



The transcription factor Relish controls *Anaplasma marginale* infection in the bovine tick *Rhipicephalus microplus*



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ABSTRACT

Rhipicephalus microplus is an important biological vector of *Anaplasma marginale*, the etiological agent of bovine anaplasmosis. The knowledge of tick immune responses to control bacterial infections remains limited. In this study, we demonstrate that transcription factor Relish from the IMD signaling pathway has an important role in the control of *A. marginale* infection in ticks. We found that RNA-mediated silencing of Relish caused a significant increase in the number of *A. marginale* in the midgut and salivary glands of *R. microplus*. In addition, the IMD pathway regulates the expression of the gene that encodes the antimicrobial peptide (AMP) microplusin. Moreover, microplusin expression was up-regulated in the midgut (2×) and salivary glands (8×) of *A. marginale* infected *R. microplus*. Therefore, it is plausible to hypothesize that microplusin may be involved in the *A. marginale* control. This study provides the first evidence of IMD signaling pathway participation on the *A. marginale* control in *R. microplus*.

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1. Introduction

Ticks are obligate ectoparasites that feed on the blood of a broad number of vertebrate hosts. As result of the hematophagy behavior, ticks acquire a wide variety of microorganisms and transmit them to vertebrate hosts, making them important vectors of several pathogens, including protozoa, viruses, and bacteria (Sonenshine and Roe, 2014). *Rhipicephalus microplus* is primarily an ectoparasite of cattle and responsible for considerable economic losses for the livestock industry in tropical and subtropical regions. *R. microplus* is the vector of the obligate intracellular Gram-negative bacteria *Anaplasma marginale*, the etiologic agent of bovine anaplasmosis (Kocan et al., 2010). This disease causes major economic losses due to temporary infertility, abortion, increased mortality, and high treatment costs (Kocan et al., 2010). *R. microplus* acquires *A. marginale* via

intake of infected blood in the acute and persistent phases of the disease (Futse et al., 2003) and become reservoirs (Kocan et al., 2010). Although transovarial transmission of *A. marginale* has not been confirmed (Esteves et al., 2015), transstadial and intrastadial transmission has been demonstrated (Aguirre et al., 1994; Samish et al., 1993). In this context, adult male ticks have a greater epidemiological importance in the transmission of *A. marginale* because of their mobility between infected and uninfected animals, allowing transmission of pathogens (Kocan et al., 2010).

As reported for invertebrate, ticks, as well as other arthropods, need to defend themselves against pathogens (Sonenshine and Hynes, 2008). For this purpose, immune mechanisms have evolved to distinguish self from non-self and produce effectors molecules against pathogens (Palmer and Jiggins, 2015). As observed in other invertebrates, the immune system of *Drosophila melanogaster* is activated by the recognition of pathogen-associated molecular patterns (PAMPs), including for instance peptidoglycan and lipopolysaccharide, that are sensed by pattern recognition receptors (PRR) (Imler et al., 2000; Royet and Dziarski, 2007). However, the knowledge of the system PAMP-PRR in ticks is very limited (Smith and Pal, 2014; Palmer and Jiggins, 2015). After pathogen recognition, distinct immune responses may occur, including

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phagocytosis by hemocytes. This response is likely mediated by a complement-like system involving lectins, that contain fibrinogen-related proteins (FREPS), thioester-containing proteins (TEPs), and convertase-like factors. Other tick defense mechanisms against pathogens involve the production of reactive oxygen species, proteases, protease inhibitors, and antimicrobial peptides (AMPs) (Hajdusek et al., 2013; Kopacek et al., 2010; Smith and Pal, 2014; Sonenshine and Hynes, 2008).

In the fruit fly, *Drosophila melanogaster*, transcription of AMP genes are regulated by intracellular signaling pathways such as Toll, Immune deficiency (IMD), and Janus kinase/signaling transducer activator of transcription (Jak/Stat). The Toll pathway is activated by Gram-positive bacteria and fungi, culminating in the production of the AMPs defensin, drosomycin, and metchnikowin whereas the IMD pathway primarily responds to Gram-negative bacteria infection and controls the expression of other AMPs, including cecropin, attacin, drosocin, and dipterin (Hoffmann, 2003). The Jak/Stat pathway is important in the anti-viral defense of *Drosophila* (Myllymaki and Ramet, 2014) and *Aedes aegypti* (Souza-Neto et al., 2009) and is involved in the control of the protozoan *Plasmodium* by the *Anopheles aquasalis* (Bahia et al., 2011).

