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# IrC2/Bf – A yeast and *Borrelia* responsive component of the complement system from the hard tick *Ixodes ricinus*☆

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ABSTRACT

Ticks possess components of a primordial complement system that presumably play a role in the interaction of the tick immune system with tick-borne pathogens and affect their transmission. Here we characterized a novel complement component, tagged as IrC2/Bf, from the hard tick *Ixodes ricinus*, the principal vector of Lyme disease in Europe. IrC2/Bf is a multi-domain molecule composed of 5–7 CCP modules, varied by alternative splicing, followed by a von Willebrand factor A domain and a C-terminal trypsin-like domain. The primary structure and molecular architecture of IrC2/Bf displays the closest homology to the C3-complement component convertases described in horseshoe crabs. The *irc2/bf* gene is mainly expressed in the tick fat body associated with the trachea and, as determined by western blotting, the protein is present in low amounts in tick hemolymph. Expression of *irc2/bf* mRNA was significantly up-regulated in response to the intra-hemocoelic injection of the yeast *Candida albicans* and all tested *Borrelia* sp. strains (*B. burgdorferi* NE5264, *B. burgdorferi* CB26, *B. garinii* MSLB, *B. afzelii* CB43), but was not affected by injection of model Gram-negative and Gram-positive bacteria or the aseptic injection control. In-line with these results, RNAi-mediated silencing of *irc2/bf* inhibited phagocytosis of *B. afzelii* and *C. albicans* but not the other bacteria. Tissue expression profiles, specific responses to microbial challenges, and patterns of phagocytic phenotypes upon RNAi silencing observed for IrC2/Bf match well with the previously reported characteristics of *I. ricinus* C3-related molecule 1 (IrC3-1). Therefore we presume that IrC2/Bf functions as a convertase in the same complement activation pathway protecting ticks against yeast and *Borrelia* infection.



## 1. Introduction

Since the discovery of the first complement components, towards the end of the 19th century, following and burgeoning studies have gradually resulted in the assembly of the currently known complex mosaic of a complement system; see (Chaplin, 2005) for review. The mammalian complement system is composed of more than 35 individual serum or cell membrane proteins that are orchestrated in a network allowing a versatile and powerful first-line defense against non-self intruders (Ricklin et al., 2010). Compared to this, the recognition of the existence of a primordial complement system in lower animals such as sea anemones or arthropods is relatively recent and is based on the knowledge of a limited number of molecules related to the central complement component C3 (Ricklin et al., 2016), C2 and/or Bf convertases or ficolin-like lectins (Kimura et al., 2009; Nonaka,

*Abbreviations:* CCP, complement control protein; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IrC2/Bf, *Ixodes ricinus* factor C2/ factor B; KD, knock-down; LPS, lipopolysaccharide; qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; TEP, thioester-containing protein.

☆ The nucleotide sequence of *irc2/bf* has been deposited in the GenBank database with Accession No. MF197880.

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2014; Pinto et al., 2007; Sekiguchi and Nonaka, 2015; Zhu et al., 2005). These findings provide solid evidence that the origin of the complement system dates back more than 1 billion years.

Our long-term research is focused on innate immunity in ticks, blood-feeding mites notorious for their versatile capacity to transmit a wide variety of pathogenic agents causing severe infections of their vertebrate hosts including humans (de la Fuente et al., 2008). In Europe, the most important arthropod disease vector is the castor bean tick *Ixodes ricinus,* especially for transmitting Lyme disease and tick borne encephalitis. The vector competence of ticks is obviously conditioned by the pathogen's ability to avoid tick defense mechanisms during pathogen acquisition, its long inter-stage persistence in the tick midgut, dissemination to the hemocoel, and finally, transmission to the naïve host via salivary glands (Hajdusek et al., 2013). One arm of tick innate immunity that the pathogens encounter and have to overcome is comprised of components of a tick primordial complement system (Kopacek et al., 2012). We have previously described several tick molecules related to components of the mammalian complement system. These include thioester-containing proteins (TEPs) (Buresova et al., 2011; Urbanova et al., 2015, 2017), ficolin-related lectins named Dorin M (or more generally Ixoderins) (Kovar et al., 2000; Rego et al., 2006), and IrFC, an injury-responsive molecule homologous to the Limulus Factor C (Urbanova et al., 2014). The latter acts as a LPS-sensitive trigger of the horseshoe crab hemolymph coagulation cascade and, additionally as a C3 convertase in activation of the horseshoe crab complement-like reactions against Gram-negative bacteria (Ariki et al., 2004, 2008; Kawabata et al., 2009). It is important to emphasize that *Limulus* Factor C and IrFC are molecules structurally different from the C2 complement components that act as a C3/C5 convertase in the classical and/or lectin pathways of vertebrate complement activation (Ricklin et al., 2010). On the other hand, C2 is highly similar to Factor B (Bf), the C3/C5 convertase involved in the alternative pathway of complement activation in vertebrates (Ricklin et al., 2010).

This study presents a characterization of an *I. ricinus* molecule that shares the signature molecular structure with the vertebrate family of C2 and/or Bf convertases: the N-terminal repeats of complement control protein (CCP) (aka sushi or SCR - short consensus repeats) modules, followed by a von Willebrand factor A (vWF) and conserved trypsin-like domains. The molecule was tagged as IrC2/Bf to accord with the nomenclature previously introduced for its homologs described in horseshoe crabs, namely CrC2/Bf from *Carcinoscorpius rotundicauda* (Zhu et al., 2005) and TtC2/Bf-1, TtC2/Bf-2 from *Tachypleus tridentatus* (Tagawa et al., 2012). Similar molecules related to Bf were also described in various invertebrates such as sea urchin *Strongylocentrotus purpuratus* (Smith et al., 1998; Terwilliger et al., 2004), sea anemones *Nematostella vectensis* (Kimura et al., 2009) and *Aiptasia pallida* (Poole et al., 2016), in the clam *Ruditapes decussatus* (Prado-Alvarez et al., 2009) or in the venom gland of the spider *Loxosceles laeta* (Myamoto et al., 2016).

We show that IrC2/Bf is involved in the interaction of the tick immune system with yeast (*Candida albicans*) and *Borrelia* sp. spirochetes but not with Gram-negative bacteria. Expression of *irc2/bf* mRNA was up-regulated upon *C. albicans or Borrelia* sp. injection into tick hemocoel and silencing of *irc2/bf* gene by RNAi (KD) resulted in significantly reduced phagocytosis of yeasts and spirochetes by tick hemocytes. To our knowledge, this is the first functional characterization of a C2/Bf-related C3 convertase from any invertebrate disease vector that partially uncovers its role in interactions with a relevant vector-borne pathogen and more generally, contributes to our understanding of the functions and evolution of the primordial complement system.

