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Full Length Article

The role of complement in the tick cellular immune defense against the entomopathogenic fungus *Metarhizium robertsii*

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ABSTRACT

Entomopathogenic fungi (EPF) have been widely explored for their potential in the biological control of insect pests and as an environmentally friendly alternative to acaricides for limiting tick infestation in the field. The arthropod cuticle is the main barrier against fungal infection, however, an understanding of internal defense mechanisms after EPF intrusion into the invertebrate hemocoel is still rather limited. Using an infection model of the European Lyme borreliosis vector *Ixodes ricinus* with the EPF *Metarhizium robertsii*, we demonstrated that ticks are capable of protecting themselves to a certain extent against mild fungal infections. However, tick mortality dramatically increases when the capability of tick hemocytes to phagocytose fungal conidia is impaired. Using RNAi-mediated silencing of tick thioester-containing proteins (TEPs), followed by *in vitro* and/or *in vivo* phagocytic assays, we found that C3-like complement components and α2-macroglobulin pan-protease inhibitors secreted to the hemolymph play pivotal roles in *M. robertsii* phagocytosis.

1. Introduction

Ticks are blood-sucking ectoparasites and vectors of many pathogens affecting humans, domestic, and wild animals (De La Fuente et al., 2008), causing enormous economic losses worldwide (Jongejan and Uilenberg, 2004). Recently, tick-borne diseases have emerged in new regions or have appeared in endemic areas, increasing concerns about public health, food security, and conservation of biodiversity (Karesh et al., 2012). Ticks are usually controlled with chemical acaricides, however their inappropriate use increases selection for resistant tick populations (Klafke et al., 2017).

Metarhizium spp. (Hipocreales: Clavicipitaceae) and *Beauveria* spp. (Hipocreales: Cordycipitaceae) are the best characterized entomopathogenic fungi (EPF) that are the most widely applied agents in the biological control of agricultural insect pests (Lacey et al., 2015). These fungi have been studied since the early 1990's as an environmentally friendly alternative to the use of chemical acaricides for tick control (Kirkland et al., 2004; Samish et al., 2008). Based on the published literature, the majority of studies have focused on the biological control of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*, the species with the highest economic impact in tropical areas (Ojeda-Chi et al., 2010; Camargo et al., 2016; Mascarin et al., 2019; Beys-da-Silva et al., 2020); Mesquita et al. (2020). Nevertheless, EPFs have also been tested against other hard tick species including *Ixodes ricinus*, the most important Lyme disease vector in Europe (Hartelt et al., 2008; Wassermann et al., 2016; Lorenz et al., 2020).

Fungal concentration, the propagule applied, the use of adjuvants, tick species, their life stage, and even different populations may result in different fungal efficacies (Aldén et al., 2001; Perinotto et al., 2017; Ment et al., 2012). Different susceptibilities of tick species to fungal infection are also driven by the cuticle barrier (Ment et al., 2012). However, unlike insects (Lu and St. Leger, 2016; Wang et al., 2016; Tawidian et al., 2019), studies about humoral and cellular immune defense of ticks against EPFs after they succeed in penetrating the hemocoel are scarce. Kurtti and Keyhani (2008) reported phagocytosis of *Metarhizium robertsii* (formerly known as *M. anisopliae*) by embryonic tick cell lines and, more recently, we have investigated the cytotoxicity of *M. robertsii* against *R. microplus* hemocytes at the ultrastructural level (Fiorotti et al., 2019). General knowledge about the immune response of tick hemocytes against invading pathogens is, however, still rather

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limited. Phagocytosis of microorganisms by tick hemocytes has been reported to be associated with the production of reactive oxygen species (Pereira et al., 2001) and involves a tick complement-like system (Buresova et al., 2009, 2011; Kopacek et al., 2012; Urbanová et al., 2015; Fogaça et al., 2021). The components of *Ixodes* sp. complement include all four phylogenetically distinct groups of thioester-containing proteins (TEPs) recognized in invertebrates (Stroschein-Stevenson et al., 2006; Buresova et al., 2011; Sekiguchi and Nonaka, 2015). In *I. ricinus*, they comprise (i) three C3-like complement components (*Ir*C3-1, *Ir*C3-2, *Ir*C3-3), (ii) three pan-protease inhibitors of α_2-macroglobulin type (*Ir*A_2M1, *Ir*A_2M2, *Ir*A_2M3), (iii) one insect-type TEP (*Ir*Tep), and (iv) two macroglobulin-complement related proteins (*Ir*Mcr1, *Ir*Mcr2) (Urbanová et al., 2015; Fogaça et al., 2021).

Previously, we demonstrated the specific roles of individual *Ir*TEPs in the phagocytosis of different microbes by tick hemocytes (Buresova et al., 2009, 2011; Urbanová et al., 2015, 2017). Here we found that *I. ricinus* hemocytes were capable of *M. robertsii* phagocytosis and that this cellular defense substantially decreased tick mortality upon infection with mild doses of the EPF. Expression of individual *Ir*TEP genes in response to *M. robertsii* immune-challenge, and their involvement in phagocytosis point to pivotal roles of *Ir*C3s and *Ir*A_2Ms molecules that are absent in insect lineages. This indicates that ticks react differently to fungal infection compared with insects.

2. Material and methods

2.1. Fungal isolate and tick collection

Metarhizium robertsii (ARSEF 2575) was obtained from the Agriculture Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) (USDA-US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). The culture was grown on Sabouraud dextrose agar (SDA) at 25 °C ± 1 °C and relative humidity (RH) ≥ 80% for 14 days. A conidial suspension in phosphate-buffered saline (PBS) plus 0.1% Tween® 80 (PBS-Tween) was used to infect the ticks.

Ixodes ricinus adult females were collected by flagging in woodlands around České Budějovice, Czech Republic. Adult females were maintained in wet chambers at 24 °C, RH ~95%, and day/night period set to 15/9 h. Females were fed naturally on guinea pigs. All laboratory animals were treated following the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 25/2018.

2.2. Infection of Ixodes ricinus females with Metarhizium robertsii

Ticks were divided into groups of 10 females and infected with *M. robertsii* by direct inoculation or immersion into a conidial suspension. For inoculation, the unfed ticks were injected using a microinjector (Drummond®) with a 138 nL suspension of five different fungal concentrations (2×10^4, 2×10^5, 2×10^6, 2×10^7 or 2×10^8 conidia/mL) or138 nL of PBS-Tween for the control group. The corresponding total numbers of injected conidia thus present ~ 2–3, ~28, ~280, ~2.800, and ~28.000, respectively. For topical infection, females were individually immersed for 3 min in 1 mL of fungal suspension of three different concentrations (2×10^6, 2×10^7 or 2×10^8 conidia/mL) or in PBS-Tween for control. The highest concentration was determined based on the concentration commonly used for topical treatments on ticks (Camargo et al., 2016). After treatment, tick females were incubated at 24 ± 1 °C and RH ≥ 90%. Tick mortality was recorded every 24 h for four weeks. The bioassay was repeated twice.

2.3. Expression of IrTEPs in response to M. robertsii infection

About 2.800 conidia in 138 nL of conidial suspension (2×10^7 conidia/mL) was injected into *I. ricinus* unfed females (wild ticks), using a microinjector (Drummond®). The control group was injected with sterile PBS, in addition to a group that did not suffer any mechanical injury (CTR). The injected females were allowed to rest for two or 6 h at room temperature. Subsequently, total RNA was extracted from the entire tick body using the NucleoSpin® RNA Kit (Macherey-Nagel, USA), following the manufacturer's instructions, its concentration being determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific, USA) and 0.5 μg of total RNA was reverse transcribed into cDNA using the High Fidelity cDNA Synthesis Transcriptor Kit (Roche®).

Expression of nine genes encoding *I. ricinus* thioester-containing proteins (IrTEPs) was quantified according to Urbanová et al. (2015) by quantitative real-time PCR (qPCR) using QuantStudio 6 Flex (Applied Biosystems®) and gene-specific primers (Table A1). The qPCR reactions were performed in technical triplicates in a final volume of 25 μL, containing 12.5 μL of the 2 × Power SYBR Green Master Mix (Thermo Scientific®), 2 μL of the 10 μM mixture of specific forward and reverse primers, 2 μL of cDNA, and 8.5 μL of nuclease-free H_2O. Relative expression of *IrTEPs* genes was normalized to *elongation factor 1* (ef-1) (Nijhof et al., 2009) using the mathematical model of Pfaffl (2001). For each experimental group, five *I. ricinus* females were injected in three independent biological triplicates.

