Infection of *Ixodes* spp. tick cells with different *Anaplasma phagocytophilum* isolates induces the inhibition of apoptotic cell death

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Abstract

Anaplasma phagocytophilum is an intracellular rickettsial pathogen transmitted by *Ixodes* spp. ticks, which causes granulocytic anaplasmosis in humans, horses and dogs and tick-borne fever (TBF) in ruminants. In the United States, human granulocytic anaplasmosis (HGA) is highly prevalent while TBF has not been reported. However, in Europe the situation is the opposite, with high prevalence for TBF in sheep and low prevalence of HGA. The origin of these differences has not been identified and our hypothesis is that different *A. phagocytophilum* isolates impact differently on tick vector capacity through inhibition of apoptosis to establish infection of the tick vector. In this study we used three different isolates of *A. phagocytophilum* of human, canine and ovine origin to infect the *Ixodes ricinus*-derived cell line IRE/CTVM20 and the *Ixodes scapularis*-derived cell line ISE6 in order to characterize the effect of infection on the level of tick cell apoptosis. Inhibition of apoptosis was observed by flow cytometry as early as 24 hours post-infection for both tick cell lines and all three isolates of *A. phagocytophilum*, suggesting that pathogen infection inhibits apoptotic pathways to facilitate infection independently of the origin of the *A. phagocytophilum* isolate and tick vector species. However, infection with *A. phagocytophilum* isolates inhibited the intrinsic apoptosis pathway at different levels in *I. scapularis* and *I. ricinus* cells. These results suggested an impact of vector-pathogen co-evolution on the adaptation of *A. phagocytophilum* isolates to grow in tick cells as each isolate grew better in the tick cell line derived from its natural vector species. These results increase our understanding of the mechanisms of *A. phagocytophilum* infection and multiplication and suggest that multiple mechanisms may affect disease prevalence in different geographical regions.

Introduction

Anaplasma phagocytophilum is an intracellular Gram-negative bacterium that is transmitted by ticks of the genus *Ixodes* and infects vertebrate host myeloid and non-myeloid cells, mainly neutrophils (Severo et al., 2015). In both invertebrate and vertebrate cells, *A. phagocytophilum* multiplies within a parasitophorous vacuole, thus evading host cell defenses (Severo et al., 2015). Infection with *A. phagocytophilum* has been shown to cause the emerging tick-borne disease human granulocytic anaplasmosis (HGA). This is one of the most prevalent tick-borne diseases in the United States (Goodman, 2005) while, although increasingly detected, it is still rare in Europe (Edouard et al., 2012). *A. phagocytophilum* is also an economically important pathogen of sheep in Europe where it causes tick-borne fever (TBF) (Stuen, 2007; Stuen et al., 2009). This disease has not been reported in the United States but cases of equine and canine granulocytic anaplasmosis caused by *A. phagocytophilum* have been recorded (Severo et al., 2015).

A. phagocytophilum intraspecific genetic variability is associated with different reservoir host and tick vector species (Stuen et al., 2013). In the United States, the main vector is *Ixodes scapularis,* with a prevalence ranging from <1% to 50% (Goltz et al., 2013). In Europe, the main vector is *Ixodes ricinus*, with pathogen-prevalence ranging from <1% to 20% although it varies between geographical areas and stages of tick development (Stuen, 2007).

The prevalence of *A. phagocytophilum* infection is very high in domestic and wild animals in Europe with up to 37.5% prevalence in sheep in Norway and 98.9% prevalence in roe deer in Germany (Stuen et al., 2013) whereas few cases of HGA are reported (Heyman et al., 2010). However, cases of HGA are common in many areas of the United States. In fact, seroepidemiological data suggest that many cases of

anaplasmosis have not been recognized and, in endemic areas of the United States, 15-36% of the human population could be infected (Dumler et al., 2005). In the United States, mortality has been estimated to be up to 5% and most deaths are associated with opportunistic infections due to immunosuppression or underlying disease (Dumler et al., 2007). In Europe, no death has been reported to be associated with HGA, suggesting that in European regions the main disease produced by *A. phagocytophilum* is TBF in sheep while in the United States HGA predominates. Cross-infection experiments indicate that *A. phagocytophilum* isolates of distinct host origin are not uniformly infectious for heterologous hosts (Jin et al., 2012). Recently, multilocus sequence typing of almost 400 *A. phagocytophilum* strains from humans and animals has shown that strains from humans, dogs, horses, wild boars and hedgehogs from Europe belong to the same clonal complex whereas cattle, sheep, roe deer, vole and shrew strains are distantly related suggesting that they are unlikely to be infectious for humans (Huhn et al., 2014).

Apoptosis is a regulated process of cell death that maintains tissue homeostasis. Control of cell death by the host can be used to manage microbial spread and enhance the induction of immunity. Different disease states, including bacterial infection, can lead to inappropriate apoptosis regulation. The manipulation of host cell death pathways is exploited by many viral and microbial pathogens as part of their life cycle. *A. phagocytophilum* is able to inhibit host cell apoptosis pathways as a mechanism to improve its survival and multiplication inside the host cell (Rikihisa, 2010). In vertebrates, the pathogen has the ability to delay host cell apoptosis by activation of an anti-apoptotic cascade since this is crucial for its intracellular survival and reproduction in the neutrophils whose life cycle is normally short (Carlyon and Fikrig, 2003; Galindo et al., 2008). Different studies have shown that *A. phagocytophilum* infection

upregulates the expression of anti-apoptotic *blc-2* genes, blocks cell surface fatty acid synthase (FAS) clustering during spontaneous neutrophil apoptosis and inhibits cleavage of pro-Caspase 8 and Caspase 8 activation as well as secretion *Anaplasma* translocated substrate-1 (Ats-1) preventing mitochondrial response to apoptotic signals (Niu et al., 2010; Rikihisa, 2010). Inhibition of apoptosis has also been observed in the *I. scapularis* cell line ISE6 in which *A. phagocytophilum* infection downregulates mitochondrial *porin* inducing mitochondrial dysfunction and thereby inhibition of the intrinsic apoptosis pathway to subvert host cell defenses and increase infection (Ayllón et al., 2013). Functional characterization using RNA interference (RNAi) demonstrated that *porin* knockdown significantly increases colonization of *I. scapularis* ticks by *A. phagocytophilum* but does not affect tick feeding, thus showing how bacterial inhibition of *porin* expression increases tick vectorial capacity for this pathogen (authors' unpublished results). These results demonstrate how *A. phagocytophilum* has evolved to use similar strategies to establish infection in both vertebrate and invertebrate hosts (Ayllón et al., 2013).

A. phagocytophilum strains isolated from a variety of host species are able to grow in both *I. scapularis* (Munderloh et al., 1999) and *I. ricinus* (Dyachenko et al., 2013) tick cell lines, suggesting that the pathogen employs similar infection and multiplication mechanisms in both tick species. Tick-*A. phagocytophilum* interactions are not as well characterized as those between the pathogen and vertebrate hosts. Understanding the mechanisms of pathogen infection and multiplication is essential to answer basic biological questions and to design new strategies for pathogen control. Despite genetic variability between *A. phagocytophilum* strains from different hosts (Huhn et al., 2014), the origin of the differences in disease prevalence between Europe and the United States has not been identified. Our hypothesis is that different *A. phagocytophilum* strains

impact differently on tick vector capacity through apoptosis inhibition to establish infection of the tick vector. If proven correct, it would indicate that pathogen strains causing HGA are well adapted to *I. scapularis* but not *I. ricinus*, while strains causing TBF are better adapted to *I. ricinus*. To address this hypothesis, the aim of this study was to characterize cell apoptosis during early *in vitro* infection of tick cell lines derived from two different *Ixodes* species with three different isolates of *A. phagocytophilum* of human, canine and ovine origin.