There is a lack of information regarding the immune system of ticks compared with the data from other arthropods (Hajdusek et al., 2013; Kopacek et al., 2010; Smith and Pal, 2014; Sonenshine and Hynes, 2008). However, the assembly of a genomic database of *Ixodes scapularis* (Gulia-Nuss et al., 2016; Megy et al., 2012), the availability of a transcriptome database of *R. microplus* ((Guerrero et al., 2005); NCBI (Biosample SAMN02463642 and Bioproject PRJNA232001)), and nucleotide sequences of other tick species deposited in Gene Bank have increased the number of tick immune signaling pathway studies. Liu et al. (2012) showed the Jak/Stat pathway in *I. scapularis* ticks has a crucial role in the control of *Anaplasma phagocytophilum*. This pathway is involved in the expression regulation of a salivary glands gene family that encodes 5.3 kDa antimicrobial peptides (Liu et al., 2012). The Jak/Stat pathway of *I. scapularis* is also involved in the expression of another AMP, Dae2, which limits the proliferation of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. Further, it was shown that the intake of cytokine IFN- γ from the host blood activates the Jak/Stat pathway, which in turn induces expression of anti-*Borrelia* AMP Dae2 (Smith et al., 2016). In addition, the Jak/Stat pathway in *I. scapularis* regulates the expression of the glycoprotein peritrofin-1, which forms the peritrophic matrix in arthropods and separates the lumen from the midgut epithelium. Silencing of Stat decreased *B. burgdorferi* colonization of *I. scapularis* probably by decreasing the level of peritrofin-1, indicating that colonization of tick midgut with this pathogen depends on integrity of the peritrophic membrane (Narasimhan et al., 2014). Recently, Shaw et al., 2017 showed that the IMD pathway controls *B. burgdorferi*, *A. phagocytophilum* and *A. marginale* colonization in *I. scapularis* and *Dermacentor andersoni* ticks. Moreover, lipids derived from these bacteria elicit IMD pathway. However, the effectors responsible for the protection against colonization have not been identified (Shaw et al., 2017). However, only a few studies have investigated the immune defenses of *R. microplus*. Our group has purified and characterized several AMPs, including hemocidins (Belmonte et al., 2012; Cruz et al., 2010; Fogaca et al., 1999), microplusin, defensin (Fogaca et al., 2004), and ixodidin (Fogaca et al., 2006) from *R. microplus*. Moreover, we have studied the mode of action of microplusin, which occurs through its capacity to chelate copper ions needed for bacterial respiration, a copper dependent process (Silva et al., 2009). We have also shown that the hemocidin Hb33-61, different from microplusin, causes bacterial membrane permeabilization (Cruz et al., 2010). *In silico* search for sequences of the Toll, IMD, Jnk and Jak/Stat signaling pathways in *R. microplus* has

recently been completed. Several components of these pathways were identified, indicating the high degree of homology of Toll, Jnk, and Jak/Stat pathways among arthropods. However, some pathway components upstream of IMD, including IMD adapter and the associated proteins Fadd, Dredd, Pirk, and Dnr1, were not found in tick databases (Rosa et al., 2016). In addition, *A. marginale* infection in BME26 cells (derived from *R. microplus* embryos) has been shown to promote a down-regulation of immune-related genes of Toll, IMD, JNK and Jak/Stat pathways (Rosa et al., 2016).

In this study, we demonstrated that transcription factor Relish from IMD pathway controls *A. marginale* infection of the midgut and salivary glands of *R. microplus*. In addition, Relish is involved in AMP microplusin regulation, which might be responsible for controlling *A. marginale* infection. In this context, understanding the tick immune system will help to elucidate the vector-pathogen interface and provide valuable data that will serve as the basis for other studies involving novel strategies for the control of bovine anaplasmosis.

2. Materials and methods

2.1. Ethics statement

All experiments involving animals were approved by the Institutional Animal Care and Use Committee and the Biosafety Committee (IACUC: 2013-66, Biosafety: B-010-13) of the University of Idaho in accordance with institutional guidelines based on the Guide for the Care and Use of Laboratory Animals developed by the U.S. National Institutes of Health (NIH).

2.2. Cattle, pathogen and tick vector

To obtain adult *R. microplus* (La Minita strain), two grams of larvae were placed into a cloth patch on the dorsal region of a spleen-intact naive Holstein calf aged 5–7 months (C48498). Prior to infestation, the calf was confirmed to be negative for *A. marginale* by nested PCR (Scoles et al., 2007) and competitive inhibition enzyme-linked immunosorbent assay (Knowles et al., 1996). After 14 days, engorged nymphs were manually removed from the calf and held in an incubator at 26 °C and 92% relative humidity to molt to the adult stage. Unfed adults were sexed and males used for experiments. Four additional calves presenting the same characteristics described above were used in this study: An *A. marginale*-infected calf (C47981): this animal was inoculated with $\sim 10^8$ *A. marginale*-infected erythrocytes (St. Maries strain) as previously described (Futse et al., 2003); and three uninfected calves (C1440, C48481, and C1439).

2.3. Double strand RNA (dsRNA) production

The genes Dorsal (GenBank: KF828755), Relish (GenBank: KF828760), and Stat (GenBank: KF828772) were selected for knockdown studies using RNA-mediated interference (RNAi). Primer sets for each gene were designed (Supplementary Table 1) using Primer3 software (Untergasser et al., 2012). The target fragments of DNA were amplified using complementary DNA (cDNA) produced from *R. microplus* midgut RNA and primers containing the T7 polymerase promoter sequence. The amplified DNA fragments were purified using a PCR product purification kit (Thermo Fisher Scientific, USA). dsRNAs were synthesized using the T7 Ribomax Express RNAi System (Promega) according to the manufacturer's instructions and cDNA as template. The purified dsRNA molecules were suspended in 0.1 mM EDTA, pH 8.0 (Thermo Fisher Scientific) (dsRNA buffer), quantified by spectrophotometry, analyzed by gel electrophoresis, and stored at –80 °C.

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