## 2. Materials and methods

### 2.1. Biological material

Adult females and males of *I. ricinus* were collected by flagging in woodlands around České Budějovice, the Czech Republic. Females were naturally fed in the presence of males on laboratory guinea pigs and engorged ticks were kept separately in glass vials until oviposition and larvae hatching. All developmental stages (eggs, larvae, nymphs and adults) were maintained in wet chambers with a humidity of about 95%, temperature 24 °C and day/night period set to 15/9 h. Females were fed naturally on laboratory guinea pigs. The larvae were fed on guinea pigs, allowed to molt to nymphs and, after 4–6 weeks, further fed on guinea pigs or rabbits. Adult females (pathogen free) were used for the experiments described below. All laboratory animals were treated in accordance with the Animal Protection Laws of the Czech Republic No. 246/1992 Sb., ethics approval No. 095/2012.

### 2.2. Determination of the full length IrC2/Bf sequence

The first strand cDNA synthesized from the salivary glands of semi-engorged females served as a template for PCR amplification of overlapping amplicons (Table 1 and Fig. S1). The primers for amplification of internal PCR fragments (Clone 1 and rIrC2/Bf) were designed according to the annotated *I. scapularis* gene ISCW023729 (https://www.vectorbase.org/) and an overlapping EST (GenBank, EW946017). The sequences of other amplicons, as well as N-terminal and C-terminal regions, were obtained using IrC2/Bf gene-specific primers (Table 1 and Fig. S1) and the 5′-RACE and 3′-RACE Systems for Rapid Amplification of cDNA Ends (Invitrogen), respectively. The amplification conditions were determined experimentally for each primer pair using the MasterGradient (Eppendorf) PCR cycler. PCR products were purified by Agarose Gel DNA Extraction Kit (Roche, Germany), cloned into pCR™4-TOPO® vector using TOPO® TA Cloning® kit (Invitrogen) and sequenced in both directions by an automated DNA sequencer ABI Prism 3130 XL (Applied Biosystems). At least three different clones of each amplicon were sequenced to eliminate PCR artifacts. The correct assembly of partial sequences was verified by PCR amplification, cloning and sequencing of a control clone (2786 bps) spanning junctions of all partial clones (Table 1 and Fig. S1). The sequences were edited and assembled using the DNASTAR™ program package.

### 2.3. Expression of recombinant IrC2/Bf fragment, production of antibodies and western blotting

The amplified PCR rIrC2/Bf product was cloned into the pET-21b + vector (Novagen) using *Nde*I and *Xho*I restriction sites and expressed in *E. coli* strain BL 21 Star™ (DE3). The resulting fusion protein with C-terminal $(His)_6$ tag was purified from isolated inclusion bodies in the presence of 8M urea using a 5 mL metal-chelating column HiTrap™ IMAC FF (GE Healthcare) charged with $Co^{2+}$ - ions. The recombinant protein was refolded and used for raising antibodies in a rabbit as described previously (Grunclova et al., 2006). The immunoglobulin fraction (IgG) was enriched from the immune sera by precipitation with caprylic acid (Russo et al., 1983) and its reactivity with the corresponding recombinant antigen was verified by western blotting. The rIrC2/Bf was separated by reducing SDS-PAGE on gradient (5–17.5%) gel and electro-transferred onto the PVDF membrane (Immobilon, Millipore). The protein profiles were stained on membranes with Coomassie Brilliant Blue R-250. The western blots were detected with IgG fraction diluted 1:500, secondary goat Anti-Rabbit IgG

**Table 1**
The oligonucleotides used in this work.

| Amplicon name | Forward primer 5′ - 3′ | Reverse primer 5′ - 3′ | cDNA fragment/length (bp)[a] | Remark |
|---|---|---|---|---|
| Clone 1 | CCCGGCGACGTGGTGGAGTT | AACGCTCCGAGGCTTGTC | 372-1463/1092 | *I. scapularis*[b] |
| rIrC2/Bf | cgcatatgGCAAAGGCGATCACCAG | cgctcgagGGCAGCCGTTAGAAGCC | 1646-2335/690 | *I. scapularis*[c] |
| RNAi | ttgggcccGCAAAGGCGATCACCG | attctagaGGCAGCCGTTAGAAGCC | 1646-2335/690 | *I. scapularis*[c] |
| Clone 2 | TCTGCGAGGAAAATGGTGAAT | TGCGATAGCCGTCGTCTTTGCC | 826-2373/1548 | *irc2/bf gene specific* |
| Clone 3 | ACGAGCGAGGTGTTCGTCACA | TGCGATAGCCGTCGTCTTTGCC | 1558-2373/816 | *irc2/bf gene specific* |
| Control clone 4 | ATGTGCCAAGAGATGC | GGCATTTATAATCGATTCCG | 294-3079/2786 | *irc2/bf gene specific* |
| 5′-RACE | 5′RACE System (Invitrogen) | AAGTCTTCGTCACAGGAGAAC | 1-409/409 | *irc2/bf gene specific* |
| 3′-RACE | ACGGAGCAGCAAGCACACGGC | 3′-RACE System (Invitrogen) | 2130-3120/971 | *irc2/bf gene specific* |
| qPCR-IrC2/Bf | CAAAGTATGCTGAAGCCAAGG | GACGACGCCCTGCATTAG | 2767-2937/71 | *irc2/bf gene specific* |
| qPCR-el.factor 1[d] | ACGAGGCTCTGACGGAAG | CACGACGCAACTCCTTCAC | 1366-1446/81 | qPCR-house keeping |

[a] For product location see GenBank (Access. No. MF197880).
[b] Primers designed according to the sequence of *Ixodes scapularis* ISCW023729.
[c] Primers designed according to the sequence of *Ixodes scapularis* EST EW946017.
[d] *I. ricinus* elongation factor 1 – GenBank (GU074828).

conjugated with peroxidase (Sigma) (1:2000) and developed with 3,3′-diaminobenzidine and hydrogen peroxide as substrates. To increase specificity of IrC2/Bf detection in tick tissues, the rabbit antibodies were affinity-purified using 350 μg of purified rIrC2/Bf recombinant protein coupled to 1.5 mL CNBr-activated Sepharose 4B (GE Healthcare) according to the previously described protocol (Sojka et al., 2012). The sorbent was incubated with 8 mL of IgG fraction in PBS (140 μg/mL) for 4 h in a cold room, centrifuged (2000 rpm, 1 min) and the remaining supernatant was collected and tagged as nonspecific immunoglobulins (IgG-ns). The sorbent was washed 3 times with PBS and the bound specific antibodies (IgG-spec) were eluted from the sorbent using 10 mL of 0.2M L-glycin, 0.15 M NaCl, pH 2.2 and immediately neutralized to pH 7.5 with 1M Tris-base. The reducing SDS-PAGE was performed using the Criterion™ TGX Stain-Free™ Precast Gels (4–15%) and proteins were visualized using TGX stain-free chemistry (BioRad). Proteins were then transferred onto PVDF membrane using Blot Turbo™ system (BioRad). For Western blot analyses, membranes were incubated in IgG (1:500), IgG-ns (1:500) or IgG-spec (1:50), followed by goat anti-rabbit IgG-peroxidase antibody (1:10000). The signal was developed using Luminata™ Classico Western HRP Substrate (Millipore) and visualized using a ChemiDoc™ MP imager, and analysed using Image Lab™ Software (BioRad).

