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Journal

Parasitology, 146(5)

ISSN

0031-1820

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Publication Date

2019-04-01

DOI

10.1017/s0031182018001968

Peer reviewed

Published in final edited form as:

Parasitology. 2019 April ; 146(5): 569–579. doi:10.1017/S0031182018001968.

Encephalitozoon cuniculi and *Vittaforma corneae* (Phylum Microsporidia) inhibit staurosporine-induced apoptosis in human THP-1 macrophages *in vitro*

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SUMMARY / ABSTRACT

Obligately intracellular microsporidia regulate their host cell life cycles, including apoptosis, but this has not been evaluated in phagocytic host cells such as macrophages that can facilitate infection but also can be activated to kill microsporidia. We examined two biologically dissimilar human-infecting microsporidia species, *Encephalitozoon cuniculi* and *Vittaforma corneae*, for their effects on staurosporine-induced apoptosis in the human macrophage-differentiated cell line, THP1. Apoptosis was measured after exposure of THP-1 cells to live and dead mature organisms via direct fluorometric measurement of Caspase 3, colorimetric and fluorometric TUNEL assays, and mRNA gene expression profiles using Apoptosis RT^2 Profiler PCR Array. Both species of microsporidia modulated the intrinsic apoptosis pathway. In particular, live *E. cuniculi* spores inhibited staurosporine-induced apoptosis as well as suppressed pro-apoptosis genes and upregulated anti-apoptosis genes more broadly than *V. corneae.* Exposure to dead spores induced an opposite effect. *V. corneae*, however, also induced inflammasome activation via Caspases 1 and 4. Of the 84 apoptosis-related genes assayed, 42 (i.e. 23 pro-apoptosis, 9 anti-apoptosis, and 10 regulatory) genes were more affected including those encoding members of the Bcl2 family, caspases and their regulators, and members of the TNF / TNFR superfamily.

Keywords

opportunistic parasites; intracellular parasites; inflammation; TUNEL; immune evasion; caspases

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ETHICAL AND REGULATORY GUIDELINES

Not applicable – i.e. no experimental procedures were used on animals or humans.

CONFLICTS OF INTEREST

The authors declare no financial, professional, or personal relationship conflicts of interest.

INTRODUCTION

Microsporidia are ubiquitous fungi-related, obligate intracellular parasites that evolved with extremely reduced genomes and proteomes to become highly dependent upon the host cell biochemistry (Cuomo *et al.*, 2012; Peyretaillade *et al.*, 2011). This host cell dependency has been highlighted by the ability of several microsporidia species that secrete factors and appropriate host cell metabolism in favor of their infection and replication (Cuomo *et al.*, 2012; Ferguson & Lucocq, 2018; He *et al.*, 2015; Heinz *et al.*, 2014; Luallen *et al.*, 2015; Panek *et al.*, 2014; Senderskiy *et al.*, 2014; Watson *et al.*, 2015; Wiredu Boakye *et al.*, 2017). In addition, microsporidia can induce cell hypertrophy, giant cell formation, and increase lifespan of infected cells (Aoki Mdel *et al.*, 2006; Desjardins *et al.*, 2015; Leitch *et al.*, 2005; Stentiford *et al.*, 2014; Timofeev *et al.*, 2016).

Apoptosis of infected cells is a universal mechanism used by the host to eliminate pathogens and facilitate the release of pathogen antigens to then promote adaptive humoral and cell-mediated immune responses in mammals (Liu *et al.*, 2009). Signaling pathways involved in regulating the host cell cycle and apoptosis however also are primary targets of intracellular prokaryotic and eukaryotic parasites for subverting innate immune responses (Faherty & Maurelli, 2008; Hay & Kannourakis, 2002; Huang *et al.*, 2016; James & Green, 2004; Luder *et al.*, 2001). A number of intracellular parasites, including several species of microsporidia, have evolved means to subvert apoptosis mechanisms to maintain or prolong infection (del Aguila *et al.*, 2006; James & Green, 2004; Scanlon *et al.*, 1999). For example, *Anncaliia algerae* inhibited induced apoptosis in human lung fibroblasts (Scanlon *et al.*, 1999) and infection with *Encephalitozoon* spp. disrupted the host cell cycle of Vero cells (Scanlon *et al.*, 2000). *E. cuniculi* also suppressed induced apoptosis in Vero cells and modulated the host cell cycle via inhibiting tumor suppressor factor p53 (del Aguila *et al.*, 2006). *V. corneae* induced formation of multinucleated xenoma-like structures during infection of monkey kidney cells (Leitch *et al.*, 2005) and *Nosema bombycis* suppressed apoptosis in a *Bombix mori* ovarian cell line (He *et al.*, 2015). Furthermore, *Nosema ceranae* and *Nosema apis* can modulate the host cycle and suppress apoptosis of the ventricular epithelium cells of *Apis melifera* (Higes *et al.*, 2013; Huang *et al.*, 2016; Kurze *et al.*, 2016; Kurze *et al.*, 2015; Martin-Hernandez *et al.*, 2017).

Macrophages are key cells in the innate immune system of vertebrates and invertebrates and comprise the first line of defense against intracellular parasites for then transitioning to adaptive immune response in higher vertebrates. Macrophages can kill microsporidia via activation signaling processes yet also serve as host cells of infection for several species of microsporidia. Among these are the *Encephalitozoon species*, i.e. *E. cuniculi*, *E. hellem,* and *E. intestinalis*, as well as *V. corneae,* species that infect mammals, including humans, and that can be grown in tissue culture. *Encephalitozoon spp.* replicate within host cell-derived parasitophorous vacuoles while *V. corneae* replicates in direct contact with the host cell cytoplasm. Based on the earlier reports that microsporidia affect the host cell machinery and apoptosis pathways, the purpose of this study was to examine the effects of these two biologically dissimilar human-infecting microsporidia, *E. cuniculi* and *V. corneae*, on staurosporine-induced apoptosis in the human macrophage-differentiated cell line, THP-1.