Materials and Methods

Tick cell cultures and *A. phagocytophilum* isolates

The *I. ricinus* embryo-derived cell lines IRE/CTVM19 and IRE/CTVM20 (Bell-Sakyi et al., 2007) were maintained in, respectively, complete L-15 and L-15/L-15B media (Bell-Sakyi, 2004). Medium was changed once a week for both cell lines. The *I. scapularis* embryo-derived cell line ISE6 (provided by U.G. Munderloh, University of Minnesota, USA) was maintained in L-15B300 medium as described previously (Munderloh et al., 1999). The tick cell line IDE8, derived from *I. scapularis* embryos (Munderloh et al. 1994) was maintained at 32°C in L-15B medium in sealed containers as previously described (Munderloh & Kurtti 1989). Infected IDE8 cultures were propagated in a modified L-15B medium further supplemented with 0.1 % $NaHCO_3$ and 10 mM HEPES. The pH of the medium was adjusted to 7.5. This modified medium is referred to as *Anaplasma* culture medium (ACM). IRE/CTVM20 and ISE6 cells were inoculated with three different isolates of *A. phagocytophilum*: the human isolate NY18 (Asanovich et al., 1997), a dog isolate from Germany (L610) and an sheep isolate from Norway obtained in this study as described below. Infected cultures were propagated at 34°C in 25 cm^2 plastic culture flasks in 5 ml of the medium.

In vitro isolation of *A. phagocytophilum* from canine blood in IDE8 cells

An EDTA blood sample from a German dog was obtained from the Diagnostic Laboratory of the Institute for Comparative Tropical Medicine and Parasitology, University of Munich (Germany). Five hundred µl of EDTA blood were diluted into 10 ml of physiological PBS, and centrifuged for 5 min at 515 x g. The supernatant was removed and the blood cell pellet was resuspended in 9 ml of sterile, double distilled water for approximately 30 s to lyse most of the erythrocytes by hypotonic shock. Physiological tonicity was restored by the addition of 1 ml of 10-fold-concentrated Hanks' balanced salt solution. The lysate was centrifuged for 5 min at 290 x g. The supernatant was discarded and the pellet resuspended in ACM and inoculated into an IDE8 culture. The cultures were incubated at 34°C. Three ml of medium were changed twice weekly. The strain isolated here is referred to as strain L610.

Isolation of *A. phagocytophilum* from sheep blood in IRE/CTVM19 cells

Blood samples were collected from Norwegian White Sheep experimentally inoculated with sheep *A. phagocytophilum* variant2 strain from Norway (Stuen et al., 2009). The presence of *A. phagocytophilum* DNA in the blood samples was confirmed by real-time PCR (Ayllón et al., 2013). White blood cells (WBC) were harvested from the blood samples one week after collection as follows. Approximately 5 ml of blood were loaded onto 25 ml of Ficoll 1077 and centrifuged at 700 x g for 30 min at 4 °C to separate erythrocytes. WBC were collected from the plasma-Ficoll interface and suspended in 5 ml. The WBC suspension was centrifuged at 250 x g for 6 min and the cells were washed once in Hanks' balanced salt solution without calcium and magnesium. The isolated ovine WBC were resuspended in 1 ml of PBS and 200 µl aliquots were co-cultivated with IRE/CTVM19 cells in 25 cm^2 culture flasks at 34°C using 5 ml complete L-15 medium. *A. phagocytophilum*-infected tick cell lines were grown in 25 cm^2 cell culture flasks in ambient air at 34°C; the medium was additionally

supplemented with 10 mM HEPES and 0.1% NaHCO3, and buffered to pH 7.5 with 1 N NaOH. Infected cell cultures were monitored weekly by preparation and light microscopic examination of Giemsa-stained cytocentrifuge smears, prepared from gently resuspended cell suspension.

DNA isolation and phylogenetic analysis of *A. phagocytophilum* isolates

Multilocus sequence analysis was conducted using *groEL*, major surface protein 4 (*msp4*), and citrate synthase (*gltA*) genes. The nucleotide sequences of *groEL*, *msp4* and *gltA* were obtained from the *A. phagocytophilum* isolates used in this study as follows. Total DNA was extracted from *A. phagocytophilum*-infected tick cell suspensions using a DNeasy Blood & Tissue Mini kit (Qiagen, Madrid, Spain) according to the manufacturer's instructions. The *groEL*, *msp4* and *gltA* gene DNA fragments were amplified using specific oligonucleotide primers (Table 1) with proof reading polymerase and reaction conditions as previously described (de la Fuente et al., 2006b). Amplicons were purified and sequenced from both ends (Secugen SL, Madrid, Spain). The sequences obtained from *msp4*, *gltA* and *groEL* genes from L610, NY18 and sheep isolates were deposited in GenBank with accession numbers KP861634, KP861635, KP861636, KP861637, KP861638, KP861639, KP861640, KP861641, KP861642 respectively. For comparison, the same sequences were obtained for 9 isolates of which the genomes have been sequenced and are available in Genbank. The isolates CRT35 (NCBI reference sequence JFBI01000001; Massung et al., 2007; Al-Khedery et al., 2012) and CRT38 (NCBI reference sequence NZ_APHI00000000) were isolated from *I. scapularis* ticks, the isolate Dog2 was isolated from a dog (NCBI reference sequence CP006618; Al-Khedery et al., 2012), the isolate JM was obtained from a rodent (NCBI reference sequence CP006617; Al-Khedery et al., 2012), the MRK isolate was obtained from a horse (NCBI reference sequence JFBH00000000; Al-Khedery et al., 2012), the

Table 1. Oligonucleotide primers used in this study.

Gene	Genbank accession No.	Forward and Reverse Primers (5´-3´)
A. phagocytophilum 16S rRNA	CP000235	CAGAGTTTGATCCTGGCTCAGAACG GAGTTTGCCGGGACTTCTTCTGTA
Ixodes 16S rRNA	ABJB010000000	GACAAGAAGACCCTA ATCCAACATCGAGGT
A. phagocytophilum msp4	CP000235	ATGAATTACAGAGAATTGCTTGTAGG TTAATTGAAAGCAAATCTTGCTCCTATG
A. phagocytophilum gltA	CP000235	GTAATAAATTGTATTATCAGAG AATACGTGAGTTTGAAACCA
A. phagocytophilum groEL	CP006617	AAATGGCGAATGTTGTWGTYAC TTARAANCCNCCCATNCCNCCCATGCC
Ixodes bcl-2	ISCW021516	TTTGCTCGCATACCGAGGTC TACGACGAGATGCACGCCACA
Ixodes IAP	ISCW010694	AACTCCCACTTGAAGATGGC CATGGTCGGAGACACCTGG
Ixodes hexokinase	ISCW012387	CATCTGCCTCGCTGAATTGC AACGGTTTCTTAGGGGCGAG
Ixodes porin	ISCW000781	GTCGTGAAACTCGACTGCAA CCGTGTTCCACTTCTCCTTC ATGGCTCCTCCGTGCTACG** AATTGTTGACCTTCGCCCT**
Rhipicephalus sanguineus Rs86	DQ201646	GGACGCGATAAAGACCAGTAT** CACACGGAGCGGCGTAGGCGA**
Ixodes caspase	ISCW003039	CATCAACGCCTTGTGCTCAG AAGCTGCACTGTCTCGTGAT
Ixodes cytochrome c	ISCW008740	AGAGCAGCCATGGTCGAAAT TCTGTGTAGCTGAAGCCTGC
Ixodes subolesin	ISCW023283	AGCGGCCAACATTCGGGAGG ATGAGCCCCACCTGGCGGAA
Ixodes ribosomal protein S4 (rps4)	DQ066214	GGTGAAGAAGATTGTCAAGCAGAG TGAAGCCAGCAGGGTAGTTTG
Ixodes cyclophilin	ISCW008497	GCTTCGGTTACAAGGGCAGCAGCATTT TCGGGTGTGCTTCAGGATGAAGTT