2.4. Metarhizium antibody preparation

Metarhizium robertsii conidial cell wall extracts were used as antigens to produce polyclonal antibodies that recognize conidia. Petri dishes containing SDA supplemented with 0.5% yeast extract were inoculated with 1×10^6 conidia/mL and incubated at 25 °C ± 1 °C and RH ≥ 80% for 14 days. Conidia were harvested by scraping the media into sterile water, followed by centrifugation (5000×*g*, 20 min), washed once with distilled water, and suspended in 10 mL of deionized H_2O. The cell suspension was autoclaved for 15 min and the resulting suspension was mixed with incomplete Freund's adjuvants and used for the production of polyclonal antibodies in rabbits. Immunization was repeated three times at intervals of 14 days. Indirect immunofluorescence was used to test serum for the presence of antibodies recognizing *M. robertsii* (described below).

2.5. RNAi-mediated silencing of IrTEPs

Fragments of nine *IrTEPs* and *GFP* (control) were amplified from *I. ricinus* cDNA and cloned into the pll10 vector with two T7 promoters in reverse orientations according to the method developed by Levashina et al. (2001). Double-stranded RNAs were synthesized as previously described (Buresova et al., 2011) injected (0.4 μL; 3 μg/μL) into the hemocoel of unfed tick females using a Narishige® microinjector. Females injected with gene-specific dsRNA or *GFP* dsRNA for the control were allowed to rest for 24 h in a wet chamber and then fed on guinea pigs for 6 days. RNAi-mediated silencing (RNAi-KD) of each *IrTEP* was followed by an *in vitro* phagocytic assay as described below.

A group-specific RNAi-KD was performed by injection of unfed females (30 ticks per group) with the mix of *IrC3* dsRNAs (*IrC3-1, IrC3-2,* and *IrC3-3)*, the mix of *IrA_2M* dsRNAs (*IrA_2M1, IrA_2M2,* and *IrA_2M3*) or control *GFP* dsRNA. After 24 h rest and natural feeding on guinea pigs for 6 days, the semi-engorged females were inoculated with *M. robertsii* and phagocytosis of conidia by tick hemocytes was evaluated using the *in vivo* phagocytosis assay (see below).

2.6. Latex beads pre-injection

For the experiment with latex beads (LtxB), 207 nL of 4-fold diluted surfactant-free red CML LtxB 0.3 μm diameter (Interfacial Dynamics Corp.) or 207 nL PBS (control group) were injected using a Drummond® microinjector into unfed females that rested for 24 h before natural feeding on guinea pigs for 6 days and then inoculated with 138 nL of *M. robertsii* suspension (2×10^5 or 2×10^6 conidia/mL, in total 28 or 280 conidia) to test tick mortality. Phagocytosis of fungal conidia by tick hemocytes upon LtxB pre-injection was evaluated using the *in vivo*

phagocytic assay (see below).

2.7. In vitro and in vivo assays for metarhizium phagocytosis

For each *in vitro* phagocytosis experiment, the hemolymph was collected from the cut leg of 25 semi-engorged females, and mixed with L15-Leibovitz medium (L15) (Biosera®) enriched with 10% inactivated fetal bovine serum (FBS) (PAA laboratories). The number of hemocytes was counted in a Bürker chamber and adjusted to 2×10^4 in 182 μL of culture medium pipetted onto a circular coverslip placed in a 24-well culture plate. The hemocytes were allowed to adhere for 15 min at 32 °C and then 2×10^5 *M. robertsii* conidia (exceeding ten times the hemocyte count) in 68 μL were added and incubated at 32 °C for 2 h or 6 h. The cells were fixed with 4% paraformaldehyde in PBS for 30 min and washed three times in PBS. The slides were then incubated with primary *anti-Metarhizium* rabbit immune serum (1:100) in a humid chamber for 1 h, washed, and incubated with secondary antibody (Goat anti-rabbit IgG Alexa Fluor 594) (Invitrogen®) at a dilution of 1:500 for 1 h at room temperature with agitation. The slides were washed and nuclei were counterstained with DAPI for 10 min and mounted with Fluoromount medium (Sigma®). Relative phagocytosis was evaluated using a BX53 fluorescence microscope (Olympus®). The phagocytic index was expressed as the percentage of hemocytes with phagocytosed conidia per 100 hemocytes counted, using at least six slides. The relative phagocytosis in RNAi-KD experiments was calculated in relation to the number of phagocytic hemocytes in the GFP control group, taken as 100% in the respective experiment. The experiments were repeated three times.

The *in vivo* phagocytosis assays of *M. robertsii* conidia by tick hemocytes were performed with semi-engorged females from the group-specific RNAi-KD (GFP control) or LtxB pre-injected (PBS control) cohorts. The ticks were injected with 5×10^4 *M. robertsii* conidia in 345 nL using a microinjector (Drummond®). Hemolymph samples were collected from individual females at specified time intervals after conidia injection and mixed with 15 μL of L15 medium supplemented with 10% FBS on the microscopic slides. Immunostaining and calculation of the phagocytic index or the relative phagocytosis were as described above for the *in vitro* assay.

2.8. Statistical analysis

The tick survival curves were analyzed by the Log-rank test ($P \leq$ 0,05). *In vitro* phagocytic index was analyzed by the unpaired *t*-test. Expression profile data were analyzed by one-way ANOVA followed by the Tukey test for pair-wise comparisons ($P \leq 0.05$). *In vitro* relative phagocytosis after RNAi-KD was analyzed by Kruskal Wallis followed by the Dun's test. *In vivo* relative phagocytosis after RNAi-KD was analyzed by two-way ANOVA followed by the Tukey test. The phagocytosis assay after LtxB pre-injection was analyzed by the unpaired *t*-test. All data were analyzed through GraphPad Prism version 8.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Mortality of I. ricinus females upon infection with M. robertsii

Unfed *I. ricinus* females were infected with *M. robertsii* (spore germination ~ 98%) either by direct capillary injection or by immersion into the conidial suspension (natural infection). Both ways of infection resulted in a dose-dependent significant increase in tick mortality (Fig. 1A, and B, respectively). Ticks injected with the highest fungal concentration (2×10^8 conidia/mL, ~28.000 conidia) started to die two days after infection, reaching 50% mortality on the 4th day and 100% mortality on the 8th day (Fig. 1A). Ticks inoculated with 2×10^7 or 2×10^6 conidia/mL (~2.800 or ~280 conidia, respectively) reached 50% mortality five and 11 days after inoculation, respectively. Half of the ticks infected with 2×10^5 conidia/mL (~28 conidia) remained alive for the 30 days of mortality monitoring and ticks injected with few conidia (2×10^4 conidia/mL) displayed almost no mortality in comparison with the control group (untreated ticks) (Fig. 1A). The lethal fungal concentration that killed 50% of the population (LC50) evaluated five days after inoculation, was determined to be 3.5×10^6 conidia/mL corresponding roughly to 500 conidia (95% CI = 2.14–5.47 $\times 10^6$ conidia/mL). For immersion infection, ticks exposed to the highest fungal concentration (2×10^8 conidia/mL) started to die four days after treatment, reaching 50% and 100% mortality on the 6th and 8th days, respectively (Fig. 1B). The slightly slower, but still significant, mortality was observed for immersion into 2×10^7 conidia/mL when 50% ticks had died 7 days after treatment and 90%, 11 days after treatment (Fig. 1B). Ticks immersed in the lowest concentration of conidia tested (2×10^6 conidia/mL) reached 50% mortality 15 days after treatment while the other half remained alive for at least 30 days. The LC50 estimated for natural infection with *M. robertsii* can be estimated to be about one order of magnitude higher than direct conidial injection.