### 2.4. RNAi silencing

A 690bp long DNA fragment of *irc2/bf* was amplified using primers containing *Apa*I and *Xba*I restriction sites (Fig. S1 and Table 1) and directionally cloned into the modified pLL10 vector containing two T7 promoters in reverse orientation (Levashina et al., 2001). The dsRNA was synthesized as described previously (Buresova et al., 2009) using the MegascriptT7 transcription kit (Ambion). The dsRNA (3 μg/μl; 0.5 μl) was injected into the tick hemocoel of unfed *I. ricinus* females using the microinjector (Drummond). Control ticks were injected with the same volume of GFP dsRNA synthesized under the same conditions from linearized plasmid pLL6 (Levashina et al., 2001). After inoculation, ticks rested for one day in a humid chamber and then were allowed to feed naturally on guinea pigs for 6 days. The efficiency of RNA interference (RNAi) was checked by quantitative real-time PCR (qRT-PCR) using RNA isolated from trachea/fat body dissected from semi-engorged females.

### 2.5. Profiling of irc2/bf expression by quantitative real-time PCR

All tissue cDNAs were prepared independently in triplicates. Adult *I. ricinus* females were allowed to feed naturally for 6 days on guinea pigs (semi-engorged females), forcibly removed and the hemolymph was collected from a cut leg and pooled. Total RNA from hemolymph and other dissected tissues (salivary glands, ovaries, gut, tracheae/fat body, Malpighian tubules, and the remaining tissues tagged as rest) was isolated and reverse-transcribed to single-stranded cDNA as described previously (Urbanova et al., 2014). For subsequent PCR applications, cDNA was diluted 20 times in nuclease-free water. Expression of the *irc2/bf* gene was determined by qRT-PCR using the LightCycler 480 (Roche) and SYBR green chemistry. The reaction conditions were as follows: 12.5 μl of FastStart Universal SYBR Green Master (Rox) (Roche), 10 pmol of qPCR-IrFC forward and reverse primers (Table 1), 5 μl of cDNA and distilled water up to 25 μl. The amplification program consisted of denaturation at 95 °C for 5 min and then 50 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and extension at 72 °C for 10 s. Relative expression of the *irc2/bf* was normalized to *I. ricinus* elongation factor 1 (*ef1*) (Nijhof et al., 2009) using the mathematical model of Pfaffl (2001).

### 2.6. Expression response of irc2/bf to aseptic, microbial, and Borrelia sp. injection

Gram-positive *Micrococcus luteus* (CIP A270), Gram-negative *Escherichia coli* (1106), yeast *Candida albicans* (MDM8), and different species of *Borrelia burgdorferi sensu lato* complex (*B. burgdorferi* NE5264, *B. burgdorferi* CB26, *B. garinii* MSLB, *B. afzelii* CB43) were cultivated as described previously (Urbanova et al., 2014, 2017). Freshly grown microbes were diluted in sterile PBS ($OD_{600}$ = 0.001 for *E. coli* and *M. luteus*, $OD_{600}$ = 1 for *C. albicans*). *Borrelia* were diluted in PBS to contain $10^4$ spirochetes in the injection dose. Unfed, pathogen free *I. ricinus* females were surface-sterilized by immersion in 3% $H_2O_2$, 70% EtOH, and sterile distilled water. A volume of 69 nl of the microbial suspension was injected into ticks using a sterile glass capillary and microinjector (Narishige). Sterile PBS or BSK-H media were used as aseptic injection controls. After inoculation, ticks were allowed to rest for 12 h at RT and the total RNA was extracted from whole body homogenates using TRI reagent® (Sigma), treated with DNAse (Ambion), transcribed into cDNA and the relative expression of irfc2/bf was determined by qRT-PCR as described above.

### 2.7. In-vitro phagocytic assays

*In-vitro* phagocytic assays were performed as previously published for the Gram-negative *Chryseobacterium indologenes* and *E. coli* (Buresova et al., 2011), yeast *C. albicans* (Urbanova et al., 2014), and the spirochete *B. afzelii* CB43 (Urbanova et al., 2017).

For each experiment, 25 unfed *I. ricinus* females were injected into the hemocoel with *irfc2/bf* dsRNA or *gfp* dsRNA (0.5 μl; 3 μg/μl) using a microinjector (Drummond), allowed to rest for one day and then fed naturally for 6 days on guinea pigs. Hemolymph collected from a group of 25 semi-engorged females was mixed with L15-BOFES medium (L15 medium supplemented with 10% fetal calf serum, PAA Laboratories). Hemocytes ($4 \times 10^4$) in a volume of 150 μl were transferred onto round microscope cover slips in a 24-well culture plate. Thereafter 100 μl of *E. coli* or *C. indologenes* ($OD_{600} = 0.1$) was added. Bacteria were incubated with hemocytes for 105 min at 28 °C. After incubation, hemocytes were fixed and stained (Buresova et al., 2009). Phagocytosis of *C. albicans* was performed as described by adding 10 μl of ($4 \times 10^5$) FITC-labeled yeasts to 240 μl of hemocytes ($4 \times 10^4$) in L15-BOFES medium and incubated for 105 min at 31 °C (Urbanova et al., 2014). For phagocytosis of the spirochete *B. afzelii* CB43, 10 μl of spirochetes ($10^8$ cells/ml) were added to 240 μl of hemocytes in L15-BOFES medium and incubated for 120 min at 28 °C. Engulfed spirochetes were detected using the previously described method of double staining (Urbanova et al., 2017). The hemocyte nuclei were counterstained with DAPI for 10 min and phagocytic hemocytes containing engulfed bacteria or *Borrelia* were counted under a fluorescence microscope (Olympus BX 53). For each experiment, 100 hemocytes were counted on each of at least 15 slides. Relative phagocytosis was calculated in relation to the number of phagocytic hemocytes in the control group injected with *gfp* dsRNA, taken as 100% for each respective experiment performed in three independent biological replicates.

