacia Biotech). Cells were washed three times in PBS and resuspended in RPMI 1640 (Dutch modified) supplemented with 50 mg/L gentamicin, 2 mM L-glutamin, and 1 mM pyruvate. Cells were counted in a Coulter Counter ZH (Beckman Coulter), and adjusted to 5×10^6 cells/mL. Red blood cells were lysed before counting using Zap-Oglobin reagent (Beckman-Coulter, Woerden, The Netherlands). Mononuclear cells (5×10^5) in a 100 μL volume were added to round-bottom 96-wells plates (Costar, Corning, The Netherlands) and incubated with either 100 μL of medium (negative control) or *B. burgdorferi* (1×10^6 spirochetes per mL). In some experiments, PBMCs were preincubated with neutralizing antibodies or chemical inhibitors for 60 min (Syk inhibitor 1 μM, 574711, EMD Millipore, USA). After pre-incubation, *B. burgdorferi,* heat-inactivated *C. albicans* UC820, or specific TLR ligands were added, Pam3Cys or FSL-1 (10 μg/mL or 1 μg/mL, respectively). After 24 h supernatants were collected and stored at −20 °C until being assayed.

PBMCs were transfected with 25nM siRNA by the transfection reagent DF4 (Dharmacon) according to the manufacturers protocol and were used in the stimulation experiments 48 h after transfection. The following SMARTpool siRNAs were used (all from Dharmacon): Dectin-1 siRNA (L-021476-00-0005), Dectin-2 siRNA (L-032637-02-0005) and nontargeting siRNA (D-001810-10-05) as a control. Silencing of expression was verified by real-time PCR. All human experiments were conducted according to the principles expressed in the Declaration of Helsinki. Before taking blood, informed written consent of each human subject was provided. The study was approved by the ethical board of the RadboudUMC.

2.4. Cytokine measurements

Concentrations of mouse IL-1β and TNF-α were determined by specific radioimmunoassay (RIA; detection limit is 20 pg/ml) as described by Netea et al. [21]. In brief, murine cytokines were detected using biotinylated monoclonal antibodies, which are able to bind to avidin-conjugated horseradish peroxidase followed by TMB-substrate incubation. After stopping the reaction with 0.1 M acid, reactions were measured in an ELISA reader.

Concentrations of human IL-1β, IL-6, or TNF-α were determined using either specific or commercial ELISA kits (PeliKine Compact, Sanquin, Amsterdam, or R&D Systems, Minneapolis), in accordance with the manufacturers' instructions. Detection limits were 40 pg/mL.

2.5. Isolation of genomic DNA and single nucleotide polymorphism analysis

DNA was isolated using the Gentra Pure Gene Blood kit (Qiagen), in accordance with the manufacturer's protocol for whole blood. DNA was dissolved in a final volume of 100 μL buffer. Polymerase chain reaction (PCR) amplification of Dectin-1 gene fragments bearing the polymorphisms Y238X (c.714T > G, rs16910526) were performed using a pre-designed TaqMan H