3.2. Tick hemocytes can phagocytize M. robertsii in vitro

An *in vitro* phagocytic assay was performed to show that *I. ricinus* hemocytes can phagocytize conidia of *M. robertsii*. To distinguish between conidia that were ingested by tick hemocytes from those attached to the hemocyte surface, it was necessary to mutually compare phase contrast images (Fig. 2A) with the corresponding immunofluorescent images obtained after specific immunolabelling using antibody against *M. robertsii* (Fig. 2B). Only the free or adhered conidia were immunolabelled while the phagocytosed ones remained dark.

The *in vitro* phagocytosis of *M. robertsii* conidia was evaluated two or

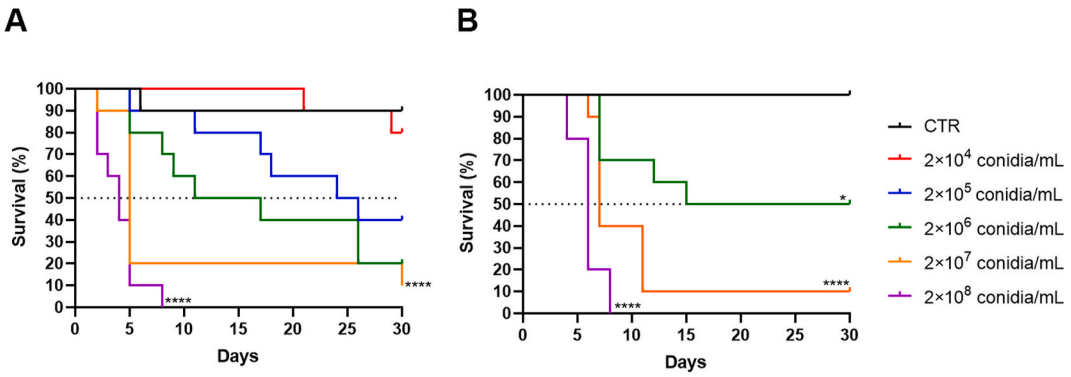

Fig. 1. Survival to fungal infection. Unfed *I. ricinus* females were inoculated with (A) or immersed in (B) a conidia suspension of different concentrations and mortality was recorded every 24 h over a 4-week period. Shown are representative experiments of a minimum of two independent repeats. The data were analyzed by the log-rank test. Asterisks indicate statistically significant differences between the control and the respective treated groups (****P < 0.0001; *$P < 0.05$).

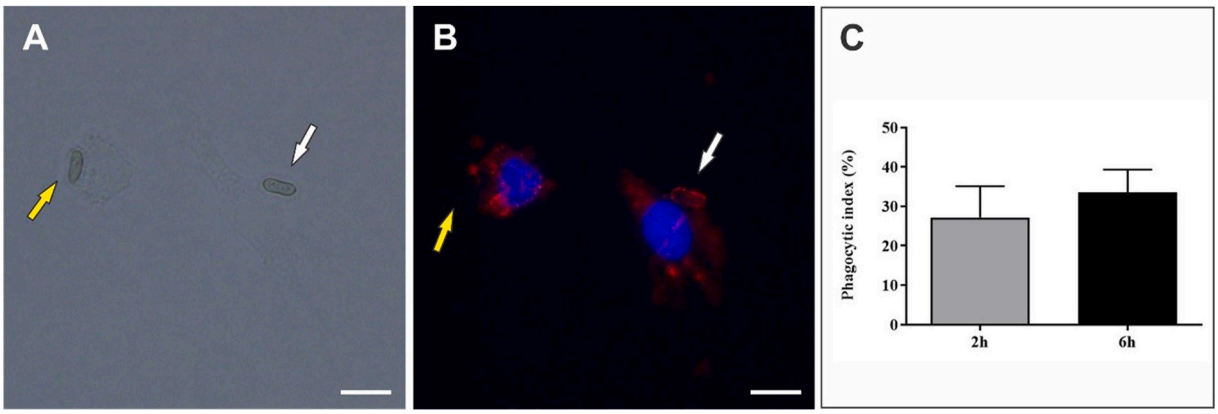

Fig. 2. Phagocytosis of *M. robertsii* conidia by tick hemocytes. Hemocytes from adult semi-engorged females were incubated with *M. robertsii* conidia *in vitro* for 2 or 6 h and immunolabelled. Phase contrast of hemocytes and conidia (A). Immunostaining of cells with *anti-M. robertsii* primary antibody and Alexa 594 secondary antibody (B). Cell nuclei were counterstained with DAPI (blue). Conidia localized outside of hemocytes are stained red (white arrow), engulfed conidia are without staining (yellow arrow). The number of phagocytic hemocytes is shown in graph (C). The phagocytic index was determined as a ratio of phagocytic and non-phagocytic hemocytes. The data were analyzed by unpaired *t*-test ($P > 0.05$). Error bars represent standard deviation. Scale bar represents 10 μm.

6 h after incubation with *I. ricinus* tick hemocytes. The percentage of hemocytes with internalized conidia increased rapidly up to ~40% phagocytic hemocytes within 6 h (Fig. 2C). Since there was no statistically significant difference between the phagocytic index observed two or 6 h after infection ($P \geq 0.05$), the 2 h interval was chosen for the following *in vitro* phagocytic assays.