### 2.8. In vivo phagocytosis of B. afzelii

Pathogen free, unfed *I. ricinus* females were injected into the hemocoel with *irc2/bf* dsRNA or control *gfp* dsRNA (5 ticks for each group and time point), allowed to rest for one day and then fed for 6 days on guinea pigs. The semi-engorged females were removed from the host and injected into the hemocoel with cultivated *B. afzelii* ($5 \times 10^4$ spirochetes in a volume of 138 nl) using a microinjector (Drummond). Hemolymph samples from individual *irc2/bf* KD or control ticks were collected at defined time points (1 h, 6 h, and 24 h) after injection of spirochetes and mixed with 10 μl of L15-BOFES medium supplemented with 10% fetal calf serum (PAA Laboratories) on microscope slides. Cells were fixed and stained as described previously (Urbanova et al., 2017). The phagocytic index was determined as the number of hemocytes with ingested *Borrelia* counted for a total 100 hemocytes in the microscopic field.

### 2.9. Statistical analysis

Data were analyzed by the non-parametric Mann-Whitney test. All statistics was performed using GraphPad Prism (version 6.00 for Windows, GraphPad Software, San Diego, CA, USA). A P value of $<0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Primary structure of IrC2/Bf

BlastN analysis of the *Ixodes scapularis* gene ISCW023729, annotated as “CUB and sushi multiple domains (CSMD) protein”, revealed its closest relation (about 30–35% sequence identity) with proteins annotated as the complement factor C2 and/or factor B (Bf) from other arthropods such as horseshoe crabs *T. tridentatus* or *C. rotundicauda*, house jumping spider *Hasarius adansoni* or the common spider *Parasteatoda tepidariorum*. The C-terminal part encoding the trypsin domain was absent in the ISCW023729 gene, however, we found it to be partially encoded by the overlapping EST sequence (GenBank EW946017). PCR primers derived from the available *I. scapularis* sequences were used to amplify, clone and sequence the internal fragments of its *I. ricinus* ortholog, tagged as IrC2/Bf, using cDNA from *I. ricinus* salivary glands as a template (Table 1 and Fig. S1). Several products of clone 1 differing in length were amplified and cloned (see below). The full coding cDNA sequence of IrC2/Bf and 5′- and 3′-untranslated regions were determined by sequencing RACE-PCR products amplified with gene-specific primers (Table 1 and Fig. S1). The complete cDNA of the longest *irc2/bf* variant was 3120 bp long and encoded a polypeptide of 996 amino-acid residues (Fig. 1A). The sequence was deposited in the GeneBank under Accession No. MF197880. The presumptive signal peptide formed by the first 23 residues was predicted by the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The theoretical mass of the whole mature IrC2/Bf was calculated to be 106,511 Da with an isoelectric point of 5.38. The IrC2/Bf sequence contained at least eight potentially N-glycosylated asparagine residues as predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) (Fig. 1A, B). The primary structure of IrC2/Bf displayed a multi-domain molecular architecture composed of a variable number (5–7) of CCP (aka sushi or SCR) modules followed by von Willebrand factor A domain, and the C-terminal trypsin domain with a conserved serine protease catalytic triad H/D/S (Fig. 1A and B). The variability occurring in the region spanning CCP3 and CCP4 modules was most likely caused by alternative splicing as we identified three different variants containing 7, 6 or 5 CCP (Fig. 1C). In addition, BLAST N analysis of the full *irc2/bf* sequence against the recently available salivary gland transcriptome shotgun assembly (TSA) obtain from individual *I. ricinus* females (Bioproject PRJNA312361, unpublished) revealed the presence of two slightly different transcripts annotated as putative complement factor b. The partial sequence of transcript Ir-256495 (GenBank GEGO01006449) is identical to the partial sequence of *irc2/bf*, while the transcript Ir-SigP-256516_FR4_1–723 (GenBank GEGO01006515) that most likely encodes another IrC2/Bf variant (lacking the C-terminal trypsin domain) was tagged as type II (Fig. 1C). Both type I and type II variants share high sequence homology with ~90% identity at both nucleotide and amino-acid levels.

### 3.2. Quantitative tissue expression profiles of irc2/bf

Analysis of *irc2/bf* expression in tissues dissected from semi-engorged *I. ricinus* females by qRT-PCR revealed that *irc2/bf* mRNA is mainly expressed in the fat body cells associated with trachea and to a much lesser extent in other tissues such as salivary glands, ovaries, hemocytes, and remaining tissues (Fig. 2).

### 3.3. Detection of IrC2/Bf in tick hemolymph by western blotting

Antibodies raised in rabbits against the recombinant IrC2/Bf fragment spanning the von Willebrand factor domain and a portion of the trypsin domain reacted strongly with the homologous recombinant fusion protein (theoretical mass 26,281 Da) (Fig. 3A). Detection of authentic IrC2/Bf in tick tissues was examined by western blotting with specific antibodies affinity purified using rIrC2/Bf-Sepharose. Despite the pre-dominant expression of *irc2/bf* gene in the trachea/fat body (Fig. 2) no band of expected size was specifically recognized on Western blots from this tissue (Fig. S2) By contrast three bands of molecular masses about 200, 160, and 120 kDa were detected in tick hemolymph (Fig. 3B), out of which the 160 kDa band disappeared upon RNAi knock down (KD) of *irc2/bf* (Fig. S2). This result implies that the 160 kDa band corresponds to the glycosylated authentic IrC2/Bf that is present in tick