The results demonstrated that viable microsporidia, but not heat-killed organisms, inhibited apoptosis of the THP-1 macrophages.

MATERIALS AND METHODS

Macrophage cell line and microsporidia.

Human monocytic leukemia THP-1 cells (ATCC TIB-202; American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml) referred to as RPMI complete medium. Culture and cryopreservation methods were performed per instructions provided by ATCC (https://www.atcc.org/Products/All/TIB-202.aspx#culturemethod). *E. cuniculi* organisms (originally isolated from rabbit; ATCC #50503), and *V. corneae* (originally isolated from human; ATCC #50505) were grown in RK-13 rabbit kidney epithelial cells (ATCC #CCL-37) in RPMI complete medium. Medium was exchanged at least twice per week and culture supernatants containing released microsporidia spores were stored in sterile flasks at 4°C until use within one month of collection.

Microsporidia spores were enriched from host cell debris as described previously (Didier *et al.*, 2010). Culture supernatants were transferred to tubes, centrifuged (400 x g 10 min at 4° C), and washed sequentially by centrifugation in sterile solutions of dH_2O, 0.3% Tween 20 in tris-buffered saline (TBS), and TBS. The pellets were resuspended in TBS, mixed with an equal volume of 100% Percoll (to achieve a final 50% Percoll vol/vol suspension), and centrifuged for 45 min at 500 x g. The pellets containing spores were washed again with TBS and suspended to the desired concentration. Suspensions of dead spores were prepared by boiling live spores for 15 min. Spore concentrations were determined by haemocytometer counting via phase contrast optics at 400 X final magnification.

Infection of THP-1 cell and induction of apoptosis.

THP-1 cells were plated at 4×10^6 cells per ml in 6- or 24-well plates for performing the caspase 3 fluorometric assay or in 8-well chamber slides (Nalge Nunc International, Naperville, IL) for the TUNEL assay. Live or dead microsporidia spores were added to wells at a parasite to host cell ratio of 3:1. Thirty minutes later, the cultures were treated with 40–80 nM (25–50 ng/ml) phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and incubated for 24 hr to differentiate the THP-1 cells to adherent macrophages. The cell cultures then were washed three times to remove PMA and fresh complete RPMI medium was added. Apoptosis was experimentally induced by treatment with 1 uM (50 ng/ml) of staurosporine (Sigma-Aldrich) at various times (as indicated in the results section).

Caspase 3 fluorometric assay.

At 1, 2, 4, or 8 days after exposure to microsporidia and 4–6 hrs after induction of apoptosis, cells were subjected to lysis buffer [250 mM HEPES, pH 7.4, 25 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 25 mM dithiothreitol (DTT); Sigma-Aldrich]. Lysates were collected in microtubes for storage at −70°C until use. Caspase 3 activity was measured according the manufacturer instructions using the 96-well

plate Caspase 3 fluorometric assay kit (Sigma-Aldrich) based on the hydrolysis of the peptide substrate Acetyl-Asp-Glu-Val-Asp-amido-4 methylcoumarin (Ac-DEVD-AMC) and cleavage of the fluorescent 7-amino-4-methylcoumarin (AMC). Optical density (OD) of the AMC cleavage product was quantified by a SPECTRAmaxM2 microplate reader and calculated as fluorescence units per mg of protein per ml lysate from the standard curve. Protein concentrations in the analyzed samples were measured by the Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

DeadEnd colorimetric and fluorometric TUNEL assays.

Apoptosis was also measured via in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL). Cultures of macrophages were grown in chamber slides, exposed to live or dead microsporidia, and induced for apoptosis at the same times points described for Caspase 3 assays. The cells were then fixed with 4% paraformaldehyde, permeabilized for 30 minutes with 0.2% Triton X-100 (Sigma-Aldrich), and assayed using either the DeadEnd Colorimetric TUNEL System (Promega, Madison WI) or the In Situ Cell Death Detection kit (TMR-red; Roche, Indianapolis, IN). In the DeadEnd assay, biotinylated nucleotides incorporated at the 3'-OH DNA ends were labeled by Streptavidin-HRP and detected using peroxidase substrate, hydrogen peroxide, and diaminobenzidine (DAB) for visualization of dark brown apoptotic nuclei. DAB-stained slides were mounted using Permount mounting medium (Fisher Scientific, Fai Lawn, NJ) and observed using a Leica (NNN) light microscope at 400X. Five fields of view per well (variant) were imaged using a digital SPOT camera attached to the microscope, and the numbers of TUNEL-positive cells relative to the total number of cell nuclei were determined. In the fluorometric TUNEL assay, nucleotides were conjugated to the TMR-red fluorescent stain allowing for direct detection of DNA strand breaks characteristic of apoptotic nuclei using fluorescent microscopy in the red range of the spectrum (with maximum of 580 nm). TMR-red-stained cells were retained on chamber slides in TBS at 4°C until further staining. Three independent sets of experiments were analyzed by each detection assay.

Immunofluorescent antibody (IFA) staining and confocal microscopy imaging to detect microsporidia infection and host cell apoptosis.

IFA was performed to ascertain and detect intracellular development of microsporidia in relation to staurosporine induction of apoptosis in individual THP-1 cells. The TMR-red-stained cells were blocked in 0.2 % v/v fish skin gelatin (FSG; Sigma-Aldrich) in TBS for 30 min and then incubated in 10% normal goat serum (NGS; GIBCO) for 40–60 min at room temp. A rabbit polyclonal antiserum raised against a combination of microsporidia species (*E. cuniculi, E. intestinalis*, *E. hellem* and *V. corneae*) was diluted 1:200 in 10% normal goat serum (NGS), centrifuged, and applied to experimental wells for incubation for 60 min at room temperature or overnight at 4 °C. The slides were washed sequentially in TBS containing 0.2% cold water fish skin gelatin (FSG; Sigma-Aldrich) and then TBS containing 0.1% Triton X-100. The cells on slides were then incubated with Alexa 488 (Molecular Probes, Eugene, OR)-conjugated goat-anti-rabbit IgG diluted 1:1000 in 10% NGS for 60 min at room temp. Slides were then rinsed with TBS and counterstained with To-Pro-3 (far red) fluorescent dye (Molecular Probes) to identify host cell nuclei. Slides were examined by inverted confocal laser scanning microscope Leica TCP SP2.