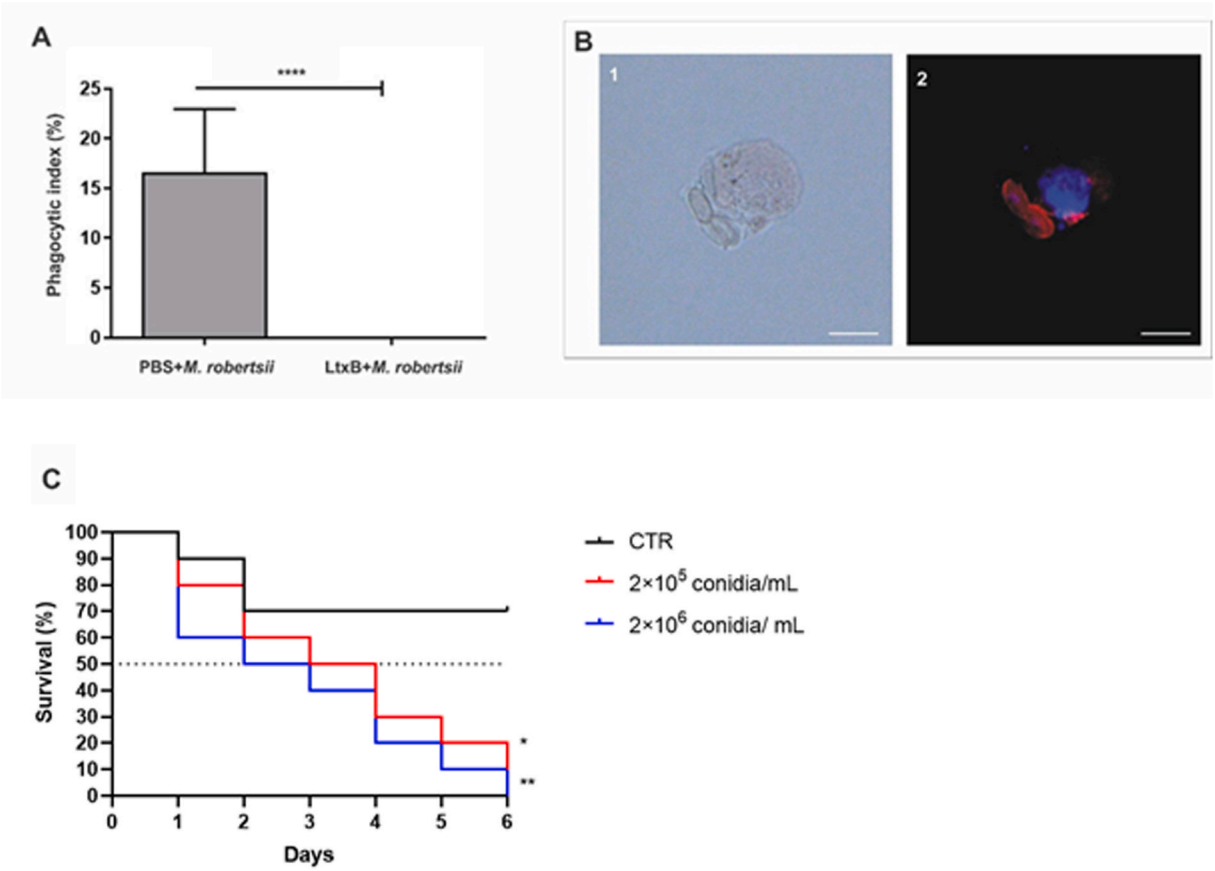

Fig. 3. Pre-injection of latex beads inhibits phagocytosis of *M. robertsii* by tick hemocytes and increases tick mortality. Phagocytosis of *M. robertsii* by tick hemocytes is eliminated by pre-injection of latex beads into the hemocoel (A). Hemolymph was collected from individual semi-engorged ticks (20 ticks for PBS and 20 ticks for latex beads) 3 h after injection of *Metarhizium*. Phagocytosis was evaluated using an *in vivo* phagocytosis assay and the phagocytic index was determined as the number of hemocytes with ingested conidia per 100 hemocytes. Data were analyzed by unpaired *t*-test. Error bar represents the standard deviation (****$P < 0.0001$). Panel B shows the detail of tick hemocyte with ingested latex beads and adhered conidia using phase contrast (1) and immunostaining of *M. robertsii* conidia (2). The scale bar is 10 μm. Pre-injection of latex beads into the tick hemocoel significantly increased tick mortality after *M. robertsii* infection (C). CTR-control ticks injected with PBS. The data were analyzed by the log-rank test. Statistically significant differences were observed when comparing 2×10^5 and 2×10^6 conidia/mL with the control group ($P = 0.0174$, $P = 0.0026$, respectively). Asterisks indicate statistically significant differences between the control and the respective treated groups (**$P < 0.01$; *$P < 0.05$). Results are representative experiments of a minimum of two independent repeats.

3.3. Elimination of *M. robertsii* phagocytosis in vivo using latex beads dramatically increases tick mortality

Our previous study demonstrated that phagocytosis of the *Borrelia* spirochetes can be efficiently eliminated by pre-injection of latex beads (LtxB) into the tick hemocoel (Urbanová et al., 2017). In the present study, unfed *I. ricinus* females were pre-injected with latex beads or PBS (control) and then allowed to feed naturally on guinea pigs for 6 days. Subsequently, females were injected with 5×10^4 conidia *M. robertsii* conidia and phagocytosis was evaluated 3 h after conidia injection using an *in vivo* phagocytosis assay. Pre-injection of LtxB resulted in a significant reduction in *Metarhizium* phagocytosis, where the phagocytic index decreased from 16% for the PBS control group to 0% for LtxB pre-injected group (Fig. 3A). Since the latex beads exhausted the phagocytic capacity of tick hemocytes, all *Metarhizium* conidia were outside of or only adhered to the cells (Fig. 3B).

To determine if the mortality caused by *M. robertsii* is affected by the tick capability to phagocytize the fungus, the following *in vivo* assay was performed: Unfed *I. ricinus* females pre-injected with LtxB were inoculated with 2×10^6 or 2×10^5 conidia/mL. These doses have only a mild impact on the mortality of ticks whose hemocytes have unimpaired phagocytic capacity (Fig. 1A). By contrast, injections of these amounts of *M. robertsii* conidia into LtxB pre-injected ticks dramatically increased their mortality as the mean survival time was only 2.5 or 3.5 days for females inoculated with 2×10^6 or 2×10^5 conidia/mL, respectively. Ticks in both experimental groups were dead six days after the treatment.

3.4. Expression of IrTEPs in response to M. robertsii infection

In our previous studies we have shown, that expression of *IrTEPs* differs specifically in response to infection with model microbes (e.g., *Escherichia coli*, *Chryseobacterium indologenes*, *Borrelia afzelii*, and *Candida albicans*) (Buresova et al., 2011; Urbanová et al., 2015, 2017). Here we examined the expression response of nine *IrTEPs* to the intra-haemocoelic injection of *M. robertsii* in whole unfed *I. ricinus* females and compared it to the injection of sterile PBS (aseptic injection control) or non-injected ticks (Fig. 4). Two hours after injection, statistically significantly up-regulation caused by *M. robertsii* infection in comparison with PBS control was observed in the expression of *IrC3-2*, *IrA$_2$M1*, *IrA$_2$M3*, *IrMcr1*, *IrMcr2*, and *IrTep*, with 6.19, 2.12, 3.92, 2.47, 2.85, and 2.19-fold changes, respectively. Expression of *IrC3-1* also seemed to be up-regulated, albeit the differences did not fulfill the criteria of our statistical test. The expression of *IrC3-3* and *IrA$_2$M2* were not affected after fungal infection (Fig. 4). The expression responses of *IrTEPs* were also evaluated 6 h after *M. robertsii* injection, where increased mRNA levels were observed only for *IrA$_2$M3,* showing an 8.38-fold change over the control PBS group (Fig. A1). Interestingly, this time interval

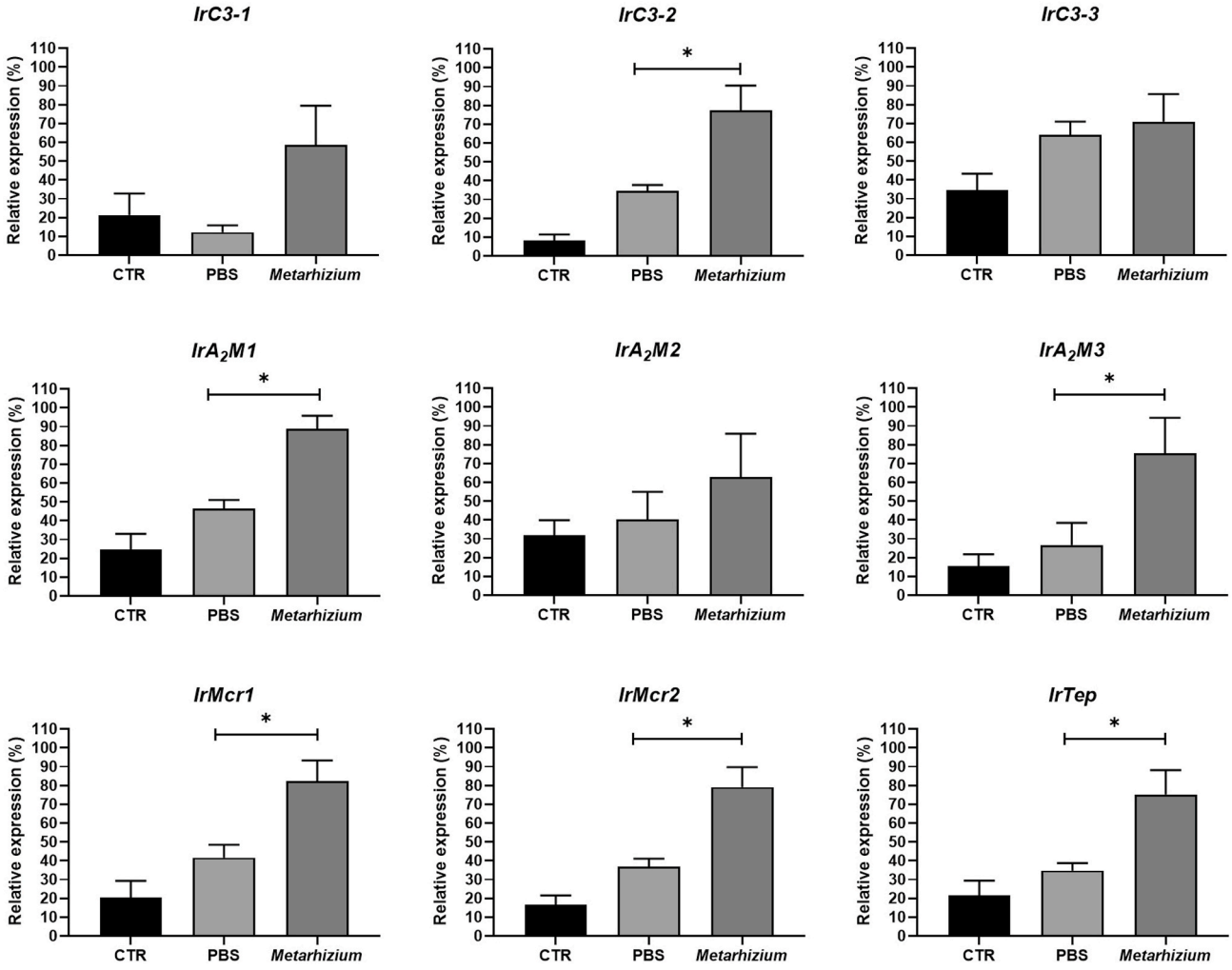

Fig. 4. Relative expression of *IrTEPs* in response to aseptic or *M. robertsii* injection. Unfed females were injected with 138 nL of sterile PBS or 138 nL of *M. robertsii* (2×10^7 conidia/mL, ~2.800 conidia) and the total RNA was isolated from the tick entire body homogenates 2 h post injection or from un-injected ticks (CTR). For gene expression analysis, the *elongation factor* (*elf-1*) was used as the reference gene. The bars represent ± SEM and asterisks indicate statistical difference by ANOVA one-way test followed by the Tukey test ($P < 0.05$).

post-injection also revealed a clear difference of *IrA$_2$M2* mRNA levels between PBS injected and non-injected females, suggesting that expression of this molecule was injury responsive.