**Primers for dsRNA synthesis contained T7 promoter sequence 5´-TAATACGACTCACTATAGGG-3 at the 5´end.

isolate Norway variant2 was isolated from a lamb (NCBI reference sequence JFBJ00000000; Al-Khedery et al., 2012) and the isolates HZ (NCBI reference sequence CP000235; Dunning et al., 2006; Al-Khedery et al., 2012) and HGE1 (NCBI reference sequence NZ_APHH01000002) were isolated from humans. The origin of the isolate HZ2 (NCBI reference sequence NC_021879) was not identified but it is likely from humans. The nucleotide sequences were concatenated and aligned with MAFFT (v7), configured for the highest accuracy (Katoh and Standley, 2013). After alignment, regions with gaps were removed and 2354 gap-free sites were used in maximum likelihood (ML) phylogenetic analysis as implemented in PhyML (v3.0 aLRT) (Anisimova and Gascuel, 2006; Guindon and Gascuel, 2003). The reliability for the internal branches was assessed using the approximate likelihood ratio test (aLRT –SH-Like) (Anisimova and Gascuel, 2006). Graphical representation and editing of the phylogenetic trees were performed with TreeDyn (v 198.3) (Chevenet et al., 2006).

Annexin V-FITC staining to detect cell apoptosis after experimental infection with

A. phagocytophilum

IRE/CTVM20 and ISE6 cells were each inoculated with the human, dog and sheep isolates of *A. phagocytophilum*. Approximately $5x10^5$-$1x10^6$ cells were collected immediately after infection (0 h) and at 24 and 48 h after infection from the same cultures. Uninfected cells were also collected from parallel cultures at the same time points to act as controls. All treatments were conducted in triplicate. Apoptosis was measured by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Immunostep, Salamanca, Spain) following the manufacturer's protocols. The kit detects changes in phospholipid symmetry analyzed by measuring Annexin V (labelled with FITC) binding to phosphatidylserine, which is exposed in the external surface of the cell membrane in apoptotic cells. Cells were stained

simultaneously with the non-vital dye propidium iodide (PI) allowing the discrimination of intact cells (Annexin V-FITC negative, PI negative) and early apoptotic cells (Annexin V-FITC positive, PI negative). All samples were analyzed on a FAC-Scalibur flow cytometer equipped with CellQuest Pro software (BD Bio- sciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The percentage of apoptotic cells was determined by flow cytometry after Annexin V-FITC and PI labeling and compared between infected and uninfected cells by Student's t-test with unequal variance ($P<0.05$).

Caspase 9 (CASP9) and Hexokinase (HK) activity assays

The apoptosis CASP9 assay was conducted using a CASP9 colorimetric assay kit (GenScript, Piscataway, NJ, USA). The assay for CASP9 is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate LEHD-pNA. The CASP9 activity was measured in uninfected and *A. phagocytophilum*-infected ISE6 and IRE/CTVM20 cells. Uninfected and infected cells were grown in sealed tubes in ambient air (approximately 7×10^5 cells per tube; 3 replicates per treatment). Infected cells were incubated for 24 h with the pathogen and cell-free complete culture medium was added to the controls. After incubation, cells were collected and processed following the manufacturer's recommendations. The pNA light emission was quantified using a microtiter plate reader at 405 nm. Relative CASP9 activity was determined by comparing the absorbance of pNA from uninfected or infected cells to that of the control.

The HK activity was measured in ISE6 and IRE/CTVM20 cells using an HK colorimetric assay kit (BioVision, CA). The assay for HK is based on spectrophotometric detection of a colored product generated by NADH produced from HK activity. The HK activity in uninfected and *A. phagocytophilum*-infected tick cells

was measured. Uninfected and infected cells were grown in sealed tubes in ambient air (approximately 7×10^5 cells per tube; N=3) and were collected and processed following the manufacturer's recommendations. The colored product was quantified using a microtiter plate reader at 450 nm, and the HK activity was calculated and expressed in mU/ml.

The results were compared between infected and uninfected cells by Student's t-test with unequal variance (P<0.05; N=3).

RNAi in tick cells

Oligonucleotide primers homologous to *I. scapularis* and *I. ricinus* genes containing T7 promoters (Table 1) were used for *in vitro* transcription and synthesis of double-stranded RNA (dsRNA) as described previously (de la Fuente et al., 2006a), using an Access RT-PCR system (Promega, Madison, WI) and an Megascript RNAi kit (Ambion, Austin, TX). The *I. scapularis* mitochondrial *porin* and the unrelated *Rs86* dsRNAs were synthesized using the same methods described previously (Ayllón et al., 2013; de la Fuente et al., 2006c) and used as positive and negative controls, respectively. The dsRNA was purified and quantified by spectrophotometry.

RNAi experiments were conducted in cell cultures by incubating tick cells with 10 μl dsRNA (5×10^{10} to 5×10^{11} molecules/ul) and 90 μl L-15B medium in 24-well plates, using 4 wells per treatment (de la Fuente et al., 2008). Control cells were incubated with the unrelated control *Rs86* dsRNA. After 48 h of dsRNA exposure, tick cells were inoculated with 1ml/well of cell-free *A. phagocytophilum* human NY18, dog L610 or sheep isolates semi-purified from approximately 5×10^6 infected tick cells (35 to 50% infected cells). Briefly, tick cells were mechanically disrupted with a syringe and a 27 gauge needle, centrifuged at 200 x g for 5 minutes to remove cells debris and intact cells and the volume adjusted to 24 ml with L-15/L-15B (IRE/CTVM20 cells) or L-15B300

(ISE6 cells) culture medium (Thomas and Fikrig, 2007) then 1ml was added per well or they were mock infected by adding 1ml/well culture medium alone. Cells were incubated for an additional 72 h, collected, and processed for DNA and RNA extraction. Total RNA was used to analyze *porin* gene knockdown by real-time RT-PCR with respect to the *Rs86* control. DNA was used to quantify the *A. phagocytophilum* infection levels by *msp4* PCR as described previously (de la Fuente et al., 2006b).

The results were compared by Student's t-test with unequal variance between *A. phagocytophilum*-infected and uninfected cells (P<0.05; N=4) and between *porin* or *Rs86* dsRNA treated cells (P<0.05; N=4).

Analysis of tick cell mRNA levels by real-time RT-PCR

Total RNA was extracted from uninfected and *A. phagocytophilum*-infected ISE6 and IRE/CTVM20 cells at 0h and 24h post-infection using TriReagent (Sigma, St. Louis, MO, USA) following the manufacturer's recommendations. Real-time RT-PCR was performed on RNA samples with gene-specific primers (Table 1) at 55°C using the iScript One-Step RT-PCR Kit with SYBR Green and the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following the manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently in the same temperature range for every sample (Ririe et al., 1997). The mRNA levels were normalized against *ribosomal protein S4* (*rps4*; Koči et al., 2013) and *cyclophilin* using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0) (Livak and Schmittgen, 2001). The results were compared by Student's t-test with unequal variance between infected and uninfected cells (P<0.05; N=3).

Determination of *A. phagocytophilum* infection by real-time PCR and immunofluorescence of tick cells

DNA was extracted from 200 μl of tick cells using TriReagent (Sigma, Madrid, Spain) following the manufacturer's recommendations. *A. phagocytophilum* infection levels were characterized by *msp4* real-time PCR with normalization against the level of tick *16S rRNA* as described previously (de la Fuente et al., 2006a) with PCR conditions of 5 min at 95℃ and 35 cycles of 15 s at 95℃, 30 s at 60℃, and 30 s at 72℃.

