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Follistatin-like Protein 1 is a Critical Mediator in the Immune Response to *Borrelia burgdorferi* Infection

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Abstract (updated)

Title: Follistatin-like Protein 1 is a Critical Mediator in the Immune Response to *Borrelia burgdorferi* Infection

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Background: *Borrelia burgdorferi* (*Bb*) causes Lyme disease (LD), the most common tick-borne illness in the US. LD manifests most commonly as acute or chronic arthritis. The host immune response to *Borrelia burgdorferi* is complex involving innate, humoral and cell-mediated pathways. The C3H/HeJ murine model of Lyme arthritis has similarities to murine model collagen-induced arthritis (CIA) in the DBA-1 mouse strain. In the CIA model, follistatin-like protein 1 (FSTL-1), a novel immune modulator, has been found to play a key role in promoting a Th1 response. We have hypothesized that FSTL-1 is an early biomarker of the inflammatory response associated with *B. burgdorferi*.

Methods: *Bb* was subcutaneously injected into 4-5 week old C3H, DBA-1 wild-type (WT) and DBA-1 FSTL-1 hypomorphic (FH) mice. Disease phenotype was evaluated by measurement of tibiotarsal joint swelling (arthritis). Infection was evaluated by bladder culture (bacterial growth), tissue DNA loads and serology (humoral response). Serum FSTL-1 levels were assayed by ELISA and FSTL-1 RNA expression in ankles was assayed by qRT-PCR.

Results: Bladder cultures from both murine strains grew *Bb*, indicating successful disseminated infection. Upon joint swelling assessment, WT mice demonstrated increased swelling when compared to FH mice (average ΔAP diameter: 0.61 ± 0.05 mm (WT) versus 0.37 ± 0.08 mm (FH)) p=0.02. Serologic response to *Bb* proteins was detectable by Western blot and ELISA but had decreased total antibody production and limited bands in FSTL-1 hypomorph serum. Serum FSTL-1 levels in C3H mice were significantly elevated at Day 1 and subsequently remained elevated. qRT-PCR revealed elevated message for FSTL-1 in ankles at Day 14 with a subsequent decrease below control by Day 42.

Conclusions: The observations that **A)** serum FSTL-1 increases early following *B. burgdorferi* infection, **B)** FSTL-1 hypomorphic mice have increased *B. burgdorferi* tissue loads despite attenuated arthritis and **C)** FSTL-1 hypomorphic mice produce fewer anti-*B. burgdorferi* antibodies in total and lack antibody specificity for some *B. burgdorferi* antigens suggests that FSTL-1 is a critical mediator for the development of the humoral response and arthritis phenotype in *Borrelia burgdorferi* infection.

Background

Borrelia burgdorferi (*Bb*) causes Lyme disease, the most common tick-borne illness in the US. Lyme Disease which manifests most commonly as arthritis with peak incidence in children between ages 5-19. The host immune response to *Borrelia burgdorferi* is complex involving innate, humoral and cell-mediated pathways of inflammation. The C3H/HeJ murine strain serves as a model of Lyme arthritis. Lyme arthritis shares phenotypic, pathologic and immunologic similarities to juvenile idiopathic arthritis (JIA). Collagen-induced arthritis (CIA), a murine model of JIA, has been studied and characterized in the DBA-1 mouse strain. In this model, follistatin-like protein 1 (FSTL-1), a novel immune modulator, has been found to play a key role. FSTL-1 is a mesenchyme-derived glycoprotein that signals via the Th1 pathway and leads to upregulation of proinflammatory cytokines IL-1β, TNF-α and IL-6. **We have hypothesized that FSTL-1 is a mediator of the inflammatory response associated with *B. burgdorferi* in murine arthritis.**

Methods

Murine infection- Spirochetes were counted using dark-field microscopy. A low passage strain of *Bb* B31 was grown in BSK media until late log phase, then diluted by to a concentration of 10^7 spirochetes/mL. 4-5week old female C3H/HeJ, 4-5week old male DBA-1 wild-type and FSTL-1 hypomorphic mice were subcutaneously injected with 10^6 spirochetes. Sham-infected controls were injected with culture media alone. Tibiotarsal joints were measured 2-3x weekly. Serum was collected prior to infection and at least weekly. All studies were performed in accordance with the University of Pittsburgh IACUC.