3.5. The role of IrTEPs in M. robertsii phagocytosis

The specific non-redundant roles of *Ir*TEPs in cellular immune responses against different model microbes, including *Borrelia* spirochetes, have been described previously (Buresova et al., 2011; Urbanová et al., 2015, 2017). The *in vitro* phagocytic assay described above for the conidia *M. robertsii* allowed us to examine the role of *Ir*TEPs in the phagocytosis of this EPF. The effect of RNAi-mediated silencing (KD) of individual *IrTEPs* genes on the phagocytic rate of *M. robertsii* by tick hemocytes is shown in Fig. 5. The most significant reduction in the relative phagocytic index was observed upon RNAi-KD of *IrA$_2$M2*, followed by *IrC3-3* KD, and *IrA$_2$M1* KD, (68%, 60%, and 57% reduction, respectively). About 50% reduction of *M. robertsii* phagocytosis was also observed for RNAi-KD of both macroglobulin complement-related encoding genes *IrMcr1* and *IrMcr2* (Fig. 5).

Our previous tissue expression and Western blot analyses (Urbanová et al., 2015), together with the transcriptomic analysis of *I. ricinus* hemocytes (Kotsyfakis et al., 2015) indicate that molecules belonging to the group of C3-complement components and α_2-macrolobulins are expressed mainly in the tick fat body and/or hemocytes. Their secretion into and presence in the tick plasma was further confirmed by the recent proteomic analysis (unpublished results). The pivotal roles of these secreted *Ir*TEPs in the phagocytosis *M. robertsii* was further confirmed using *in vivo* phagocytic assays. Cohorts of 30 unfed *I. ricinus* females were injected with either a mix of *IrC3-1,2,3* dsRNA, a mix of *IrA$_2$M1,2,3* dsRNA or *GFP* dsRNA for control. The females were allowed to feed naturally on guinea pigs and on the 6th day, the females were injected with 5×10^4 conidia. The hemolymph was collected 2, 6, and 12 h post-injection, and the number of phagocytic hemocytes was counted as described above. The result of *in vivo* phagocytosis of *M. robertsii* by tick hemocytes after the group-specific silencing is shown in Fig. 6. The number of phagocytic hemocytes was significantly reduced in both experimental groups (*IrC3s* and *IrA$_2$Ms*) compared to the GFP control group in all three time points examined. The silencing of the *IrC3s* group

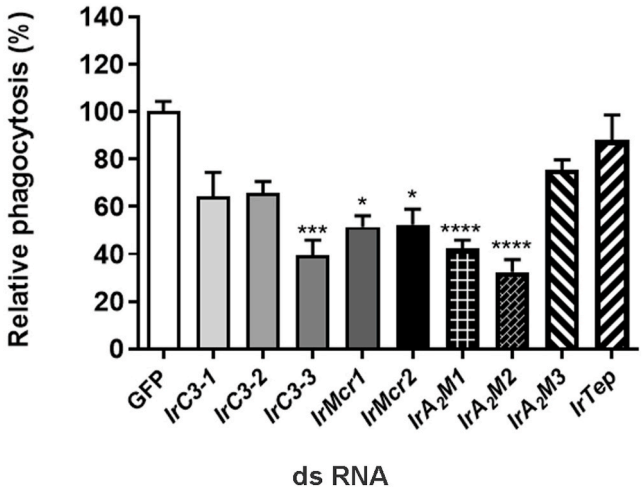

Fig. 5. The role of *Ir*TEPs in phagocytosis of *M. robertsii* by tick hemocytes. Unfed *I. ricinus* females were injected with individual *IrTEP* dsRNAs or *GFP* dsRNA for control. Hemolymph collected from semi-engorged females was incubated with *M. robertsii* conidia *in vitro*. The number of phagocytic hemocytes was related to that obtained for the *GFP* control group taken as 100% in the respective experiments. The experiment was repeated three times. Error bars represent standard error. Asterisks indicate statistically significant differences between the *GFP* and the respective *IrTEPs* (****$P < 0.0001$; ***$P < 0.001$, *$P < 0.05$) by Kruskal-Wallis test followed by Dunn's test.

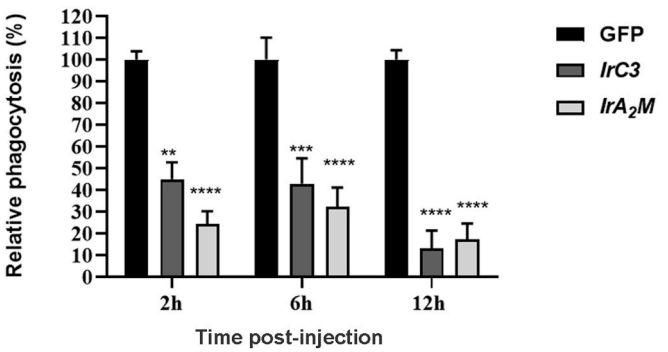

Fig. 6. *In vivo* reduction of *M. robertsii* phagocytosis after *IrC3s* and *IrA$_2$Ms* group-specific RNAi-KD. Semi-engorged females with group-specific gene silencing were injected with 5×10^4 of *M. robertsii* conidia. Hemolymph samples from different time points after injection of conidia were collected from individual ticks (30 ticks per time point). Relative phagocytosis was evaluated using an *in vivo* assay, where the *GFP* control group was taken as 100% in the respective experiment. Error bars represent standard error from three independent treatments. Asterisks indicate statistically significant differences between the GFP and the respective silenced groups in each time point (****$P < 0.0001$; ***$P < 0.001$, **$P < 0.01$) by Two-way ANOVA followed by Tukey test.

resulted in approximately 55%, 55%, and 85% reduction in phagocytosis in 2 h, 6 h, and 12 h, respectively. An even more significant effect on fungal phagocytosis by tick hemocytes was achieved upon group silencing of *IrA$_2$Ms,* resulting in the reduction of phagocytosis index by 75%, 70% and 80% in 2 h, 6 h, and 12 h, respectively (Fig. 6).

4. Discussion

Entomopathogenic fungi (EPF) are the most studied and well established microorganisms used in biological control programs against agricultural arthropod pests (Quesada-Moraga et al., 2004; Santi et al., 2011). EPF are highly virulent for several insects species (Wang and St. Leger, 2006; Keppanan et al., 2018; Jiang et al., 2020). Even though there are studies with excellent results using fungi to control ticks (Camargo et al., 2016; Marciano et al., 2020; Mesquita et al., 2020), the efficacy against tick engorged females in the field should be improved to meet the results that are expected by the market and the regulatory agencies. Here, *M. robertsii* was virulent on *I. ricinus* adult females at the highest concentrations (2×10^8 and 2×10^7 conidia/mL, meaning ~28.000 and ~2.800 conidia injected per tick, respectively), the doses commonly used for biological tests with ticks. Lower doses (2×10^5 and 2×10^6 conidia/mL, meaning dozens to several hundred conidia per injection) did not cause high tick mortality, suggesting that ticks could resolve a low fungal infection. Here, the fungus at a lower concentration (2×10^6 conidia/mL) took four weeks to kill 50% *I. ricinus* unfed females when the ticks were immersed in a conidial suspension. A similar mortality to *M. robertsii* infection was reported for other hard tick species Butt et al. (2016) and Kirkland et al. (2004). In addition to the virulence of the fungal isolate to the arthropod host, studies have reported that different tick species have different compounds in their cuticle, which makes them more or less tolerant to fungal penetration (Ment et al., 2012; Butt et al., 2016). It is also already known that different tick populations of the same tick species may have different susceptibilities to the fungus (Perinotto et al., 2017; Webster et al., 2017), suggesting that there are many factors involved in the tick tolerance to EPF.