For the immunofluorescence assays, 200 μl of tick cells were harvested at 0, 7, 24 and 48 h after inoculation. Tick cell smears were prepared using a cytocentrifuge then fixed with 10% neutral buffered paraformaldehyde for 1 h at room temperature. The cells were permeabilized with 0.3% Triton X-100 in PBS for 30 min, blocked with 2.5% bovine serum albumin in PBS for 1 h at room temperature, and incubated over- night at 4℃ with purified MSP4 antibodies (24 μg/ml). After washing with PBS, slides were incubated in 100 μl PBS with FITC-goat anti-rabbit IgG (Sigma)-labeled antibody (diluted 1/160) for 75 min at room temperature. Finally, the slides were washed with PBS and mounted with ProLong Gold antifade reagent (Invitrogen). Images were acquired on a Nikon Eclipse Ti-U microscope with a 60x objective and a Nikon Digital Sight DS Vi1 camera.

Western Blot analysis

Cell pellets from uninfected and *A. phagocytophilum*-infected ISE6 and IRE/CTVM20 cells were collected at 24 h post-infection by centrifugation at 700 x g for 5 min and resuspended in 500 μl of Lysis Buffer (10 mM phosphate buffer saline (PBS), pH 7.4, supplemented with 1% Triton X-100 and complete mini protease inhibitor cocktail). To lyse the cells, samples were passed through a 27G needle several times, followed by sonication and vortexing cycles for 30 min. The resulting homogenates were centrifugated at 200 x g for 5 min to remove cellular debris. The supernatants were collected and protein concentration was determined by BCA Protein Assay (Thermo

Scientific, San Jose, CA, USA) using BSA as standard. Forty five μg from each sample were resuspended in Laemmli Sample buffer and separated on a 15% SDS-PAGE gel under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with SuperBlock blocking buffer in TBS (Thermo Scientific) for an hour at room temperature and incubated overnight at 4°C with a 1:200 dilution of rabbit polyclonal anti-Cytochrome c (Santa Cruz Biotechnology) or anti-Porin (Ayllón et al., 2013) antibodies. To detect the antigen-bound antibody, membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:20,000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Immunoreactive proteins were detected by chemoluminescence using the SuperSignal West Pico chemoluminescent substrate (Thermo Scientific) and visualized with an ImageQuant 350 Digital Imaging System (GE Healthcare, Pittsburgh, PA, USA).

Results and Discussion

A. phagocytophilum isolated from human, canine and ovine hosts infects *I. ricinus* and *I. scapularis* tick cells

Rickettsial inclusions in the tick cells were first recognised after 41 (dog isolate) or 30 days (sheep isolate) of incubation with *A. phagocytophilum* isolated from either canine or ovine blood samples. The infected cells contained one or more large vacuoles filled with numerous bacteria. Examination of DNA extracted from infected cell suspensions by quantitative real-time PCR confirmed multiplication of *A. phagocytophilum* by decrease of Ct values over time. Once established, the *A. phagocytophilum* isolates were subsequently subcultured every 3-4 weeks. For this purpose, 500 μl aliquots of cell suspension containing a high proportion of infected cells were transferred into new 25 cm^2 flasks containing uninfected IRE/CTVM19 cells in 5 ml cell culture medium (final
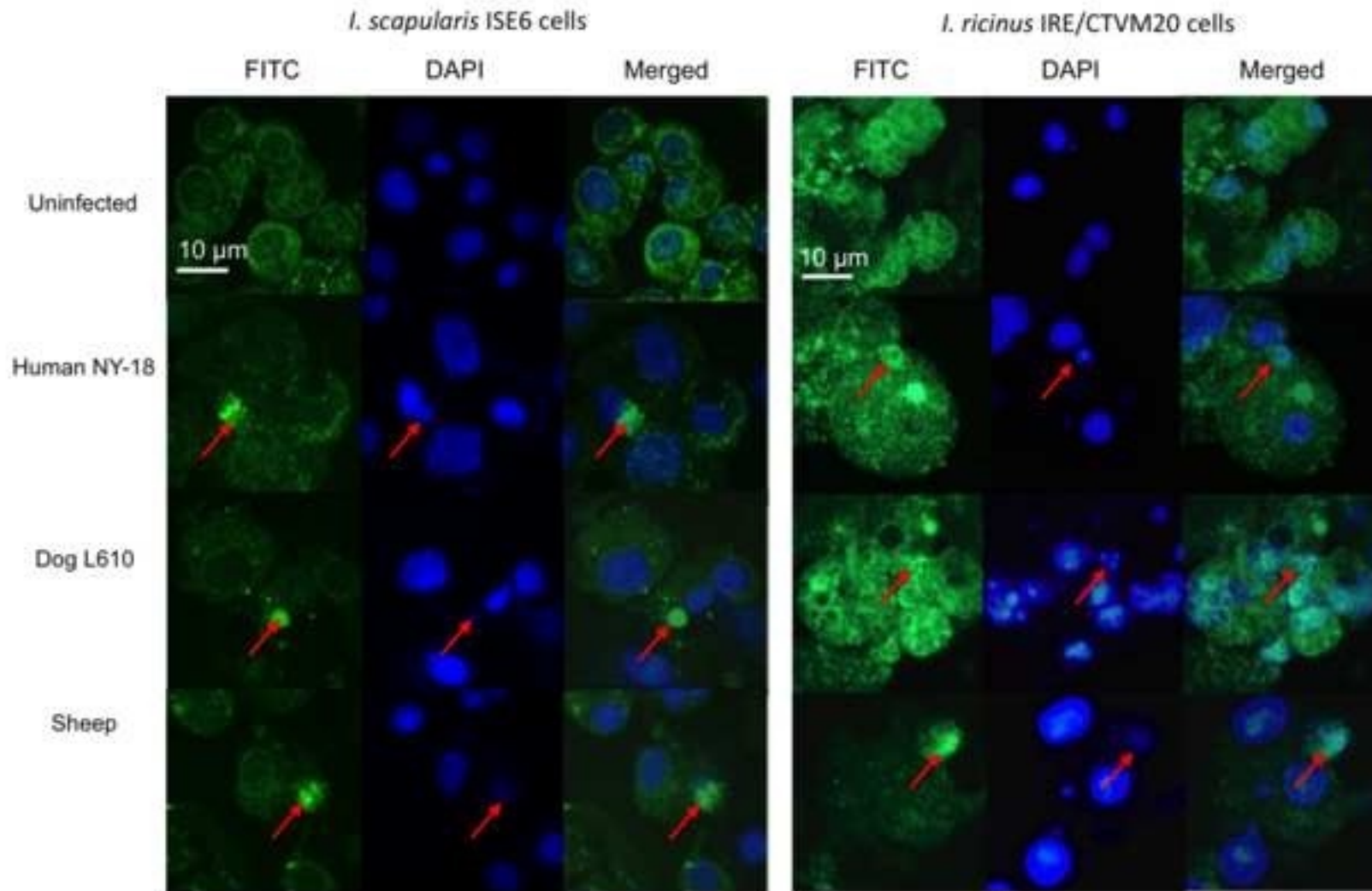
dilution 1:10). The *I. scapularis* ISE6 and *I. ricinus* IRE/CTVM20 cell lines were also inoculated with cell-free supernatants from IRE/CTVM19 cultures infected with the sheep *A. phagocytophilum* isolate. The infection resulted in rapid development of inclusions containing *Anaplasma* organisms in the cells and finally in lysis of the cells. When equal volumes of inoculum were subcultured into fresh IRE/CTVM20 and ISE6 cells simultaneously, the development of bacterial inclusions was observed firstly in IRE/CTVM20 cells (approximately 2-3 weeks after inoculation) and then in ISE6 cells about one week later. No differences in microscopic appearance were observed between the three isolates. Both tick cell lines supported multiplication of the three bacterial isolates (Fig. 1).