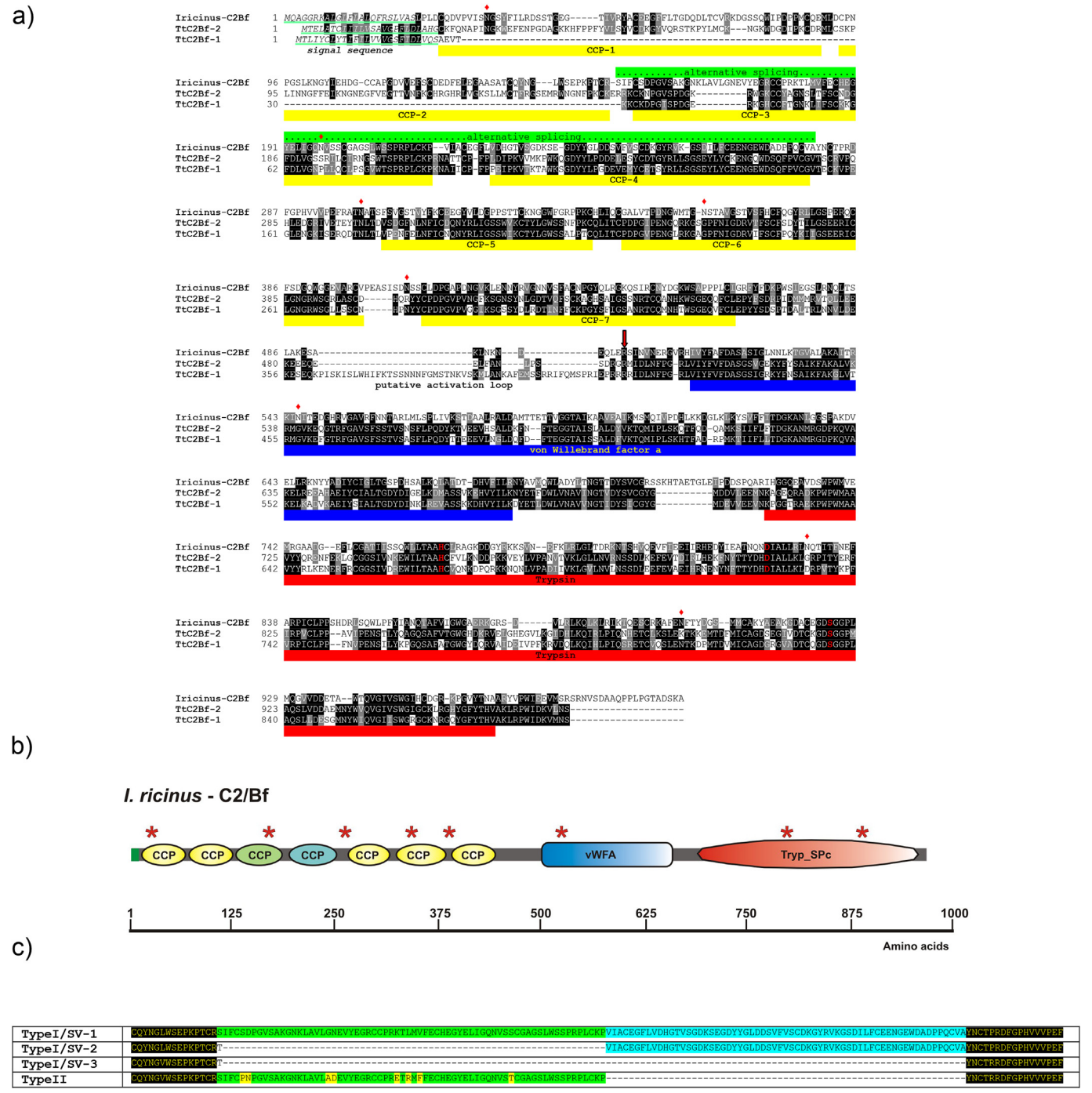


**Fig. 1.** Primary structure of IrC2/Bf. **Panel A** – amino-acid sequence alignment of IrC2/Bf-type I (GenBank MF197880) with TtC2/Bf-1 and TtC2/Bf-2 from the horseshoe crab *Tachypleus tridentatus* (GenBank AB353280 and AB353281, respectively); the CCP (1–7) modules, von Willebrand factor domain, and trypsin domains are depicted according to the TtC2Bf-2 (Tagawa et al., 2012); signal peptides are in italics; red bold letters in the trypsin domain indicate the conserved catalytic triad residues H/D/S; the red diamonds indicate the predicted N-glycosylation sites in the IrC2/Bf sequence; the green bar over the IrC2/Bf sequence indicates the alternatively spliced region; the red arrow points to the putative cleavage site. **Panel B** – Schematic structure of the IrC2/Bf domain architecture. Asterisks denote the predicted sites of N-glycosylation; the green and cyan color of CCP domains 3 and 4 correspond to the alternative splicing variants shown in Panel C. **Panel C** – Alternative splicing variants of IrC2/Bf identified in the region spanning the CCP3 and CCP4 modules. Three splicing variant were identified for the IrC2/Bf-type I. The corresponding sequence segment of IrC2/Bf type II was found in the GenBank (Acc. No. GEGO01006515). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hemolymph in trace amounts.

### 3.4. Expression response of irc2/bf upon injection of model microbes and Borrelia sp. spirochetes

Surface sterilized unfed *I. ricinus* females were injected with *E. coli, M. luteus*, and *C. albicans* representing Gram-negative bacteria, Gram-positive bacteria, and yeast, respectively. Injection of sterile PBS served as an aseptic injection control. Total RNA was isolated 12 h after injection from the whole body homogenates and the level of *irc2/bf* mRNA was determined by qRT-PCR. Injection of sterile PBS, *E. coli* and *M. luteus* did not significantly affect *irc2/bf*

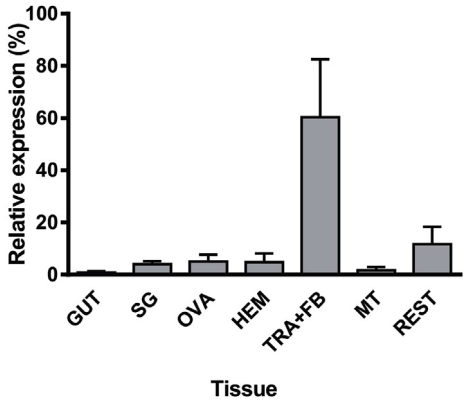


**Fig. 2.** Relative expression profile of *irc2/bf* mRNA in tissues dissected from partially fed females were determined by qRT-PCR and related to the *elongation factor-1* as a housekeeping gene. Gut – midgut; SG – salivary glands; OVA – ovaries; HEM – hemocytes; TRA + FB – trachea/fat body complex; MT – Malpighian tubules, REST – the remaining tissues. The error bars represent standard errors from three independent biological replicates.