During fungal infection, ticks and other arthropods try to eliminate the pathogen through the cellular and humoral immune responses. The most important cellular immune response against foreign intruders of ticks is phagocytosis, mediated mainly by granulocytes and plasmatocytes (Munderloh and Kurtti, 1995; Fiorotti et al., 2019; Fogaça et al., 2021). The phagocytosis of EPF in the arthropod hemocoel as a defence mechanism is frequently mentioned but experimental evidences for this

process are rather rare (Zimmermann, 2007; Kurtti and Keyhani, 2008; Dostálová et al., 2017).

Here, the *in vitro* phagocytosis test with *I. ricinus* hemocytes and *M. robertsii* conidia demonstrated an immediate response and apparent phagocytic activity, reaching up to 40% phagocytic hemocytes in 6 h (Fig. 2C). Similar results were reported by (Kurtti and Keyhani, 2008), using *I. scapularis* embryonic cells with the same fungal isolate. Here, the specific immune sera raised against *M. robertsii* cell walls clearly labeled the conidia present in the plasma or adhered to the hemocytes but weakly labeled the internalized conidia. A possible explanation for the weak label inside the hemocytes is phagosome acidification caused by the increased influx of chloride, in the initial phase of phagocytosis, which quickly degrades the fluorescent signal (Zhang et al., 2020). The other possibility is that the conidia engulfed in the hemocytes rapidly alter their surface carbohydrate epitopes, as previously demonstrated by the lectin mapping of *Beauveria bassiana* isolated from the hemolymph of infected moth larvae (Wanchoo et al., 2009).

The key role of phagocytosis in protection of ticks against low doses of *M. robertsii* infection was demonstrated here by tick pre-injection with latex beads. It was earlier shown that injection of latex or polystyrene beads suppresses phagocytosis in fruit fly (Nehme et al., 2011) or ticks (Liu et al., 2011). We have previously reported, that pre-injection of LtxB into *I. ricinus* dramatically reduces the number of circulating hemocytes, resulting in complete abolishing of *B. afzelii* phagocytosis, yet without any effect on the transmission of spirochetes to the host (Urbanová et al., 2017). In this study we used the LtxB pre-injection to demonstrate that elimination of hemocyte capacity to phagocytose *M. robertsii* results in significantly increased mortality of ticks inoculated with sublethal doses of fungi (Fig. 3).

Inspired by the mammalian system of complement that is involved in foreign recognition, opsonisation and phagocytosis of the microbial intruders (Ricklin et al., 2010), we have been investigating, for more than a decade, components of a tick complement system and their roles in phagocytosis of model microbes and tick-borne pathogens (Buresova et al., 2011; Kopacek et al., 2012; Fogaça et al., 2021). The present study, focused on the *I. ricinus* TEPs, allowed us to compare the results achieved for the entomopathogenic *M. robertsii* with the model fungi, the yeast *Candida albicans* (Urbanová et al., 2015). In response to fungal injection, *C. albicans* significantly up-regulated expression of only *IrC3-1,* while most *Ir*TEPs (specifically *IrC3-2*, *IrA$_2$M1*, *IrA$_2$M3, IrMcr1*, *IrMcr2,* and *IrTep)* were responsive to *M. robertsii* 2 h post-injection (Fig. 4). However, it is important to take into consideration the dynamic changes of the immune response as only the *IrA$_2$M3* gene remained up-regulated 6 h post-injection (Fig. A1).

RNAi-KD of individual *IrTEPs* followed by the *in vitro* phagocytic assay demonstrated that *M. robertsii* phagocytosis was significantly reduced when the *IrC3-3*, *IrMcr1, IrMcr2, IrA$_2$M1* and *IrA$_2$M2* genes were silenced, reaching the highest 68% reduction in relative phagocytosis in the case of *IrA$_2$M2* RNAi-KD (Fig. 5). For phagocytosis of *C. albicans*, the maximal suppression (~40%) was achieved upon *IrC3-3* RNAi-KD (Urbanová et al., 2015). The crucial role in phagocytosis of *M. robertsii* by *I. ricinus* was confirmed by the group-specific RNA-KD of all *IrC3s* or *IrA$_2$Ms* that almost eliminated phagocytosis of the fungi, when evaluated by an *in vivo* assay 12 h post-fungal injection (Fig. 6).

Interestingly, the *Ir*A$_2$M1, *Ir*A$_2$M2 and *Ir*C3-3 identified here as the most important for phagocytosis of *M. robertsii* were also found to be the main *Ir*TEPs involved in the phagocytosis of another *I. ricinus* pathogen, namely the Gram-negative bacterium *C. indologenes* (Buresova et al., 2009, 2011). The common denominator of both pathogens are the secreted Zn-dependent metalloproteases. The metalloprotease from *C. indologenes* (Venter et al., 1999) was proposed to be the major virulence factor of this pathogen (Pan et al., 2000). The recently described Zn-dependent metalloproteases from *M. robertsii* (Mrmep1 or Mrmep2) were suggested to be involved in the digestion of hemolymph proteins and binding to danger-sensing receptors (Zhou et al., 2018). Metalloproteases associated with EPF were reported to facilitate fungal development in the insect host (*Galleria mellonella*) and also inhibit the phagocytic activity of its hemocytes (Griesch and Vilcinskas, 1998; Mukherjee and Vilcinskas, 2018). The molecular interactions of the pathogen-derived metalloproteases with α_2-macroglobulins and/or C3-like complement components circulating in the tick hemolymph are not yet elucidated and warrant further investigation.

Knowledge about the immune response of arthropods has progressed remarkably. Our study provided an analysis of survival strategies adopted by ticks challenged with EPF, as well as insights into tick immunology in general. Many fungal isolates used in biological control do not produce satisfactory results against ticks, and host susceptibility may be one of the factors involved, not only triggered by the arthropod's cuticle composition but also because it involves the arthropod's immune response. Insect versus EPF interactions are much more frequently studied. Ticks usually are assumed to follow the same rules as insects when inferring their interactions with fungal pathogens. Still, these systems may have contrasting processes and assuming similarities may raise critical consequences. Knowledge about the immune response after challenging with EPF is therefore very relevant for the development of alternate techniques for tick control.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104234.

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References

Aldén, L., Demoling, F., Bååth, E., 2001. Rapid method of determining factors limiting bacterial growth in Soil. Appl. Environ. Microbiol. 67, 1830–1838. https://doi.org/10.1128/AEM.67.4.1830-1838.2001.

Beys-da-Silva, W.O., Rosa, R.L., Berger, M., Coutinho-Rodrigues, C.J.B., Vainstein, M.H., Schrank, A., Bittencourt, V.R.E.P., Santi, L., 2020. Updating the application of *Metarhizium anisopliae* to control cattle tick *Rhipicephalus microplus* (Acari: ixodidae). Exp. Parasitol. 208, 107812. https://doi.org/10.1016/j.exppara.2019.107812.

Buresova, V., Hajdusek, O., Franta, Z., Loosova, G., Grunclova, L., Levashina, E.A., Kopacek, P., 2011. Functional genomics of tick thioester-containing proteins reveal the ancient origin of the complement system. J. Innate Immun. 3, 623–630. https://doi.org/10.1159/000328851.

Buresova, V., Hajdusek, O., Franta, Z., Sojka, D., Kopacek, P., 2009. IrAM-An α2-macroglobulin from the hard tick *Ixodes ricinus:* characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. Dev. Comp. Immunol. 33, 489–498. https://doi.org/10.1016/j.dci.2008.09.011.