Approximately 12–14 optical sections at intervals of 0.3–0.5 μm were recorded for the presence of microsporidia.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) microarray.

Expression of apoptosis pathway-related genes was examined four days after exposure to live or dead spores or medium (control) and four hrs after apoptosis induction in THP-1 cells using the RT^2 Profiler PCR Array system (SABiosciences, Qiagen, Germantown, MD) per manufacturer's instructions. Briefly, cells were washed three times in TBS and lysed by Tryzol reagent (Invitrogen) and subjected to Qiagen clean-up columns. Total RNA (1 μg) was used to generate cDNA via incubation with random oligo-hexamers (Invitrogen) and reverse transcription (Superscript III; Invitrogen). Samples were treated with *E. coli* RNAse H (Invitrogen) for clearance of RNA-DNA helices and qRT-PCR was performed using the Apoptosis RT^2 Profiler PCR Array kit (SABiosciences - Qiagen) in a Strategene mx3000 thermocycler for amplification of 84 apoptosis pathway-associated genes in 96-well platform. These included 47 genes that encoded pro-apoptosis proteins, 21 genes that encoded anti-apoptosis proteins, and 15 genes encoding regulatory proteins (that may either trigger or suppress apoptosis). In addition to the "Functional gene grouping" table provided with the Apoptosis RT2 Profiler PCR Array kit (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-012A.html), anti- or pro-apoptotic gene functions were also verified using public databases for gene ontology (http://www.genecards.org/; http://www.geneontology.org/. Gene expression levels (E) were considered to be up- or down-regulated based on at least 2-fold differences over that of control untreated macrophages (i.e. $-2 \geq \Delta E \geq +2$, ΔE). Replicate samples were assayed in triplicate and were normalized for expression relative to the housekeeping gene, beta-2-microglobulin (one of the five housekeeping genes included in the kit and that was expressed at the same level in the experimental and control samples). Results were analyzed using Cluster software version 3 (de Hoon *et al.*, 2004; Eisen *et al.*, 1998) and hierarchical clustering to generate dendograms was performed with TreeView software version 2 (Saldanha, 2004). Canonical pathways were examined using Ingenuity Pathway Analysis (Qiagen).

Statistical analysis.

Statistical analyses and graphing were performed using Graphpad PRISM 7.01 (GraphPad Software, San Diego, CA, 2003). One-way ANOVA with Tukey pairwise post hoc test or unpaired *t*-test were used for analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Microsporidia infection of THP-1 macrophages inhibited staurosporine-induced caspase 3 activity.

THP-1 macrophages were inoculated with live or dead microsporidia and induced for apoptosis by treatment with staurosporine (or medium as a control) followed by measuring caspase 3 activity (Fig. 1). Results were most consistent for experiments at the 4-day infection time point which may reflect a sufficiently established infection that could be detected prior to cell rupture/leakage and parasite release that would have produced secondary infections. Thus, the results throughout this study were presented after 4 days'

infection. Exposure of THP-1 cells with live or dead spores of *E. cuniculi* and *V. corneae* did not independently induce caspase 3 activity. Interestingly, infection of the macrophages with microsporidia (i.e. after exposure to live spores) of both species did significantly inhibit staurosporine-induced caspase 3 activity compared to macrophages not incubated with live microsporidia and induced with staurosporine. Conversely, incubation of THP-1 macrophages with dead *V. corneae* spores significantly increased staurosporine-induced caspase 3 activity compared to the THP-1 macrophages induced with staurosporine in the absence of microsporidia. Incubation of the macrophages with dead *E. cuniculi* and then induced with staurosporine also increased caspase 3 activity over the non-microsporidia-treated apoptosis-induced macrophages, but this was not statistically significantly different.

Infection with viable *E. cuniculi*, but not *V. corneae* inhibited expression of TUNEL in staurosporine-induced THP-1 macrophages.

The DeadEnd colorimetric system was used to detect TUNEL-staining macrophages in cultures treated with live and dead *E. cuniculi* or *V. corneae*, with and without staurosporine-induction (and comparable medium controls). Macrophages incubated with live *E. cuniculi* and induced by staurosporine exhibited significantly fewer TUNEL-positive cells compared to macrophages incubated without microsporidia and induced with staurosporine (Fig 2A). Conversely, cultures incubated with dead *E. cuniculi* spores and induced with staurosporine exhibited significantly higher numbers of TUNEL-positive macrophages than macrophages only treated with staurosporine. Interestingly, staurosporine-induced THP-1 macrophages treated with either live or dead *V. corneae* spores exhibited higher percentages of TUNEL-expressing cells than macrophages only induced with staurosporine. Representative images in Fig. 2B show the relatively lower and higher numbers of TUNEL-positive staurosporine-induced macrophages incubated with live and dead *E. cuniculi*, respectively, compared to macrophages only induced with staurosporine.

TUNEL expression was absent in *E. cuniculi*-infected cells but occurred in *V. corneae*-infected cells.