Borrelia burgdorferi isolation from mice- At sacrifice, bladders were sterilely obtained and placed into BSK media. Cultures were evaluated twice weekly for four weeks by dark-field microscopy, the presence of any viable spirochete was determined to be a positive culture.

Tibiotarsal joint qPCR for OspC- Total DNA was extracted from tissues using the method of Weis *et al*. Quantitative PCR was used to enumerate spirochetes using the OspC gene as a *Bb* standard, and the actin gene for murine DNA calibration. Triplicate experiments were performed using the iQ SYBR Green kit on a Bio-Rad MyiQ2 thermal cycler/imaging system. Standard curves and full melting temperature profiles were performed on each plate for standardization.

Serologic response- Proteins were separated with 12.5% SDS-PAGE. Proteins were transferred to nitrocellulose by the Towbin method. Membranes were blocked with 5% milk in TBS-0.5% Tween-20, and probed using pooled chronically infected murine serum from at 1:500 dilution for one hr. After washing, they were secondarily probed using 1:3000 dilution of goat-α-mouse-HRP for one hr. Reactive bands were visualized using chemiluminescence.

Borrelia ELISA-96well polystyrene ELISA plates were coated with 100uL 5ug/ml *Borrelia* cell lysate in 0.1M NaHCO3 pH 8.6 0 overnight at 4 degress. Block with 5% dry milk in TBS-T20 for 1 hour. Probe with mouse serum 1:100 dilution in %5 dry milk + TBS-T20 for 1 hour. Detect with 1:5000 goat anti-mouse IgG-alkaline phosphatase. Develop with –Step pNPP for 20 minutes with agitation. Add 50ul/well 2N NaOH as stop reagent. Measure OD on ELISA plate reader at 405nm wavelength. Wash with 0.05% TBS-T20 5x between each step. All but first step performed at ambient temperature.

ΔTibiotarsal size in mice- Mice were evaluated under anesthesia with 1% Isoflurane 2-3x weekly for the duration of the experiment. Tibiotarsal diameter in the Anterior-Posterior (AP) and Medi-Lateral (ML) orientation was determined using metric calipers. Mean and standard error were calculated for each group. Tibiotarsal joint swelling was calculated using the formula: Area =π(0.5) M-L diameter * (0.5) AP diameter.

Tibiotarsal FSTL-1 qRT-PCR- Total RNA isolated from murine tissues was analyzed by qRT-PCR using FSTL-1 primers as noted previously. Values were calculated using the comparative C_T method, and changes in fluorescence were monitored with the MyiQ single-color real-time PCR detection system using Sybr green (Bio-Rad).

Serum FSTL-1 levels- FSTL-1 was assayed by coating Nunc ELISA plates with monoclonal rat anti-FSTL-1 overnight at 4°C. Plates were washed with PBS-0.05% Tween-20 and blocked for 1h, then washed. Diluted samples were added for 2 hrs, followed by biotin-labeled polyclonal rabbit anti-FSTL-1. Plates were washed and incubated with streptavidin-HRP and developed with TMB. Absorbance was read at 450 nm.

Statistics-Individual groups were analyzed using Mann-Whitney or unpaired t-tests.

Table 1. *Bb* growth from murine bladder

Murine strain	C3H/HeJ	DBA-1
+ bladder outgrowth	7	5
total	15	10
% with outgrowth	47%	50%