Butt, T.M., Wood, M., Taylor, J.W.D., Bakirci, S., Hazir, C., Ulug, D., Hazir, S., 2016. Differential susceptibility of *Hyalomma excavatum* adults and nymphs to the entomopathogens *Metarhizium anisopliae* ARSEF 4556 and Steinernema carpocapsae. Int. J. Pest Manag. 62, 261–266. https://doi.org/10.1080/09670874.2016.1181287.

Camargo, M.G., Nogueira, M.R.S., Marciano, A.F., Perinotto, W.M.S., Coutinho-Rodrigues, C.J.B., Scott, F.B., Angelo, I.C., Prata, M.C.A., Bittencourt, V.R.E.P., 2016. *Metarhizium anisopliae* for controlling *Rhipicephalus microplus* ticks under field conditions. Vet. Parasitol. 223, 38–42. https://doi.org/10.1016/j.vetpar.2016.04.014.

De La Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M., Sonenshine, D.E., 2008. Overview: ticks as vectors of pathogens that cause disease in humans and animals. Front. Biosci. 13, 6938–6946. https://doi.org/10.2741/3200.

Dostálová, A., Rommelaere, S., Poidevin, M., Lemaitre, B., 2017. Thioester-containing proteins regulate the Toll pathway and play a role in *Drosophila* defence against microbial pathogens and parasitoid wasps. BMC Biol. 15, 1–16. https://doi.org/10.1186/s12915-017-0408-0.

Fiorotti, J., Menna-Barreto, R.F.S., Gôlo, P.S., Coutinho-Rodrigues, C.J.B., Bitencourt, R. O.B., Spadacci-Morena, D.D., Da Costa Angelo, I., Bittencourt, V.R.E.P., 2019. Ultrastructural and cytotoxic effects of *Metarhizium robertsii* infection on *Rhipicephalus microplus* hemocytes. Front. Physiol. 10 https://doi.org/10.3389/fphys.2019.00654.

Fogaça, A.C., Sousa, G., Pavanelo, D.B., Esteves, E., Martins, L.A., Urbanová, V., Kopáček, P., Daffre, S., 2021. Tick immune system: what is known, the interconnections, the gaps, and the challenges. Front. Immunol. 12, 1–23. https://doi.org/10.3389/fimmu.2021.628054.

Griesch, J., Vilcinskas, A., 1998. Proteases released by entomopathogenic fungi impair phagocytic activity, attachment and spreading of plasmatocytes isolated from haemolymph of the greater wax moth *Galleria mellonella*. Biocontrol Sci. Technol. 8, 517–531. https://doi.org/10.1080/09583159830036.

Hartelt, K., Wurst, E., Collatz, J., Zimmermann, G., Kleespies, R.G., Oehme, R.M., Kimmig, P., Steidle, J.L.M., Mackenstedt, U., 2008. Biological control of the tick *Ixodes ricinus* with entomopathogenic fungi and nematodes: preliminary results from laboratory experiments. Int. J. Med. Microbiol. 298, 314–320. https://doi.org/10.1016/j.ijmm.2007.10.003.

Jiang, W., Peng, Y., Ye, J., Wen, Y., Liu, G., Xie, J., 2020. Effects of the entomopathogenic fungus *Metarhizium anisopliae* on the mortality and immune response of *Locusta migratoria*. Insects 11, 1–12. https://doi.org/10.3390/insects11010036.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129. https://doi.org/10.1017/S0031182004005967.

Karesh, W.B., Dobson, A., Lloyd-Smith, J.O., Lubroth, J., Dixon, M.A., Bennett, M., Aldrich, S., Harrington, T., Formenty, P., Loh, E.H., MacHalaba, C.C., Thomas, M.J., Heymann, D.L., 2012. Ecology of zoonoses: natural and unnatural histories. Lancet 380, 1936–1945. https://doi.org/10.1016/S0140-6736(12)61678-X.

Keppanan, R., Sivaperumal, S., Ramos Aguila, L.C., Hussain, M., Bamisile, B.S., Dash, C. K., Wang, L., 2018. Isolation and characterization of *Metarhizium anisopliae* TK29 and its mycoinsecticide effects against subterranean termite *Coptotermes formosanus*. Microb. Pathog. 123, 52–59. https://doi.org/10.1016/j.micpath.2018.06.040.

Kirkland, B.H., Westwood, G.S., Keyhani, N.O., 2004. Pathogenicity of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to ixodidae tick species *Dermacentor variabilis, Rhipicephalus sanguineus*, and *Ixodes scapularis*. J. Med. Entomol. 41, 705–711. https://doi.org/10.1603/0022-2585-41.4.705.

Klafke, G., Webster, A., Dall Agnol, B., Pradel, E., Silva, J., de La Canal, L.H., Becker, M., Osório, M.F., Mansson, M., Barreto, R., Scheffer, R., Souza, U.A., Corassini, V.B., dos Santos, J., Reck, J., Martins, J.R., 2017. Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul state, Southern Brazil. Ticks Tick. Borne. Dis. 8, 73–80. https://doi.org/10.1016/j.ttbdis.2016.09.019.

Kopacek, P., Hajdusek, O., Buresova, V., 2012. Tick as a model for the study of a primitive complement system. Adv. Exp. Med. Biol. 710, 83–93. https://doi.org/10.1007/978-1-4419-5638-5_9.

Kotsyfakis, M., Kopáček, P., Franta, Z., Pedra, J.H.F., Ribeiro, J.M.C., 2015. Deep sequencing analysis of the *Ixodes ricinus* haemocytome. PLoS Neglected Trop. Dis. 9, 1–22. https://doi.org/10.1371/journal.pntd.0003754.

Kurtti, T.J., Keyhani, N.O., 2008. Intracellular infection of tick cell lines by the entomopathogenic fungus *Metarhizium anisopliae*. Microbiology 154, 1700–1709. https://doi.org/10.1099/mic.0.2008/016667-0.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., Kafatos, F.C., 2001. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. Cell 104, 709–718. https://doi.org/10.1016/S0092-8674(01)00267-7.

Liu, L., Narasimhan, S., Dai, J., Zhang, L., Cheng, G., Fikrig, E., 2011. *Ixodes scapularis* salivary gland protein P11 facilitates migration of *Anaplasma phagocytophilum* from the tick gut to salivary glands. EMBO Rep. 12, 1196–1203. https://doi.org/10.1038/embor.2011.177.

Lorenz, S.C., Humbert, P., Patel, A.V., 2020. Chitin increases drying survival of encapsulated *Metarhizium pemphigi* blastospores for *Ixodes ricinus* control. Ticks Tick. Borne. Dis. 11, 101537. https://doi.org/10.1016/j.ttbdis.2020.101537.

Lu, H.L., St Leger, R.J., 2016. Insect immunity to entomopathogenic fungi. Adv. Genet. 94, 251–285. https://doi.org/10.1016/bs.adgen.2015.11.002.

Marciano, A.F., Golo, P.S., Coutinho-Rodrigues, C.J.B., Camargo, M.G., Fiorotti, J., Mesquita, E., Corrêa, T.A., Perinotto, W.M.S., Bittencourt, V.R.E.P., 2020. *Metarhizium anisopliae* sensu lato (s.l.) oil-in-water emulsions drastically reduced *Rhipicephalus microplus* larvae outbreak population on artificially infested grass. Med. Vet. Entomol. 34, 488–492. https://doi.org/10.1111/mve.12445.

Mascarin, G.M., Lopes, R.B., Delalibera, Í., Fernandes, É.K.K., Luz, C., Faria, M., 2019. Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. J. Invertebr. Pathol. 165, 46–53. https://doi.org/10.1016/j.jip.2018.01.001.

Ment, D., Churchill, A.C.L., Gindin, G., Belausov, E., Glazer, I., Rehner, S.A., Rot, A., Donzelli, B.G.G., Samish, M., 2012. Resistant ticks inhibit *Metarhizium* infection prior to haemocoel invasion by reducing fungal viability on the cuticle surface. Environ. Microbiol. 14, 1570–1583. https://doi.org/10.1111/j.1462-2920.2012.02747.x.

Mesquita, E., Marciano, A.F., Corval, A.R.C., Fiorotti, J., Corrêa, T.A., Quinelato, S., Bittencourt, V.R.E.P., Golo, P.S., 2020. Efficacy of a native isolate of the entomopathogenic fungus *Metarhizium anisopliae* against larval tick outbreaks under semifield conditions. BioControl 65, 353–362. https://doi.org/10.1007/s10526-020-10006-1.