To determine if the cells undergoing apoptosis were infected or uninfected after addition of live microsporidia spores as performed in studies of Fig. 2, we instead applied fluorometric TUNEL-staining assay in combination with immunostaining of microsporidia, and TO-PRO far red-staining of host cell nuclei. Of the cultures incubated with live *E. cuniculi*, none of the infected macrophages concurrently expressed TUNEL at 4 days after infection and 3.3% of the uninfected macrophages were TUNEL-positive (Fig. 3A). Among the cultures incubated with live *V. corneae*, 3.7% of the macrophages were concurrently infected and TUNEL-positive, and 9.7% of the uninfected macrophages were TUNEL-positive. Representative images show *E. cuniculi*-infected THP-1 cells (green) distinct from apoptotic uninfected macrophages (TUNELTMR-red) (Fig. 3B.a). Among cultures incubated with *V. corneae,* infected macrophages occasionally were observed to stain positively with TUNEL (Fig. 3B.b). Cultures incubated with dead *E. cuniculi* commonly exhibited the presence of apoptotic macrophages containing remnants of phagocytized spores (Fig. 3A.c). These results suggested that *E. cuniculi* inhibited apoptosis within cells infected for four days while *V. corneae*-inhibited apoptosis was lower and not distinctively selective between infected and neighboring uninfected cells.

Microsporidia-infected THP-1 macrophages exhibited an apoptosis inhibition profile.

The Qiagen SaBiosciences RT^2 profiler array for apoptosis-related gene expression (i.e. mRNA) was applied to THP-1 macrophages incubated with live versus dead microsporidia for four days and induced with staurosporine for comparison to control macrophages incubated in medium and induced with staurosporine. Heat maps using hierarchical clustering provide a visualized overview for the relative expression of the regulatory as well as pro- and anti-apoptosis-related genes of this array in macrophages infected with live microsporidia or exposed to dead spores (Fig. 4). Apoptosis inhibitory genes tended to be upregulated in infected macrophage cultures but downregulated in the cultures incubated with dead microsporidia. Conversely, pro-apoptosis genes were down-regulated in the cultures inoculated with live microsporidia and upregulated in the macrophages with dead microsporidia. The overall gene expression patterns indicated that live microsporidia spores producing infection inhibited apoptosis while dead spores of microsporidia promoted apoptosis of the macrophages, but that this appeared to be more strongly evident for *E. cuniculi* than *V. corneae*.

Among the genes expressed by macrophages incubated with dead *E. cuniculi* spores, 42–44 of the 47 pro-apoptosis genes were upregulated while only 3 of 21 anti-apoptosis genes were upregulated (Table S1). Conversely, only 4–9 of the 21 anti-apoptosis genes were upregulated and 12–15 genes were down-regulated, indicating that dead *E. cuniculi* spores promoted gene expression in macrophages that favored apoptosis. When relating percentages of up- versus-down-regulated genes within each group of pro-and anti-apoptosis genes, over 90% of the upregulated genes were pro-apoptotic in macrophages treated with dead *E. cuniculi* spores. Among the anti-apoptosis genes, the majority was down-regulated (i.e. nearly 64%). Macrophages treated with dead *V. corneae* spores however, did not exhibit such a strong difference in that approximately half of each group of pro- and anti-apoptosis genes was up- and down-regulated.

Macrophages incubated with live *E. cuniculi* exhibited up-regulation of 11–17 of the 21 anti-apoptosis genes and down-regulation of the 29–40 of the 47 pro-apoptosis genes. This also was associated with up-regulation of only 7–19 of the 47 pro-apoptotic genes. In macrophages incubated with live *E. cuniculi* spores, 69.4% of anti-apoptotic genes were up-regulated and 72.5% of pro-apoptotic genes were down-regulated. Again, the percentages of up- versus down-regulated pro- and anti-apoptosis genes were approximately 50% in macrophages treated with live *V. corneae*. Thus, the overall percentage of genes up- or down-regulated in macrophages after incubation with live or dead *E. cuniculi* shifted more than did the genes in macrophages after exposure to live or dead *V. corneae* spores.

Of the 84 apoptosis-related genes assayed in this array kit, 42 comprised the top ten genes that were up- or down-regulated in at least one of the four treatment groups (i.e. with live or dead microsporidia of two species). These included 23 pro-apoptosis, 9 anti-apoptosis, and 10 regulatory genes and encoded three major groups of proteins; the Bcl2 family (11 genes), caspases and their regulators (8 genes), and proteins of the TNF and TNFR superfamilies (11 genes) (Table S2).

Spores of both species studied here affected the expression of the anti-apoptosis *Bcl2* gene similarly; live spores of both species upregulated its expression while dead spores downregulated *Bcl2* expression. Interestingly, the gene for the pro-apoptotic Bax protein, known as *Bcl2* antagonist, was slightly upregulated by live (+1.75) and dead *E. cuniculi* (+1.04) spores, as well as by dead *V. corneae* spores (+1.10), whereas live *V. corneae* spores induced its upregulation 7.67-fold. Furthermore, *Bax* expression was the fourth highest upregulated gene in cells treated with live *V. corneae* after genes for tumor protein *p53* (+100), *Bad,* another pro-apoptotic member of Bcl2 family (+10.72), and *Casp1* (+8.17). After exposure of macrophages to live *E. cuniculi*, however, *Bad* and *Casp1* were down-regulated. Overall, exposure to live *E. cuniculi* resulted in downregulation of most pro-apoptosis members of Bcl2 family that conversely were upregulated after introduction of macrophages to dead *E. cuniculi* spores, but this was more variable after macrophages were exposed to live or dead *V. corneae* spores (Table S2, Figure 5).

Genes of the caspases *Casp1, Casp3, Casp4,* and *Casp9* that were among the ten most affected (Table S2) were downregulated by exposure to live *E. cuniculi* spores. Of these, exposure to live *V. corneae* spores induced upregulation of *Casp1* and *Casp4* that encode "inflammatory caspases" involved in inflammasome activation (Sollberger et al., 2012). Dead *V. corneae* spores, unlike dead *E. cuniculi* spores downregulated these genes. *Casp9* was the most significantly down-regulated gene after introduction of live *E. cuniculi* (−5.33, Table S2) but was only slightly affected by live *V. corneae* (−0.66). Live *V. corneae* down-regulated *Casp3* (−3.83), as did *E. cuniculi,* but to a lesser extent (− 1.88).