Multilocus sequence analysis using *gltA*, *groEL* and *msp4* was conducted to confirm the identitity of the three isolates used in this study (Fig. 2). As expected, the results showed that the sheep isolate clustered together with Norway variant2 isolate obtained from a lamb. The L610 dog isolate clustered together with isolates from horses, ticks, humans, rodents and dogs but was different from the previously reported Dog2 isolate (Al-Khedery et al., 2012). Finally, as expected, the human NY18 isolate clustered together with other human isolates.
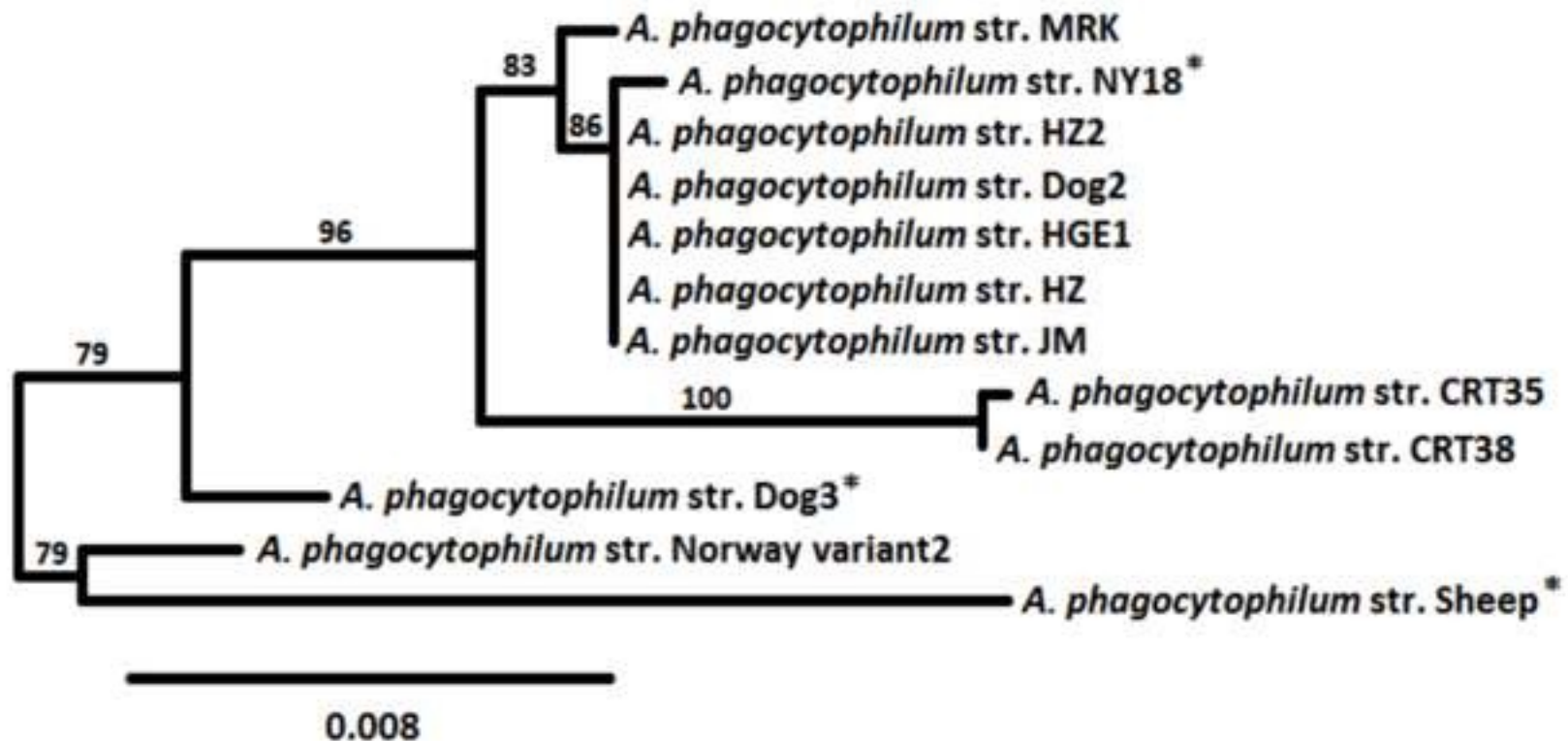
All three *A. phagocytophilum* isolates inhibit apoptosis of tick cells to promote their survival

An increase in *A. phagocytophilum* DNA levels was observed for all three isolates after infection of both *I. scapularis* ISE6 and *I. ricinus* IRE/CTVM20 cells, thus confirming bacterial multiplication (Fig. 3A). However, human NY18 and dog L610 isolates were able to multiply faster and reached higher infection levels in *I. scapularis* cells while the sheep isolate grew faster in *I. ricinus* cells (Fig. 3A). Apoptosis of tick cells was analyzed by flow cytometry at 24 h post-infection and showed a decrease when
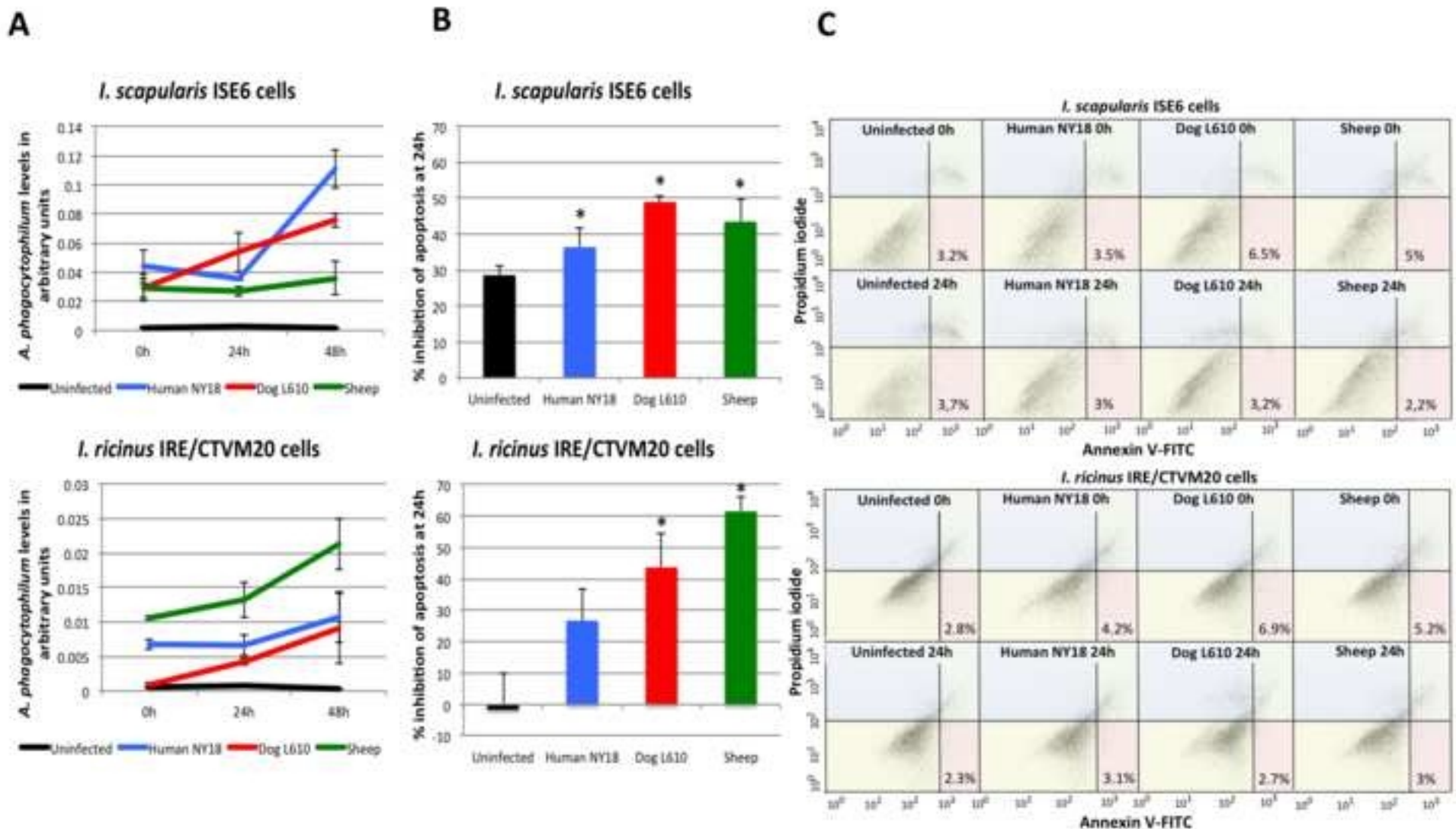
Figure 1. A. phagocytophilum infection of tick cells. Microphotography of the immunofluorescence analysis of tick cells demonstrated the presence of A. phagocytophilum inside tick cells 24 h after infection with all three isolates in ISE6 and IRE/CTVM20 cells. Tick cells were stained with rabbit anti-MSP4 antibodies (green, FITC) or DAPI (blue). Anaplasma morulae (arrows) were observed 24 h after infection with all 3 isolates. Bar, 10 µm.