Mukherjee, K., Vilcinskas, A., 2018. The entomopathogenic fungus *Metarhizium robertsii* communicates with the insect host galleria mellonella during infection. Virulence 9, 402–413. https://doi.org/10.1080/21505594.2017.1405190.

Munderloh, U.G., Kurtti, T.J., 1995. Cellular and molecular interrelationships between ticks and prokaryotic tick-borne pathogens. Annu. Rev. Entomol. 40, 221–243. https://doi.org/10.1146/annurev.en.40.010195.001253.

Nehme, N.T., Quintin, J., Cho, J.H., Lee, J., Lafarge, M.C., Kocks, C., Ferrandon, D., 2011. Relative roles of the cellular and humoral responses in the *Drosophila* host defense against three gram-positive bacterial infections. PloS One 6. https://doi.org/10.1371/journal.pone.0014743.

Nijhof, A.M., Balk, J.A., Postigo, M., Jongejan, F., 2009. Selection of reference genes for quantitative RT-PCR studies in *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus appendiculatus* ticks and determination of the expression profile of Bm86. BMC Mol. Biol. 10, 1–14. https://doi.org/10.1186/1471-2199-10-112.

Ojeda-Chi, M.M., Rodriguez-Vivas, R.I., Galindo-Velasco, E., Lezama-Gutiérrrez, R., 2010. Laboratory and field evaluation of *Metarhizium anisopliae* (Deuteromycotina: hyphomycetes) for the control of *Rhipicephalus microplus* (Acari: ixodidae) in the Mexican tropics. Vet. Parasitol. 170, 348–354. https://doi.org/10.1016/j.vetpar.2010.02.022.

Pan, H.J., Teng, L.J., Chen, Y.C., Hsueh, P.R., Yang, P.C., Ho, S.W., Luh, K.T., 2000. High protease activity of *Chryseobacterium indologenes* isolates associated with invasive infection. J. Microbiol. Immunol. Infect. 33, 223–226.

Pereira, L.S., Oliveira, P.L., Barja-Fidalgo, C., Daffre, S., 2001. Production of reactive oxygen species by hemocytes from the cattle tick *Boophilus microplus*. Exp. Parasitol. 99, 66–72. https://doi.org/10.1006/expr.2001.4657.

Perinotto, W.M.S., Angelo, I.C., Golo, P.S., Camargo, M.G., Quinelato, S., Sá, F.A., Coutinho Rodrigues, C.J.B., Marciano, A.F., Monteiro, C.M.O., Bittencourt, V.R.E.P., 2017. In vitro pathogenicity of different *Metarhizium anisopliae* s.l. isolates in oil formulations against *Rhipicephalus microplus*. Biocontrol Sci. Technol. 27, 338–347. https://doi.org/10.1080/09583157.2017.1289151.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 16–21. https://doi.org/10.1093/nar/29.9.e45.

Quesada-Moraga, E., Santos-Quirós, R., Valverde-García, P., Santiago-Álvarez, C., 2004. Virulence, horizontal transmission, and sublethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: blattellidae). J. Invertebr. Pathol. 87, 51–58. https://doi.org/10.1016/j.jip.2004.07.002.

Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 11, 785–797. https://doi.org/10.1038/ni.1923.

Samish, M., Ginsberg, H., Glazer, I., 2008. Anti-tick biological control agents: assessment and future perspectives. Ticks Biol. Dis. Control 36, 447–469. https://doi.org/10.1093/jmedent/36.6.733.

Santi, L., e Silva, L.A.D., da Silva, W.O.B., Corrêa, A.P.F., Rangel, D.E.N., Carlini, C.R., Schrank, A., Vainstein, M.H., 2011. Virulence of the entomopathogenic fungus *Metarhizium anisopliae* using soybean oil formulation for control of the cotton stainer bug, *Dysdercus peruvianus*. World J. Microbiol. Biotechnol. 27, 2297–2303. https://doi.org/10.1007/s11274-011-0695-5.

Sekiguchi, R., Nonaka, M., 2015. Evolution of the complement system in protostomes revealed by de novo transcriptome analysis of six species of Arthropoda. Dev. Comp. Immunol. 50, 58–67. https://doi.org/10.1016/j.dci.2014.12.008.

Stroschein-Stevenson, S.L., Foley, E., O'Farrell, P.H., Johnson, A.D., 2006. Identification of *Drosophila* gene products required for phagocytosis of *Candida albicans*. PLoS Biol. 4 https://doi.org/10.1371/journal.pbio.0040004, 0087–0099.

Tawidian, P., Rhodes, V.L., Michel, K., 2019. Mosquito-fungus interactions and antifungal immunity. Insect Biochem. Mol. Biol. 111, 103182. https://doi.org/10.1016/j.ibmb.2019.103182.

Urbanová, V., Hajdušek, O., Mondeková, H.H., Šíma, R., Kopáček, P., 2017. Tick thioester-containing proteins and phagocytosis do not affect transmission of *Borrelia afzelii* from the competent vector ixodes ricinus. Front. Cell. Infect. Microbiol. 7, 1–11. https://doi.org/10.3389/fcimb.2017.00073.

Urbanová, V., Šíma, R., Šauman, I., Hajdušek, O., Kopáček, P., 2015. Thioester-containing proteins of the tick *Ixodes ricinus*: gene expression, response to microbial challenge and their role in phagocytosis of the yeast *Candida albicans*. Dev. Comp. Immunol. 48, 55–64. https://doi.org/10.1016/j.dci.2014.09.004.

Venter, H., Osthoff, G., Litthauer, D., 1999. Purification and characterization of a metalloprotease from *Chryseobacterium indologenes* Ix9a and determination of the amino acid specificity with electrospray mass spectrometry. Protein Expr. Purif. 15, 282–295. https://doi.org/10.1006/prep.1998.1020.

Wanchoo, A., Lewis, M.W., Keyhani, N.O., 2009. Lectin mapping reveals stage-specific display of surface carbohydrates in in vitro and haemolymph-derived cells of the entomopathogenic fungus *Beauveria bassiana*. Microbiology 155, 3121–3133. https://doi.org/10.1099/mic.0.029157-0.

Wang, C., St Leger, R.J., 2006. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc. Natl. Acad. Sci. U.S.A. 103, 6647–6652. https://doi.org/10.1073/pnas.0601951103.

Wang, J.B., St Leger, R.J., Wang, C., 2016. Advances in genomics of entomopathogenic fungi. Adv. Genet. 94, 67–105. https://doi.org/10.1016/bs.adgen.2016.01.002.

Wassermann, M., Selzer, P., Steidle, J.L.M., Mackenstedt, U., 2016. Biological control of *Ixodes ricinus* larvae and nymphs with *Metarhizium anisopliae* blastospores. Ticks Tick. Borne. Dis. 7, 768–771. https://doi.org/10.1016/j.ttbdis.2016.03.010.

Webster, A., Pradel, E., Souza, U.A., Martins, J.R., Reck, J., Schrank, A., Klafke, G., 2017. Does the effect of a *Metarhizium anisopliae* isolate on *Rhipicephalus microplus* depend on the tick population evaluated? Ticks Tick. Borne. Dis. 8, 270–274. https://doi.org/10.1016/j.ttbdis.2016.11.012.

Zhang, X., Mao, F., Wong, N.K., Bao, Y., Lin, Y., Liu, K., Li, J., Xiang, Z., Ma, H., Xiao, S., Zhang, Y., Yu, Z., 2020. CLIC2α chloride channel orchestrates immunomodulation of

hemocyte phagocytosis and bactericidal activity in Crassostrea gigas. iScience 23, 101328. https://doi.org/10.1016/j.isci.2020.101328.

Zhou, R., Zhou, X., Fan, A., Wang, Z., Huang, B., 2018. Differential functions of two metalloproteases, Mrmep1 and Mrmep2, in growth, sporulation, cell wall integrity, and virulence in the filamentous fungus *Metarhizium robertsii*. Front. Microbiol. 9, 1–11. https://doi.org/10.3389/fmicb.2018.01528.

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Sci. Technol. 17, 879–920. https://doi.org/10.1080/09583150701593963.