Live spores of both species noticeably suppressed macrophages expression of the gene for *LTA* (Lymphotoxin-α) that encodes Tumor Necrosis Factor-β (*E. cuniculi,* −2.66; *V. corneae*, −5.85). In contrast, exposure to dead spores induced *LTA* expression (*E. cuniculi,* +2.29; *V. corneae*, +5.85. Pro-apoptosis genes for *Fas, FADD, TNFRSF11B, TRADD,* and the genes for cytokine receptors of the TNFR superfamily and associated proteins were down-regulated by live *E. cuniculi* spores and up-regulated by dead *E. cuniculi*. However, live spores of *V. corneae* up-regulated *TNFRSF11B* (+3.94) and slightly *Fas* (+0.30), whereas dead *V. corneae* spores down-regulated both of these genes. Genes for anti-apoptotic and regulatory TNFR family members, *CD40LG* and *CD27*, were up-regulated by live *E. cuniculi*, spores (Table S2). The most dramatic effect was the 100-fold up-regulation of the gene for tumor protein TP53 by live spores of *V. corneae*, and conversely, its 46-fold down-regulation after exposure to dead spores. In comparison, live and dead spores of *E. cuniculi* only moderately increased *TP53* expression (2.5- and 1.59-fold after exposure to live and dead spores, respectively).

DISCUSSION

Suppression of cysteine aspartic acid protease (caspase) 3 activity in THP-1 macrophages after application of live, but not dead spores of *E. cuniculi* and *V. corneae* suggested microsporidia infections contributed to the arrest of the final stages of the cell death apoptosis pathways. The data presented here corroborate previously published results that *E. cuniculi* infection arrested Caspase 3 cleavage in infected Vero cells using western blot immunodetection (del Aguila *et al.*, 2006). Immunocytochemistry results demonstrated the

depletion of Caspase 3 in the ventricular epithelial cells of honeybees infected with *N. ceranae* (Higes *et al.*, 2013). In addition, blockade of Caspase 3 activation was demonstrated in cells infected with *Toxoplasma gondii* and other intracellular protists (Payne *et al.*, 2003).

Activation of Caspase 3 by Caspases 8, 9 or 10 at the "execution" stage of apoptosis leads to degradation of nuclei and other organelles via autophagy and proteasomal digestion. All three apoptosis signaling pathways, mitochondrial (intrinsic), death receptor (extrinsic), and perforin/granzyme pathways converge at the level of Caspase 3 (Bruchhaus *et al.*, 2007). TUNEL staining allows for visualization of DNA strand breaks characteristic for degrading nuclei. A significant reduction in the number of TUNEL-positive nuclei was previously shown in gut epithelial cells of honey bees in response to *N. apis* and *N. ceranae* infections (Huang *et al.*, 2016; Kurze *et al.*, 2015; Martin-Hernandez *et al.*, 2017). Results shown in the present study now demonstrate that human 0–1 macrophages exposed to live *E. cuniculi* also were inhibited from expressing TUNEL-positive nuclei after induction of apoptosis suggesting that the arrest of the final stages of apoptosis may be a common signature of microsporidia pathogenesis, even in phagocytic cells of the innate immune system. Interestingly, however, live *V. corneae* spores failed to demonstrate a substantial decrease in TUNEL-positive macrophages. Moreover, studies using both fluorescent TUNEL staining in combination with immunostaining of spores revealed that >20% of macrophages containing *V. corneae* spores displayed TUNEL-positive nuclei suggesting they were undergoing apoptosis while none of the macrophages infected with *E. cuniculi* expressed TUNEL staining.

The variable approaches these microsporidia species use for controlling host cell death pathways may reflect their differences in biology and mechanisms of infection (Cali & Takvorian, 2014). *E. cuniculi* develops within a membrane-bound parasitophorous vacuole (PV) within a host cell and may infect neighboring cells through polar filament extrusion while still located within the initial host cell PV. In addition, infected macrophages traffic into multiple organs to disseminate infections in new loci (Didier & Weiss, 2006). Thus, inhibition of host cell apoptosis or programmed cell death would be beneficial for maintaining *E. cuniculi* infection. In contrast, *V. corneae* infection and replication occurs in direct contact within the host cell cytoplasm, and the replicating and differentiating organisms eventually fill the entire host cell cytoplasm. *V. corneae* spores must exit the host cell and then triggered by environmental stimuli before they can extrude their polar filaments to infect new host cells. Another distinguishing feature about *V. corneae* infection has been the formation of bi- and multi-nucleated cells (Cali & Takvorian, 2014; Leitch *et al.*, 2005) which does not occur during *E. cuniculi* infections. Thus, host cell death and destruction after a period of *V. corneae* infection and replication may be a means for spores to escape and infect new host cells, especially after the entire host cell cytoplasm becomes laden with organisms. As such, protection from programmed cell death or apoptosis appears more beneficial to *E. cuniculi* than to *V. corneae* after initial infection is established.

Analysis of expression profiles of apoptotic-related genes further revealed profound differences between two species. Infection with *E. cuniculi* and *V. corneae* often induced different modulations in apoptosis-related gene expression. Among the 42 most affected genes (Table 2S, Figure 5), 12 were affected in opposite directions by *E. cuniculi* and *V.*

corneae. After introduction with live *E. cuniculi,* pro-apoptosis genes such as *Bad* (−1.6*),* *Bcl-2L1* (−1.5), and *BclL-2L11* (−1.8) were down-regulated in the THP-1 macrophages, and anti-apoptosis genes such as *Birc2* (+0.52) and *CD40LG* (+2.74) were slightly up-regulated. Alternatively, the same pro-apoptosis genes were essentially upregulated including *Bad* (+10.72), *Bcl-2L1* (+4.4), and *Bcl-2L11* (+4.1) in macrophages treated with *V. corneae* spores, while the anti-apoptosis genes such as *Birc2* (−2.4) and *CD40LG* (−4.33) were down-regulated (Fig. 5; Table S2). Thus, the spectrum of up- or down-regulated genes suggests that infection with *E. cuniculi* more strongly protects macrophages from apoptosis at the four-day post infection time point assayed here (which is within the first infection cycle prior to secondary host cell infection). *V. corneae* infection, in contrast, caused upregulation of many pro-apoptotic genes suggestive of some positive signaling or induction of apoptosis at this same time point that may favor its mechanism of dissemination (Table S2, Figure 5).