Figure 2. Phylogenetic analysis of A. phagocytophilum isolates. Multilocus sequence analysis of A. phagocytophilum isolates. The nucleotide sequences of the selected genes (gltA, groEL and msp4) were concatenated and aligned to construct a phylogenetic tree using maximum likelihood. Numbers of internal branches were obtained using the approximate likelihood ratio test (aLRT – SH-Like). The isolates obtained in this study are marked with an asterisk. For comparison, the same sequences were obtained from isolates, CRT35 (JFBI01000001), CRT38 (NZ_APHI00000000), Dog2 (CP006618), JM (CP006617), MRK (JFBH00000000), Norway variant2 (JFBJ00000000), HZ (CP000235), HGE1 (NZ_APHH01000002), and HZ2 (NC_021879) and included in the analysis.
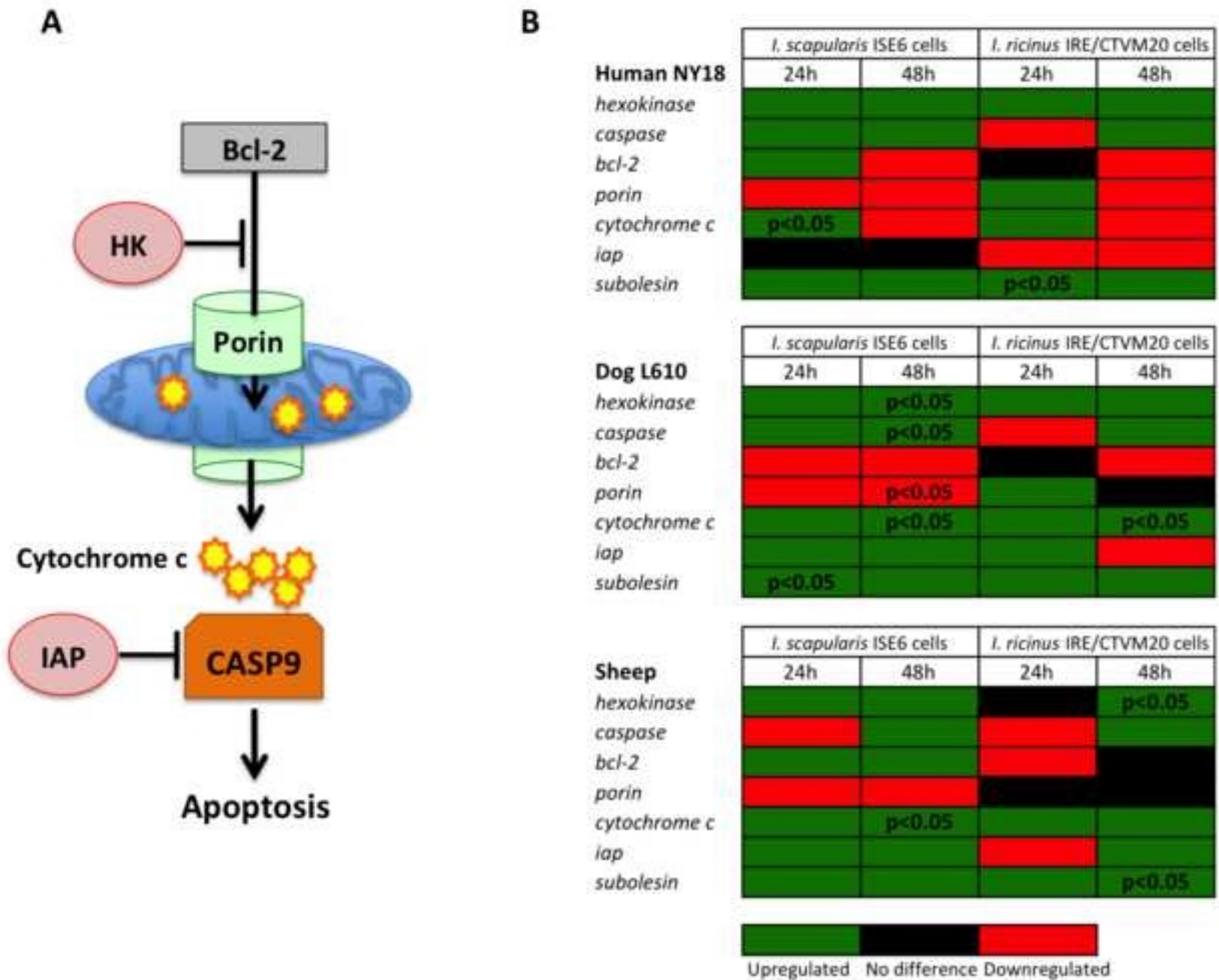
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Figure 3. Apoptosis in infected and uninfected tick cells. A. phagocytophilum inhibited apoptosis of ISE6 and IRE/CTVM20 cells within 24 h of infection. (A) Tick cells were infected with A. phagocytophilum at 0 h and grown for 48 h. A. phagocytophilum DNA levels were determined by PCR and represented as mean ± SD in arbitrary units (N=3). (B) The percentage of apoptotic cells at 24 h post-infection was determined by flow cytometry after Annexin V-FITC and PI labeling and compared between infected and uninfected cells by Student' s t-test with unequal variance (*P<0.05; N=3). The percent inhibition of apoptosis was calculated by comparing cells collected at 0 and 24 h post-infection and represented as average ± SD. (C) Flow cytometry analysis of ISE6 and IRE/CTVM20 cells; percentages shown indicate the number of Annexin V positive, PI negative and Annexin V, PI double positive cells. Data is representative of three experiments.

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compared to uninfected controls (Figs. 3B and 3C). The percentage inhibition of apoptosis between 0 and 24 h post-infection was higher for the dog and sheep isolates when compared to the human NY18 isolate (Fig. 3B), possibly reflecting adaptation to tick cells due to the fact that the NY18 isolate has been in culture for much longer than the dog and sheep isolates. Each isolate grew faster in the tick cells derived from their natural vector species.

Infection with *A. phagocytophilum* isolates inhibits the intrinsic apoptosis pathway at different levels in *I. scapularis* and *I. ricinus* cells

Previous results suggested that mitochondrial Porin is involved in inhibition of the intrinsic apoptosis pathway by *A. phagocytophilum* infection of tick cells (Ayllón et al., 2013). The intrinsic apoptosis pathway is induced by Bcl-2 proteins through Porin resulting in Cytochrome c release and activation of CASP9, and is inhibited by HK and inhibitors of apoptosis (IAP) (Fig. 4A). Therefore, levels of gene and protein expression for these molecules were characterized in infected and uninfected tick cells.