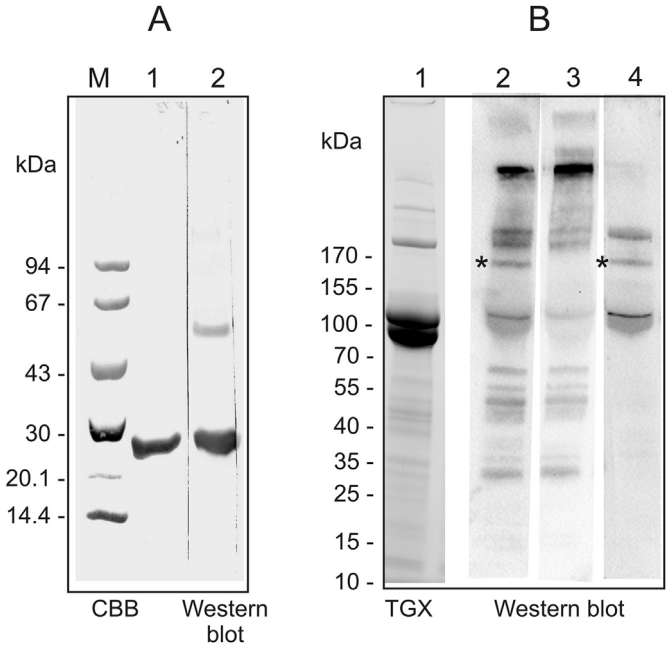


**Fig. 3.** Detection of IrC2/Bf by Western blotting. **Panel A** – Preparation of recombinant IrC2/Bf fragment and specific polyclonal antibodies. Recombinant fragment of IrC2/Bf was expressed in *E. coli* as a $(His)_6$-tagged fusion protein, purified using $Co^{2+}$ chelating chromatography, refolded and used as an antigen to raise antibodies in a rabbit. M – Low molecular weight standards (GE Healthcare); lanes 1, 2 – purified recombinant IrC2/Bf fragment. M and lane 1 stained with Coomassie (CBB); lane 2 – western blot with IgG fraction from serum of immunized rabbit (1:500) and secondary antibody goat anti-rabbit IgG-peroxidase (1:2000). Developed with 3,3′-diaminobenzidine and hydrogen peroxide as substrates. **Panel B** – Detection of authentic IrC2/Bf in tick hemolymph. Positions of PageRuler Prestained Protein Ladder (Thermo Scientific) are indicated on the left. Lanes 1, 2, 3, 4 – hemolymph collected from semi-engorged control ticks pre-injected with *gfp* dsRNA (15 μl, 10 times diluted in reducing SDS-PAGE buffer per lane). Lane 1 – protein profile visualized using TGX Stain-Free™ technology; Lane 2 – Western blot using IgG fraction from serum of immunized rabbit (1:500); Lane 3 – Western blot using nonspecific IgG fraction not bound to rIrC2/Bf-Sepharose (1:500); Lane 4 – Western blot using specific IgG fraction eluted from rIrC2/Bf-Sepharose (1:50). Western blots were developed using secondary antibody goat anti-rabbit IgG-peroxidase (1:10000), Luminata™ Classico Western HRP Substrate (Millipore) and visualized using a ChemiDoc™ MP imager. Asterisks indicate the band that disappeared upon IrC2/Bf KD (see also Fig. S2).

expression, as compared to the non-injected ticks. In contrast, injection of the yeast *C. albicans* resulted in 6.1-fold up-regulation of *irc2/bf* mRNA expression in relation to the aseptically injected ticks (Fig. 4A). We further examined the expression of *irc2/bf* upon the injection of cultivated *Borrelia* sp. spirochetes and BSK-medium alone as a mock. Expression of *irc2/bf* increased upon injection of all tested *Borrelia* genospecies compared to the injection of sterile PBS or BSK-H medium (Fig. 4B). The average increase in expression achieved in this experiment was 2.75- and 2.4-fold for *B. burgdorferi* NE5264; 3.1- and 2.7-fold for *B. burgdorferi* CB26; 4.0- and 3.5-fold for *B. garinii* MSLB and 2.45- and 2.1-fold for *B. afzelii* CB43, in relation to the PBS and BSK-H injection controls, respectively.

### 3.5. The role of IrC2/Bf in phagocytosis of different microbes

Using RNAi-mediated gene-specific silencing followed by *in vitro* phagocytic assays, we previously demonstrated that individual components of the tick primordial complement system played specific roles in the phagocytosis of different microbes by tick hemocytes (Buresova et al., 2011; Urbanova et al., 2017, 2014, 2015). Similar experiments were performed in order to investigate the role of IrC2/Bf in this cellular immune response. Alignment of *irc2/bf* fragment nucleotide sequences used for preparation of dsRNA (Fig. S1) with the corresponding sequence of the irc2/bf type II identified in the *I. ricinus* salivary gland transcriptome (GenBank GEGO01006515) revealed a sufficient number of identical oligonucleotide segments that should warrant simultaneous silencing of both genes. Unfed *I. ricinus* females were injected with gene-specific *irc2/bf* dsRNA or *gfp* dsRNA as a negative control. Ticks were allowed to feed naturally for 6 days, then the hemolymph was collected from semi-engorged females and used for a phagocytic assay *in vitro*. Dissected trachea/fat body tissues from these females were used to check *irc2/bf* mRNA levels by qRT-PCR that displayed RNAi efficiency higher than 95%. As shown in Fig. 5, *irc2/bf* KD led to a decrease in phagocytosis of the yeast *C. albicans* and the spirochete *B. afzelii*, by about 40% and 30%, respectively, compared to the GFP control, while phagocytosis of Gram-negative bacteria *E. coli* and *C. indologenes* were not affected.

### 3.6. In vivo phagocytosis of B. afzelii upon irc2/bf RNAi-silencing

Unfed tick females were injected with *irc2/bf* dsRNA or *gfp* dsRNA as a control and allowed to feed on guinea pigs for 6 days. *B. afzelii* spirochetes were injected into semi-engorged females, and phagocytosis was examined in hemolymph collected at different time intervals, post injection. The *Borrelia* phagocytosis by tick hemocytes was reduced in IrC2/Bf KD ticks compared to GFP control at all time points (Fig. 6). The highest phagocytic activity was observed 6 h after *Borrelia* injection (30% phagocytic hemocytes in GFP group vs. 14% in IrC2/Bf KD group).

## 4. Discussion

For the comparative studies on tick innate immunity, horseshoe crabs present the phylogenetically closest invertebrate (Chelicerate) model. This has been already evidenced by characterization of several tick immune molecules, e.g. fibrinogen-related lectins (Kovar et al., 2000; Rego et al., 2006), $\alpha_2$-macroglobulins (Buresova et al., 2009; Kopacek et al., 2000; Saravanan et al., 2003) or IrFC related to the Limulus Factor C (Urbanova et al., 2014). In this work, we have cloned and characterized IrC2/Bf from the tick *I. ricinus*, and proposed its function as a C3 convertase given its close homology with the described horseshoe crab convertases CrC2/Bf from *C. rotundicauda* (Le Saux et al., 2008; Zhu et al., 2005) or TtC2/Bf-1 and TtC2/Bf2 from *T. tridentatus* (Tagawa et al., 2012).