A few genes, however, were regulated similarly by live spores of both species. For example, the anti-apoptotic *Bcl-2* gene was upregulated, whereas pro-apoptosis *LTA, CARD8 TP53BP2, DARC1,* and *BCLAAF 1* genes were downregulated. Given that dead spores of *E. cuniculi* and *V. corneae* modulated expression of these genes in the opposite manner from live spores (Fig. 5), it is likely that some aspects of apoptosis inhibition are actively induced by both microsporidia during the course of their intracellular developmental stages. In the case of *V. corneae* infection, conspicuous upregulation of Bad (+10.7), a BH3-only protein among the key regulators of mitochondrial signaling pathway, together with elevated expression of the protein kinase Akt1 (+4.38) may indicate that infection triggers phosphorylation of Bad as a means to inhibit apoptosis. Increased phosphorylation of Bad, for example, correlated with inhibition of apoptosis associated with *Trypanosoma cruzi* and *Leishmania* spp. infections, and the activity of pro-apoptotic Bad was shown to be negatively regulated via Akt/PKB-mediated phosphorylation (Aoki Mdel *et al.*, 2006; Ruhland *et al.*, 2007).

That these microsporidia would affect the expression of the Bcl2 proteins was expected based on previous studies. This family includes pro- and anti-apoptotic regulators of the intrinsic apoptosis pathway via modulated release of Cytochrome C and other pro-apoptotic factors from the intramembrane space of mitochondria (Luo *et al.*, 1998). Bcl2 proteins are deregulated also by many intracellular infectious agents (Faherty & Maurelli, 2008; Graumann *et al.*, 2009). Specifically, infections with *Toxoplasma gondii* (Channon *et al.*, 2002; Goebel *et al.*, 2001; Graumann *et al.*, 2009; Molestina *et al.*, 2003; Orlofsky *et al.*, 2002), *Theileria parva* (Dessauge *et al.*, 2005), *Cryptosporidium parvum* (Liu *et al.*, 2009) each induced expression of anti-apoptotic Bcl2 family proteins. Also, up-regulation of the pro-survival “buffy”, a *Drosophila* Bcl2 protein, has been recently reported in ventricular epithelium cells infected with microsporidia, *N. ceranae* and *N. bombycis* (Martin-Hernandez *et al.*, 2017).

Caspase 9 that was shown here to de noticeably downregulated (−5.53) by *E. cuniculi*, also has been linked to intrinsic apoptosis pathway by triggering pro-caspase 3 cleavage and the downstream cascade resulting in cell disintegration and death (Bratton & Salvesen, 2010). Downregulation of Caspase 3 and Caspase 9 along with activation of anti-apoptotic *Bcl2*

indicated that *E. cuniculi* as well as *V. corneae* infections influenced the mitochondrial apoptosis pathway (Figure S1). This signaling pathway was demonstrated to be negatively regulated by many other intracellular parasites (Graumann *et al.*, 2009), including several microsporidia species such as *Anncaliia algerae* (Scanlon *et al.*, 1999), *Nosema ceranae*, *N. apis* (Huang *et al.*, 2016; Martin-Hernandez *et al.*, 2017) and *N. bombycis* (He et al., 2015).

In addition to their role in modulating apoptosis, Bcl2 proteins can function as crucial regulators of inflammatory responses such as those associated with infections by various pathogens (Faherty & Maurelli, 2008; Hay & Kannourakis, 2002; James & Green, 2004; Luder *et al.*, 2001). For example, infection of mice with *T. gondii* results in an increase in inflammatory neutrophils and macrophages coinciding with upregulation of the pro-apoptotic Bcl2 family protein, A1 (Orlofsky *et al.*, 2002). Similarly, upregulation of pro-apoptotic Bcl2L1 and Bcl2L11 after *V. corneae*, but not *E. cuniculi* infection suggests that *V. corneae* may elicit a stronger inflammatory response through Bcl2 family signaling. Regulating inflammatory responses is necessary for enabling parasite infection and replication, and the results presented here indicate that microsporidia may modulate apoptosis-related genes affecting inflammation to secure their survival albeit through different mechanisms. Here, we found that genes for Caspase 1 and Caspase 4 were downregulated by live *E. cuniculi* (−4.78 and − 2.24 respectively) but strongly upregulated by live *V. corneae* spores (+8.17 and +3.32). Caspase 1 may serve as a substrate for Caspase 4, and their formation of immune complexes or inflammasomes thereby activate secretion of cytokines IL-1β and IL-18 from mammalian macrophages (Sollberger *et al.*, 2012). *E. cuniculi* naturally infects macrophages and thus may have evolved mechanisms to suppress inflammatory caspases whereas *V. corneae* is not typically observed to infect macrophages. On the other hand, *E. cuniculi* infection was associated with overexpression of genes for CD27, CD40LG, TNFRSF1A, and TNFRSF21, which were downregulated in macrophages infected in vitro with *V. corneae*. These Tumor Necrosis Factor Receptor (TNFR) superfamily members exhibit pro-inflammatory effects (Hehlgans & Pfeffer, 2005; Locksley *et al.*, 2001). Positive regulation of these genes by *E. cuniculi* may contribute to increased trafficking or recruitment of immune competent cells such as macrophages, leukocytes, and mononuclear inflammatory cells that provide new host cells for infection or to form the multifocal granulomas that can protect the microsporidia in vertebrate hosts (Snowden & Shadduck, 1999; Sokolova *et al.*, 2016; Weiss, 2014).