Gene expression analysis in response to *A. phagocytophilum* infection in tick cells showed a tendency towards upregulation for most genes (Fig. 4B). However, statistically significant (P<0.05) differences between infected and uninfected cells were only obtained for some of the genes (Fig. 4B). Nevertheless, results showed differences between both *A. phagocytophilum* isolates and tick cell lines. For example, as previously reported for the NY18 isolate in ISE6 cells (Ayllón et al., 2013), *porin* expression was downregulated in response to infection with the dog L610 isolate in ISE6 cells, but this downregulation was not seen in IRE/CTVM20 cells. In contrast, *cytochrome c* mRNA levels increased in response to infection with the dog L610 isolate in both tick cell lines. The expression of *cytochrome c* was significantly upregulated at either 24 or 48 h post-infection for all bacterial isolates in ISE6 cells but only for the

Figure 4. Mitochondrially induced apoptosis pathway. (A) Schematic representation of the intrinsic apoptosis pathway. (B) Characterization of differential expression of genes involved in the intrinsic apoptosis pathway in response to A. phagocytophilum infection of tick cells at 24 h and 48 h post-infection. Up- or downregulation was calculated as the ratio with respect to uninfected cells using normalized Ct values from real time RT-PCR and analyzed statistically Student' s t-test with unequal variance (P<0.05).

L610 isolate in IRE/CTVM20 cells. However, *caspase* expression was significantly upregulated in ISE6 cells with the dog L610 isolate only, and was downregulated at 24 h in IRE/CTVM20 cells infected with all three *A. phagocytophilum* isolates. Subolesin has been shown to be involved in tick cell response to pathogen infection (de la Fuente et al., 2008) and as expected was upregulated by infection with all three *A. phagocytophilum* isolates (Fig. 4B).

To further characterize the effect of pathogen infection on the tick intrinsic apoptosis pathway, CASP9 levels (Fig. 5A), HK activity (Fig. 5B), Cytochrome c levels (Fig. 5C) and Porin levels (Fig. 5D) were measured in uninfected and *A. phagocytophilum*-infected tick cells at 24 h post-infection. The results showed that infection with *A. phagocytophilum* significantly reduced CASP9 levels in IRE/CTVM20 but not in ISE6 cells (Fig. 5A), suggesting a difference between the two cell lines in how their apoptosis response is affected by infection.

HK activity was lower in *I. ricinus* IRE/CTVM20 cells infected with the European *A. phagocytophilum* isolates (dog L610 and sheep) than in uninfected cells and cells infected with the North American NY18 isolate. In contrast, with *I. scapularis* ISE6 cells HK activity was higher in cells infected with the sheep isolate than in uninfected cells or cells infected with the human and dog isolates (Fig. 5B). As in previous experiments using the human NY18 isolate of *A. phagocytophilum* (Ayllón et al., 2013), these results suggested an impact of vector-pathogen co-evolution on the adaptation of *A. phagocytophilum* isolates to grow in tick cells.

Cytochrome c protein levels detected by Western blot decreased in ISE6 cells infected with the human and sheep isolates, but not the dog isolate when compared to the uninfected controls (Fig. 5C). In contrast, in uninfected IRE/CTVM20 cells Cytochrome c protein levels were almost undetectable, but increased in *A.*

Figure 5. Apoptosis pathway proteins in A. phagocytophilum-infected and uninfected tick cells. (A) CASP9 levels (OD450nm values; average ± SD) were determined at 24 h post-infection using a CASP9 colorimetric assay. (B) Hexokinase activity (mU/ml; average ± SD) were determined at 24 h post-infection using an HK colorimetric assay. (C) Cytochrome c levels were determined at 24 h post-infection by Western blot and a representative image is shown. The Cytochrome c protein (ı12 kDa) is indicated with an arrow. (D) Porin levels were determined at 24 h post-infection by Western blot and a representative image is shown (Expected Porin size 30 kDa). In all the experiments, results from uninfected and A. phagocytophilum-infected ISE6 and IRE/CTVM20 cells were compared by Student's t-test with unequal variance (*P<0.05; N = 3).
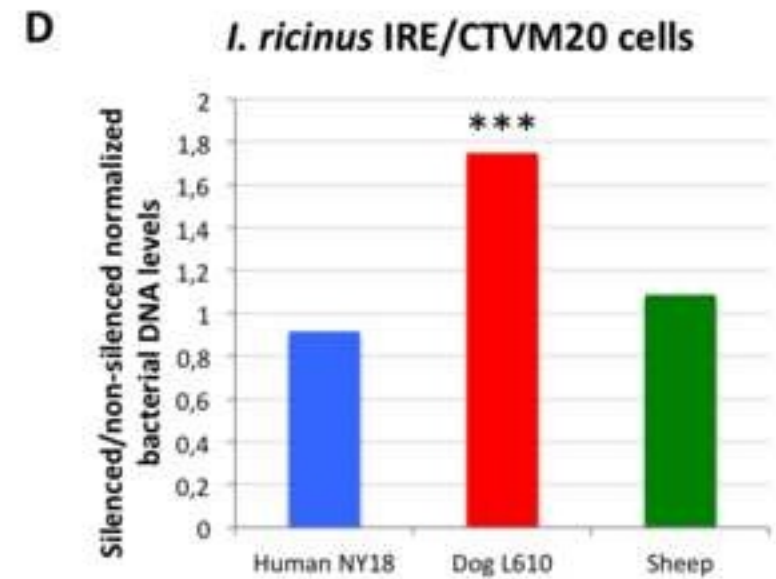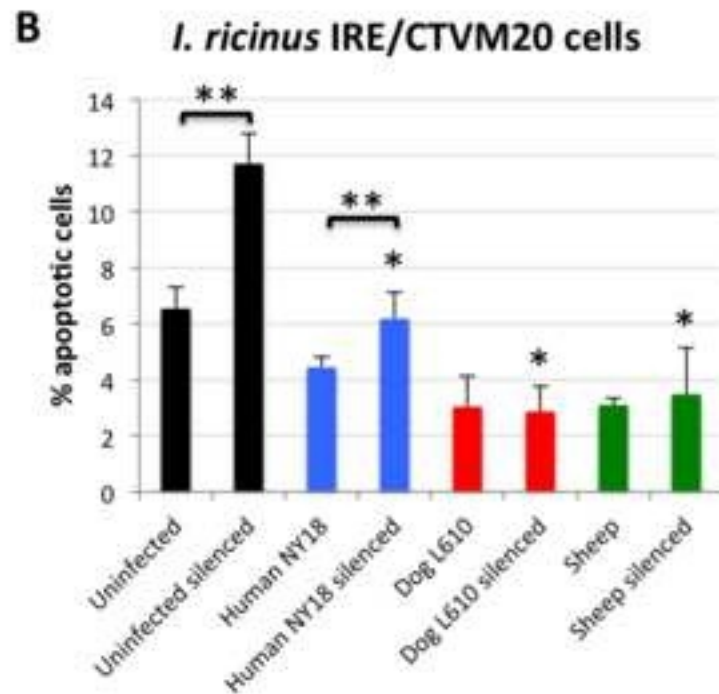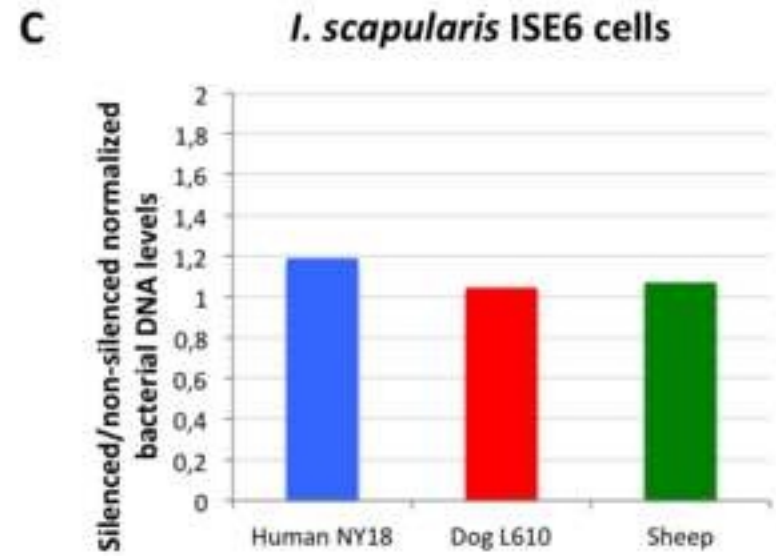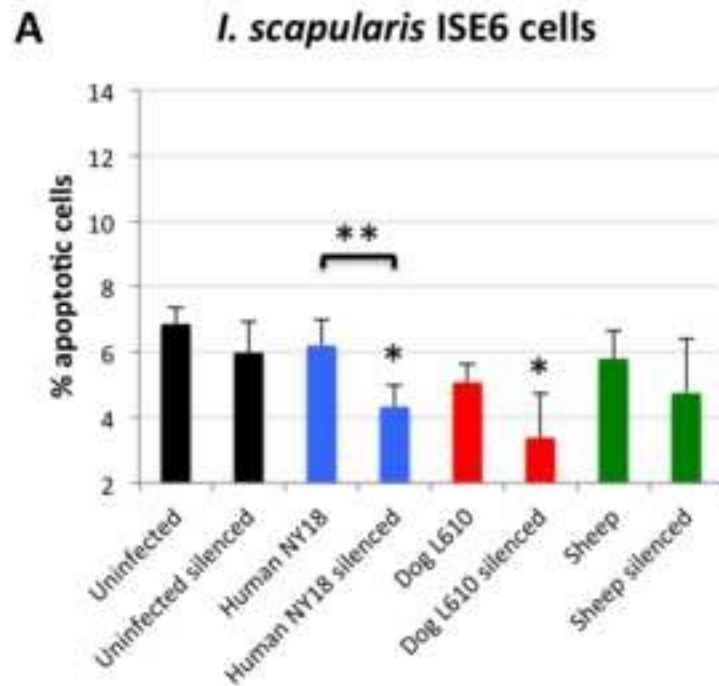