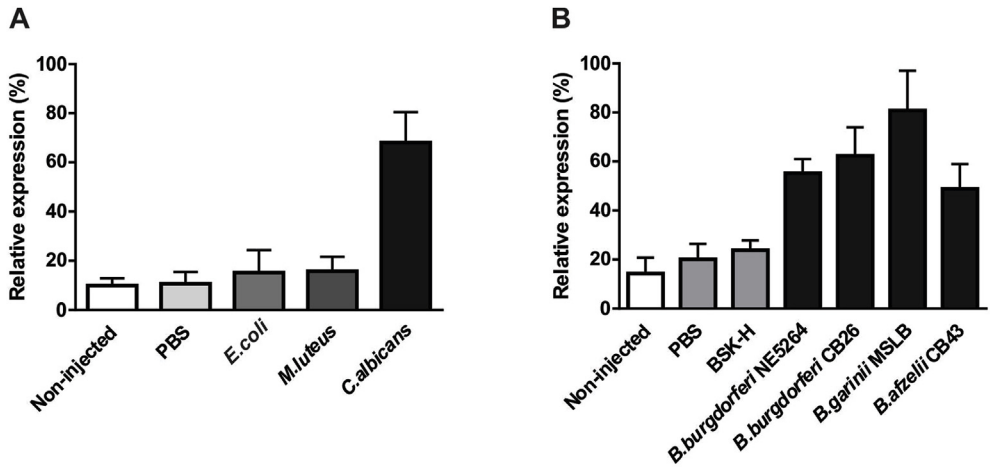


**Fig. 4.** *Irc2/bf* expression responses to the injection of model microbes and *Borrelia* sp. spirochetes.
Unfed *I. ricinus* females were injected with specified microbes (Panel A) or with four different species of *Borrelia burgdorferi* s.l. complex: *B. burgdorferi* NE5264, *B. burgdorferi* CB26, *B. garinii* MSLB and *B. afzelii* CB43 (Panel B). Sterile PBS and/or BSK-H medium were used as aseptic injection controls. Total RNA was isolated from the tick whole body homogenates 12 h after the challenge. Expression is shown in relation to the *elongation factor-1* as a housekeeping gene. The error bars represent the standard errors from three independent biological replicates.

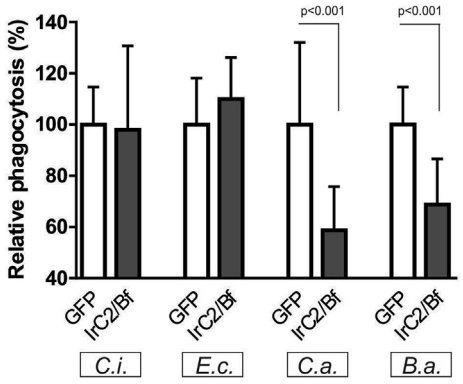


**Fig. 5.** The effect of *irc2/bf* RNAi silencing on phagocytosis of model microbes and spirochetes in an *in-vitro* assay. The hemolymph collected from 25 partially fed females pre-injected with *irc2/bf* dsRNA (IrC2/Bf) or *gfp* dsRNA (GFP) for control, was incubated with indicated microbe for 2 h. C. i. – *C. indologenes*, E. c. – *E. coli*, C. a. – *C. albicans*, B. a. – *B. afzelii*. The number of phagocytic hemocytes in individual RNAi experiments was related to that obtained for the *gfp* dsRNA injected control group, taken as 100% in the respective experiments. The error bars represent standard deviations of three independent experiments. Data were analyzed by Mann-Whitney test.

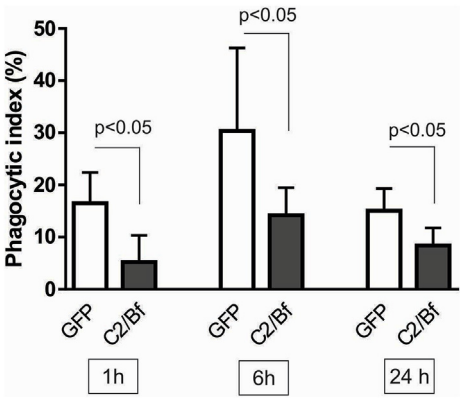


**Fig. 6.** *In vivo* inhibition of *B. afzelii* phagocytosis upon *irfc2/bf* RNAi-silencing. Semi-engorged females were pre-injected with *irc2/bf* dsRNA (C2/Bf) or *gfp* dsRNA (GFP) for control and injected with a dose of $5 \times 10^4$ *B. afzelii* CB43 2 h later. Hemolymph from different time points after injection of *Borrelia* were collected from individual ticks (5 ticks per time point) and the phagocytic index was determined as a number of hemocytes with ingested *Borrelia* counted for a total of 100 hemocytes in the microscopic field. Data were analyzed by Mann-Whitney test.

We found that the IrC2/Bf possesses a variable number (5–7) of CCP modules at its N-terminal part. The higher number of CCP modules (most frequently five) were reported for C2/Bf homologs identified in invertebrate animals, e.g. the NvBf-2 from the sea anemone *N. vectensis* (Kimura et al., 2009), the SpBf from the sea urchin *S. purpuratus* (Smith et al., 1998) or the above mentioned horseshoe crab convertases CrC2/Bf (Zhu et al., 2005), and TtC2/Bf-1 and TtC2/Bf-2 (Tagawa et al., 2012). The structural organization of the longest variant of IrC2/Bf with 7 CCP modules matches the best with TtC2/Bf-2 that contains two additional N-terminal CCP1 and CCP2 modules compared to TtC2/Bf-1 but lacks the extra sequence of about 40 amino acid residues between the CCP7 and vWF domains (Fig. 1A) (Tagawa et al., 2012). So far the highest number of seven CCP modules identified was reported to be present also in the Bf homologs from other arthropods such as the spiders *Ammothea* sp. (FB-1), *H. adansoni* (FB-1) or the centipede *Scolopendra subspinipes* (FB-1) (Myamoto et al., 2016). The variability in the number of CCP modules identified in IrC2/Bf is most likely due to an alternative splicing event. We have identified three variants of IrC2/Bf type I, where either none, CCP2 or CCP2+CCP3 modules were spliced out and resulted in mRNAs encoding 7, 6, and 5 CCP modules, respectively (Fig. 1B and C). Similar alterations in the CCP number caused by an alternative splicing was also reported for the sea urchin SpBf, yielding in total, 4 different splice variants: one with 5, two with 4, and one with 3 CCP modules (Smith et al., 2001; Terwilliger et al., 2004). The SpBf variant with 3 CPP modules, similar to NvBf-1 from *N. vectensis*, was suggested to represent the ancestors of the vertebrate C2/Bf structure, whereas invertebrate Bfs with higher CCP numbers are supposed to experience domain duplication or shuffling (Kimura et al., 2009). The CCP modules, as well as their inter-domain linkers, are important for specific protein-protein interactions within the complement system (Lehtinen et al., 2004). The function of individual CCP modules of the CrC2/Bf from the horseshoe crab *C. rotundicauda* was elegantly studied using a two-pronged strategy based on yeast two-hybrid and pull down assays (Le Saux et al., 2008). The authors

deciphered how the five CCP modules of CrC2/Bf specifically interact with core components of the pattern recognition receptor (PRR), composed of galactose-binding protein, carcinolectin-5 and C-reactive protein. Proteolytic activity of activated CrC2/Bf was shown to promote the assembly of PRR on the bacterial surface (Le Saux et al., 2008).