A striking observation in these results was the more than 100-fold increased expression of the *p53* gene induced in macrophages incubated with live *V. corneae.* In contrast, exposure to live or dead *E. cuniculi* only moderately induced upregulation of the p53 family proteins (p53 and p73). Previously, it was demonstrated that *Encephalitozoon spp.* infections of Vero cells blocked p53 phosphorylation and translocation to the nucleus, thus inhibiting its transcriptional functions (del Aguila *et al.*, 2006). The p53 pathway, along with the NF-Kβ pathway, are evolutionarily-conserved and contribute to ensure genome stability, inhibit neoplastic processes, and provide overall cell homeostasis (Cooks *et al.*, 2014; Fridman & Lowe, 2003). Functions of p53 as a transcriptional factor and modulator of innate immune responses date back to early invertebrates. For example, p53 was shown to activate TLR genes in T lymphocytes and alveolar macrophages (Menendez *et al.*, 2011). Many stress signals activate expression of p53 which is further affected by numerous posttranslational

modifications including ubiquitination, phosphorylation, and acetylation that then influence outcomes and targets of *p53* induction. On role of p53 is to protect the cell and organism from inflammatory stress associated with pathogen infections and for reducing development of cancers. Conversely, some oncogenic viruses and bacteria such as *Helicobacter pylori* have evolved mechanisms to inactivate or modulate inhibitory effects of p53 (Cooks *et al.*, 2014). The elevated expression of p53 in the macrophages incubated with live *V. corneae* may reflect this stress response. Noticeably, two other genes, *Bax, a* transcriptional target of p53 in the apoptosis pathway, and *Akt*, the kinase that potently phosphorylates p53, were significantly upregulated, as well. It's therefore possible that the overexpression of p53 and activation of p53 pathway might be more predominant for *V. corneae* than *E. cuniculi* given the biology that this microsporidium affects the host cell cycle for promoting multinucleation and cell cycle arrest (Leitch *et al.*, 2005) not observed for *E. cuniculi* infection.

Overall, the results of this study demonstrated that two species of microsporidia infecting humans modulated apoptosis of host cell macrophages. Under natural conditions, *E. cuniculi* readily infects macrophages and epithelial cells while *V. corneae* is less likely to infect macrophages. However, macrophages function in innate immune responses and would likely encounter both species under natural infections, either during initial stages of infection, during the course of immune responses, or for phagocytizing organisms killed by immune responses. Also, macrophages are key regulators of inflammation, and thus, it was not surprising that the live *E. cuniculi* spores generally suppressed pro-apoptosis genes and upregulated anti-apoptosis genes on a broader and more consistent basis than did *V. corneae* in an effort to facilitate infection, host cell parasitism, and cell-to-cell infection. However, while more common to *Encephalitozoon* spp., *V. corneae* also may produce disseminated infections which most likely occurs via trafficking monocytes / macrophages (Deplazes *et al.*, 1998; Didier & Weiss, 2006). In addition, *V. corneae*, may benefit from earlier host cell death to allow release of mature spores into the environment for infecting new host cells and may not have evolved as strong an anti-apoptosis modulation since it does not routinely infect macrophages. Conversely, incubation of macrophages with dead organisms seemed to promote or induce host cell apoptosis, perhaps as a means to induce phagocytosis of residual organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FINANCIAL SUPPORT

This work was supported by the Tulane University Research Enhancement Fund and the National Institutes of Health for the Tulane National Primate Research Center (OD 011104).

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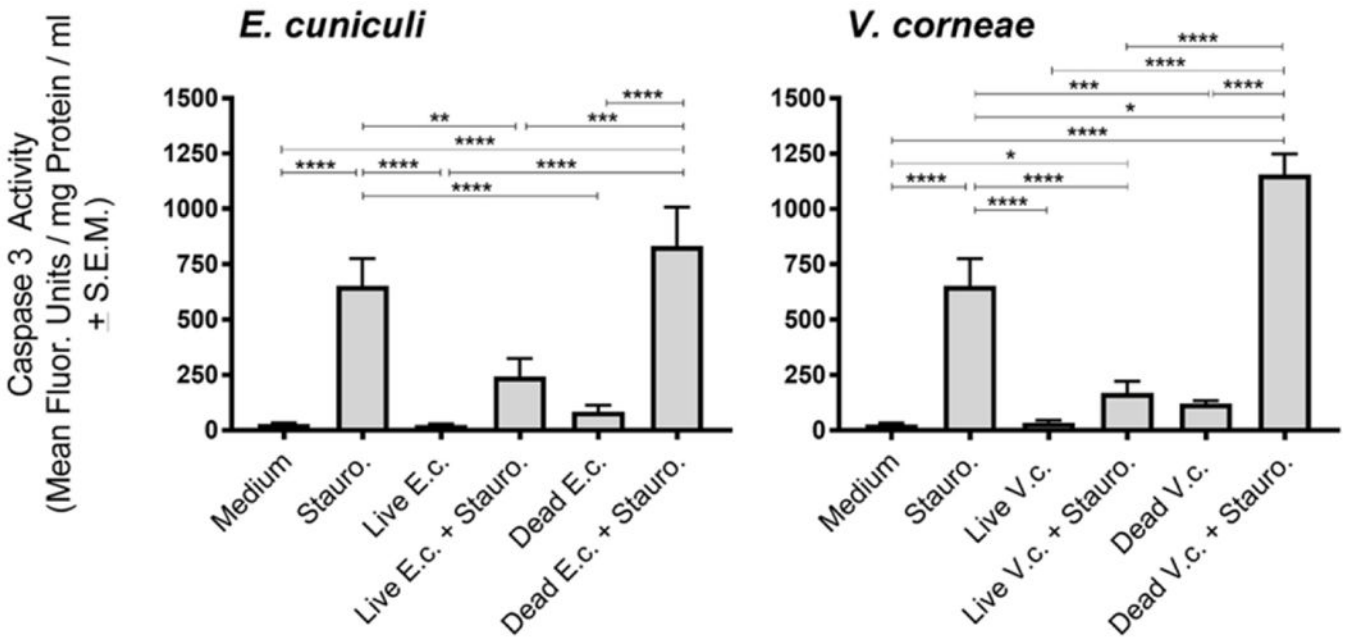

Figure 1. Caspase 3 activity in THP-1 macrophages exposed to *E. cuniculi* and *V. corneae* spores.