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phagocytophilum-infected cells, particularly in the cells infected with the L610 and NY18 isolates (Fig. 5C). Porin protein levels appeared to be slightly lower in infected *I. scapularis* ISE6 cells when compared to the uninfected control but were unchanged in *I. ricinus* IRE/CTVM20 cells (Fig. 5D).

Taken together, the results of the analyses at the mRNA and protein levels at 24 h post-infection suggested that, as previously reported in *I. scapularis* tick cells (Ayllón et al., 2013), *A. phagocytophilum* infection of ISE6 cells downregulated *porin* expression resulting in lower Cytochrome c protein levels as a mechanism to inhibit the intrinsic apoptosis pathway. However, in *I. ricinus* IRE/CTVM20 cells the inhibition of apoptosis appeared to be regulated by lower CASP9 protein levels in infected tick cells. These mechanisms for the inhibition of cell apoptosis occurred independently of the origin of the *A. phagocytophilum* isolates.

Effect of T2 porin knockdown on *A. phagocytophilum* infection levels in ISE6 and IRE/CTVM20 cells

Recently, Ayllón et al. (2015) showed that *A. phagocytophilum* (NY18) inhibits the intrinsic apoptosis pathway through *porin* downregulation to prevent Cytochrome c release in *I. scapularis* salivary glands. To characterize this mechanism *in vitro*, gene knockdown by RNAi was used for functional characterization of the *porin* gene during *A. phagocytophilum* infection of ISE6 and IRE/CTVM20 cells. Significant gene knockdown (45-68% gene silencing) was obtained in both ISE6 and IRE/CTVM20 cells. Similar to results in tick salivary glands (Ayllón et al., 2015), silencing of *porin* expression inhibited apoptosis in ISE6 cells infected with the human NY18 isolate when compared to control cells treated with the unrelated *Rs86* dsRNA (Fig. 6A). However, this effect was not significant for the dog and sheep isolates in ISE6 cells (Fig. 6A). Furthermore, infection with the human and dog isolates did inhibit apoptosis when

Figure 6

Figure 6. Effect of *porin* gene knockdown on apoptosis and pathogen infection of tick cells. (A) *A. phagocytophilum* infection significantly inhibited apoptosis in *I. scapularis* cells treated with *porin* dsRNA and infected with the human and dog isolates (*p<0.05). The porin gene knockdown significantly inhibited apoptosis in I. scapularis cells infected with the human isolate (**p<0.05). (B) A. phagocytophilum infection significantly inhibited apoptosis in I. ricinus cells treated with porin dsRNA and infected with the human, dog and sheep isolates (*p<0.05). The porin gene knockdown significantly inhibited apoptosis in I. ricinus cells infected with the human isolate (**p<0.05). (C) The porin gene knockdown did not affect rickettsial DNA levels in infected I. scapularis cells. (D) The porin gene knockdown resulted in higher levels of rickettsial DNA for the dog L610 isolate in I. ricinus cells when compared to controls teated with control Rs86 dsRNA (***p<0.05). The percent of apoptotic cells were compared by Student' s t-test with unequal variance between A. phagocytophilum-infected and uninfected cells treated with porin dsRNA (*P<0.05; N=4) and between porin (silenced) or Rs86 dsRNA treated cells (**P<0.05; N=4). Rickettsial DNA levels were compared between porin and Rs86 dsRNA treated cells by Student' s t-test with unequal variance (***P<0.05; N=4).

compared to uninfected cells (Fig. 6A). In IRE/CTVM20 cells, *porin* knockdown increased the percentage of apoptotic cells in both uninfected and *A. phagocytophilum* (NY18)-infected cells by an unknown mechanism (Fig. 6B). However, as in ISE6 cells, infection with the human, dog and sheep isolates inhibited apoptosis when compared to uninfected cells (Fig. 6B). Despite the effect of infection on tick cell apoptosis, only the levels of the dog isolate of *A. phagocytophilum* increased after porin gene knockdown in *I. ricinus* but not in *I. scapularis* cells (Figs. 6C and 6D). These results reinforced the view that infection with *A. phagocytophilum* inhibits the intrinsic apoptosis pathway at different levels in *I. scapularis* and *I. ricinus* cells infected with different rickettsial isolates suggesting that the tick cell response to infection through other apoptosis pathways (Ayllón et al., 2015) may be limiting rickettsial infection in these cells. These results highlighted differences not only between *I. scapularis* and *I. ricinus* tick cells and between different isolates of *A. phagocytophilum*, but also between *in vitro* and *in vivo* results due to apoptotic tick tissue-specific response to infection (Ayllón et al., 2015).

Conclusions

The results showed that infection with different and unrelated isolates of *A. phagocytophilum* inhibits the intrinsic apoptosis pathway of tick cells early in infection, but with a clear effect on pathogen infection. Nevertheless, infection with *A. phagocytophilum* isolates inhibited the intrinsic apoptosis pathway at different levels in *I. scapularis* and *I. ricinus* tick cells. These results suggest that vector-pathogen co-evolution has an impact on the adaptation of *A. phagocytophilum* isolates to grow in tick cells, as each isolate grew faster in the tick cells derived from their natural vector species. These results increase our understanding of *A. phagocytophilum* infection and

multiplication and suggest that several mechanisms may affect disease prevalence in different geographical regions.

Acknowledgements

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