Tick IrC2/Bf is mainly synthesized in the fat body associated with trachea and a small amount is also expressed in other tissues such as salivary glands (Fig. 2), whereas the injury-responsive IrFC, is almost exclusively expressed in tick hemocytes (Urbanova et al., 2014). The tissue expression profile of IrC2/Bf matches best with three *I. ricinus* TEPs, namely IrC3-1, IrC3-3, and IrAM2-1 (Urbanova et al., 2015). The amount of IrC2/Bf in tick hemolymph is quite small, barely detectable by western blotting (Fig. 3B). This result contrasts with a relative abundancy of TtC2/Bf-1 and TtC2/Bf-2 detected in the plasma of the horseshoe crab *T. tridentatus,* where hemocytes are the major expression tissue (Tagawa et al., 2012). Similarly, body fluid cells – coelomocytes, were reported to express Bf-related molecules in other marine invertebrates such as sea urchins *S. purpuratus* (Smith et al., 1998) or the sea cucumber *Apostichopus japonicus* (Zhong et al., 2012).

Expression of *irc2/bf* was clearly up-regulated upon injection of the yeast *C. albicans* and all examined *B. burgdorferi* s.l. strains but not upon challenge with Gam-negative (*E. coli*) or Gram-positive (*M. luteus*) bacteria (Fig. 4A and B). This pattern of immune responses to microbial challenge resembled that previously reported for the IrC3-1 molecule (Urbanova et al., 2015, 2017). Expression of *irc2/bf* did not respond to aseptic injection, which was, by contrast, observed for the IrFC that is hypothesized to play a similar role as Limulus Factor C in hemolymph coagulation (Urbanova et al., 2014). Constitutive expression, not responsive to the injection of sterile sea water or different LPS from Gram-negative bacteria, was published for the gene encoding sea urchins SpBf (Terwilliger et al., 2004). Fluctuating mRNA levels encoding AjBf-2 at different time points following injection of LPS (*E. coli*) was observed in coelomocytes and body walls of the sea cucumber *A. japonicus* (Zhong et al., 2012). Most recently, it was shown that the expression of FactorB related molecules Ap_Bf-1 and Ap_Bf-2 from the sea anemone *A. pallida* respond differently to the challenge by living Gram-negative *Serratia marcescens* depending on the symbiotic or aposymbiotic state of the animal (Poole et al., 2016).

The volume of hemolymph that could be collected from one semi-engorged *I. ricinus* female is in the range of 1–2 μl, which substantially restricts most functional studies at the protein level, and are not comparable with those performed in horseshoe crabs. Therefore our functional genomics studies of complement-related molecules in ticks must rely on RNAi KD of individual molecules followed by evaluation of the *in vitro* and/or *in vivo* phagocytic phenotype (Buresova et al., 2011; Kopacek et al., 2012). RNAi silencing of IrC2/Bf performed in this work was successful as injection of *irc2/bf* dsRNA into unfed females reduced the corresponding mRNA level in the trachea/fat body from semi-engorged females by more than 95% and resulted in the disappearance of a ~160 kD band from tick hemolymph recognized by specific antibodies (Fig. 3B and Fig. S2). Reactivity with two other bands of 200 kDa and 120 kDa can be most likely explained as a cross-reactivity of antibodies with another tick proteins bearing a von Willebrand factor domain such as vitellogenins or carrier proteins abundantly present in tick hemolymph (Perner et al., 2016). IrC2/Bf KD resulted in the phagocytic inhibition of the yeast *C. albicans* and the spirochete *B. afzelii,* but not Gram-negative *E. coli* or *C. indologenes* (Fig. 5). More recently, we have examined the role of all nine *I. ricinus* TEPs in the phagocytosis of yeast *C. albicans* and spirochete *B. afzellii* and found that phagocytosis of *C. albicans* was significantly reduced upon RNAi-KD of IrC3-1,2,3 and IrMcr-2 (Urbanova et al., 2015), while RNAi-KD of IrC3-2,3 decreased the phagocytosis of *B. afzelii* (Urbanova et al., 2017). Taking together our previously published functional studies, we can summarize as follows: (i) phagocytosis of the metalloprotease secreting, Gram-negative *C. indologenes,* which is pathogenic to ticks (Buresova et al., 2006), involves protease inhibitors IrAM1, IrAM2 and IrC3-3 that are most likely associated with the convertase activity of IrFC (Buresova et al., 2009; Urbanova et al., 2014); (ii) phagocytosis of *E. coli* is affected by RNAi-KD of Ir-Tep, IrC3-3, and IrFC (Buresova et al., 2009; Urbanova et al., 2014); (iii) phagocytosis of the model yeast *C. albicans* depends mainly on IrC3-1, IrMcr-2 (Urbanova et al., 2015), and IrC2/Bf (this work); (iv) phagocytosis of the spirochete *B. afzelii* is reduced upon RNAi silencing of IrC3-2 and 3 (Urbanova et al., 2017), and IrC2/Bf (this work). The specificity of IrC2/Bf in phagocytosis of *C. albicans* and *B. afzelii,* together with the above specific responses to challenge by these microbes, as well as expression in the trachea/fat body complex, are almost identical to the previously published characteristics of IrC3-1 (Urbanova et al., 2017, 2014, 2015). Therefore we assume that IrC2/Bf functions as a convertase in the complement activation pathway protecting the tick against yeast and *Borrelia* infection, while IrFC is involved in complement-like reactions leading to the elimination of Gram-negative bacteria. This hypothesis matches well with the proposed model of recognition and elimination of microbes in the horseshoe-crab hemolymph, where Factor C is bound via its interaction with LPS on the surface of Gram-negative bacteria whereas factor C2/Bf is associated mainly with fungi and Gram-positive bacteria (Tagawa et al., 2012).

Even though expression of *irc2/bf* responses to the injection of *Borrelia* sp. and silencing its expression by RNAi inhibits phagocytosis of spirochetes, it is unlikely that the complement-like reactions within the tick hemocoel play a significant role in *Borrelia* transmission from infected ticks to the naïve host. We have recently demonstrated that neither RNAi silencing of tick TEPs nor the total inhibition of phagocytosis by injection of latex beads had any significant impact on the spirochetal burden in mice tissues infested with infected nymphs (Urbanova et al., 2017). Therefore it seems that tick complement might play a role in the tick defense against *Borrelia* spirochetes that succeed in penetrating from the midgut to the hemocoel (Coleman et al., 1997; Dunham-Ems et al., 2009), but have little if any impact on the tick competence to transmit Lyme disease. On the other hand, tick complement may still affect transmission of other tick-borne pathogens such as the malaria-like *Babesia* sp.; this is our current research focus.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2017.10.012.

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