Figure 1. Tibiotarsal swelling

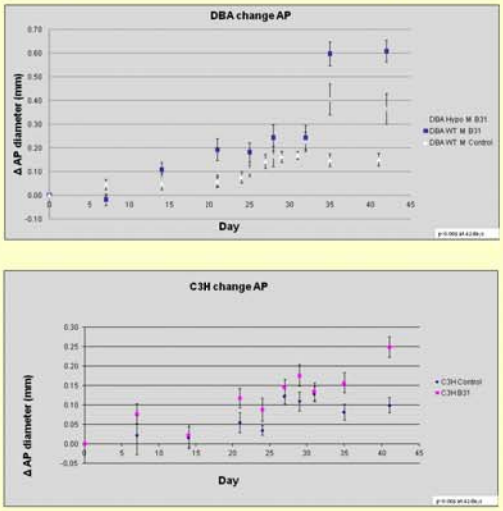


Figure 2. Serologic response

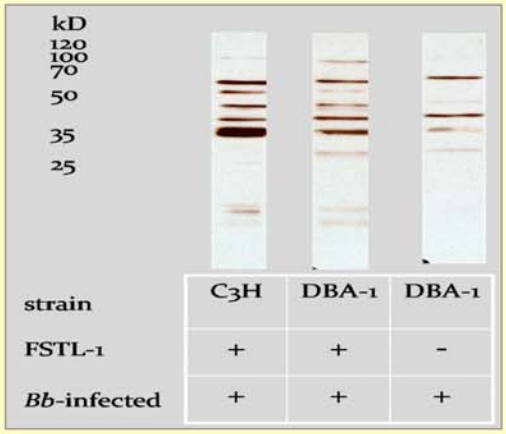


Figure 3. Borrelia ELISA

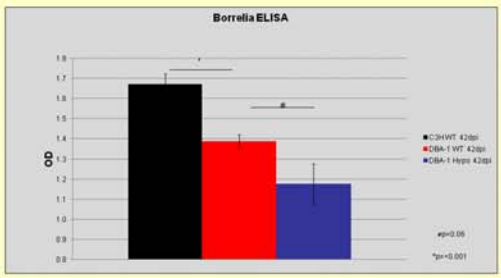


Figure 4. OspC tissue loads of *Bb*

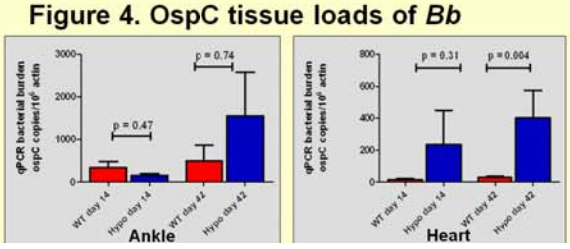


Figure 5. Ankle Joint FSTL-1 RNA

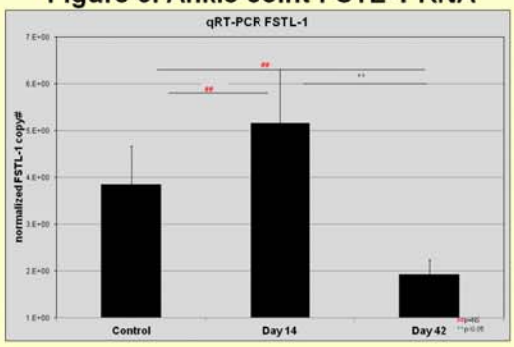
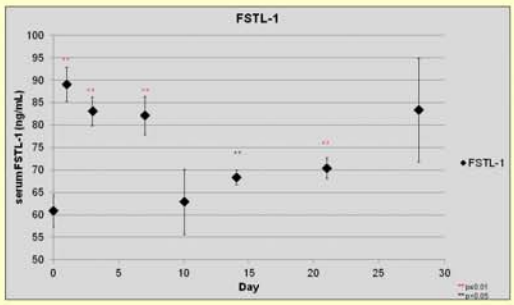


Figure 6. Serum FSTL-1 levels



Conclusions

- The DBA-1 murine strain is susceptible to *Borrelia burgdorferi* infection, and develops arthritis similar to the C3H/HeJ strain, a model strain for murine borreliosis
- Serum FSTL-1 is increased early following *B. burgdorferi* infection in mice suggesting that FSTL-1 serves as mediator of Lyme disease.
- FSTL-1 appears to be important for the development of ankle swelling and production of antibody to *B. burgdorferi* infection.
- The DBA-1 strain represents a novel murine model for the study of *Borrelia burgdorferi* infection and FSTL-1 involvement in *Bb* infection

Acknowledgements

This research was supported in part by NIH K08 AI072365-01A2 (AN) and NIH R01 AI073556-03, NIH R01 AR056959 and 5T32AR052282-08 (RH, BC) as well as the Children's Hospital of Pittsburgh Department of Pediatrics.

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