Four days after addition of live or dead *E. cuniculi* (E.c) or *V. corneae* (V.c.) spores, cell cultures of THP-1 macrophages were induced for apoptosis by addition of staurosporine (Stauro.). Caspase 3 activity was measured by fluorometry 4 hours later. Results represent the means of three experiments with at least 3 replicates each. ANOVA was performed as was significant for E.c. and V.c. experiments ($P < 0.0001$ and $P < 0.0001$, respectively). Tukey's post hoc test was the applied for pairwise comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

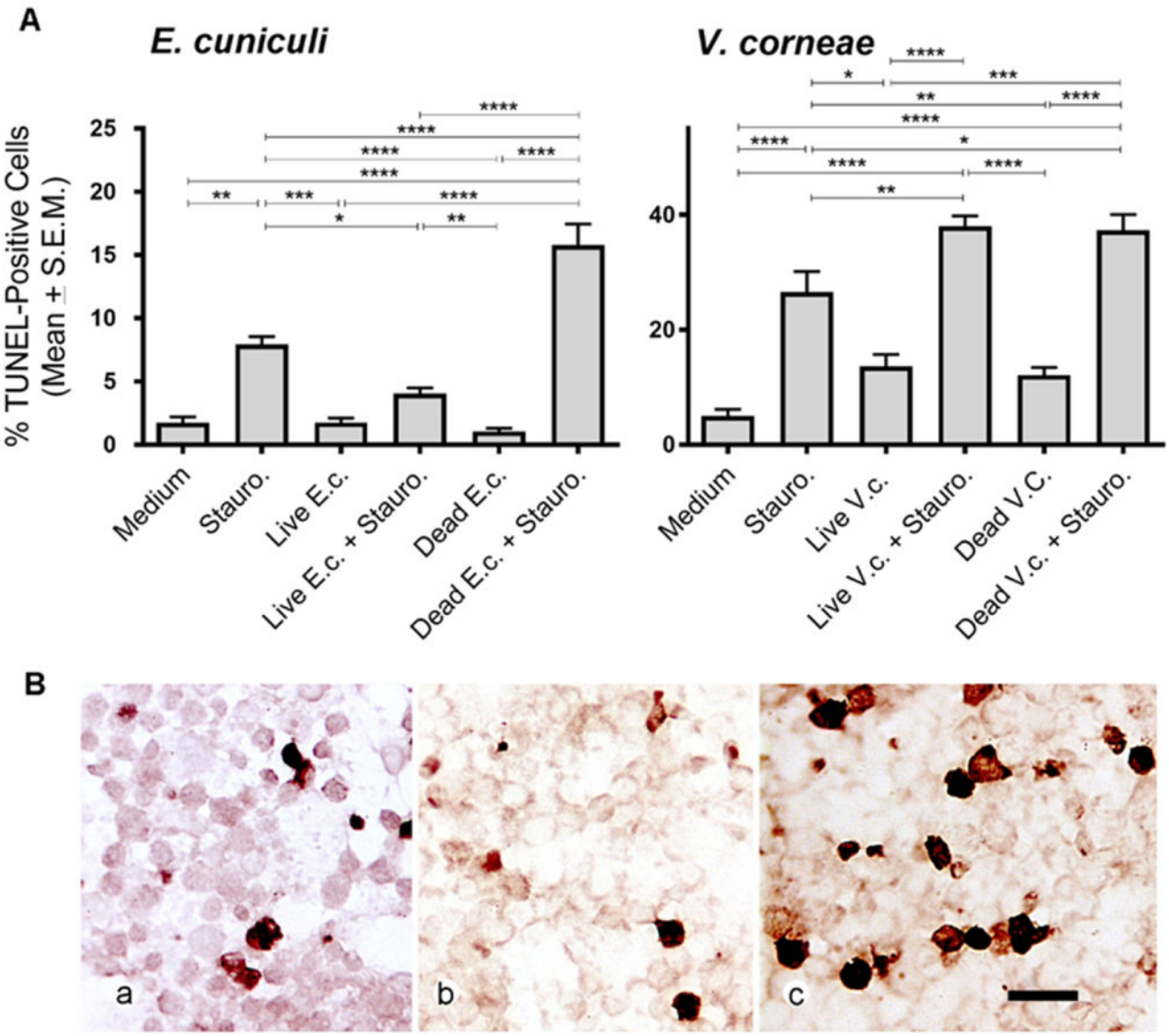

Figure 2. TUNEL expression in THP-1 macrophages after exposure *E. cuniculi* or *V. corneae* (V.c.).

THP-1 macrophages were treated as described in Fig. 1 and then were fixed and stained for TUNEL (DeadEnd colorimetric system, Promega) four hours after staurosporine induction of apoptosis. **A.** TUNEL-positive cells were counted under bright-field microscopy at 400X. Results shown represent the means from 5 fields of view of each of 10 slides per experimental treatment group. ANOVA was performed and was significant for E.c. ($P <$ 0.0001) and V.c ($P < 0.0001$) experimental groups. Tukey's post hoc test was then applied for pairwise comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. **B.** Representative images are shown demonstrating TUNEL staining of THP-1 cells incubated in medium (a), with live *E. cuniculi* spores (b) or dead *E. cuniculi* spores (panel c). Scale bar = 20 μm.

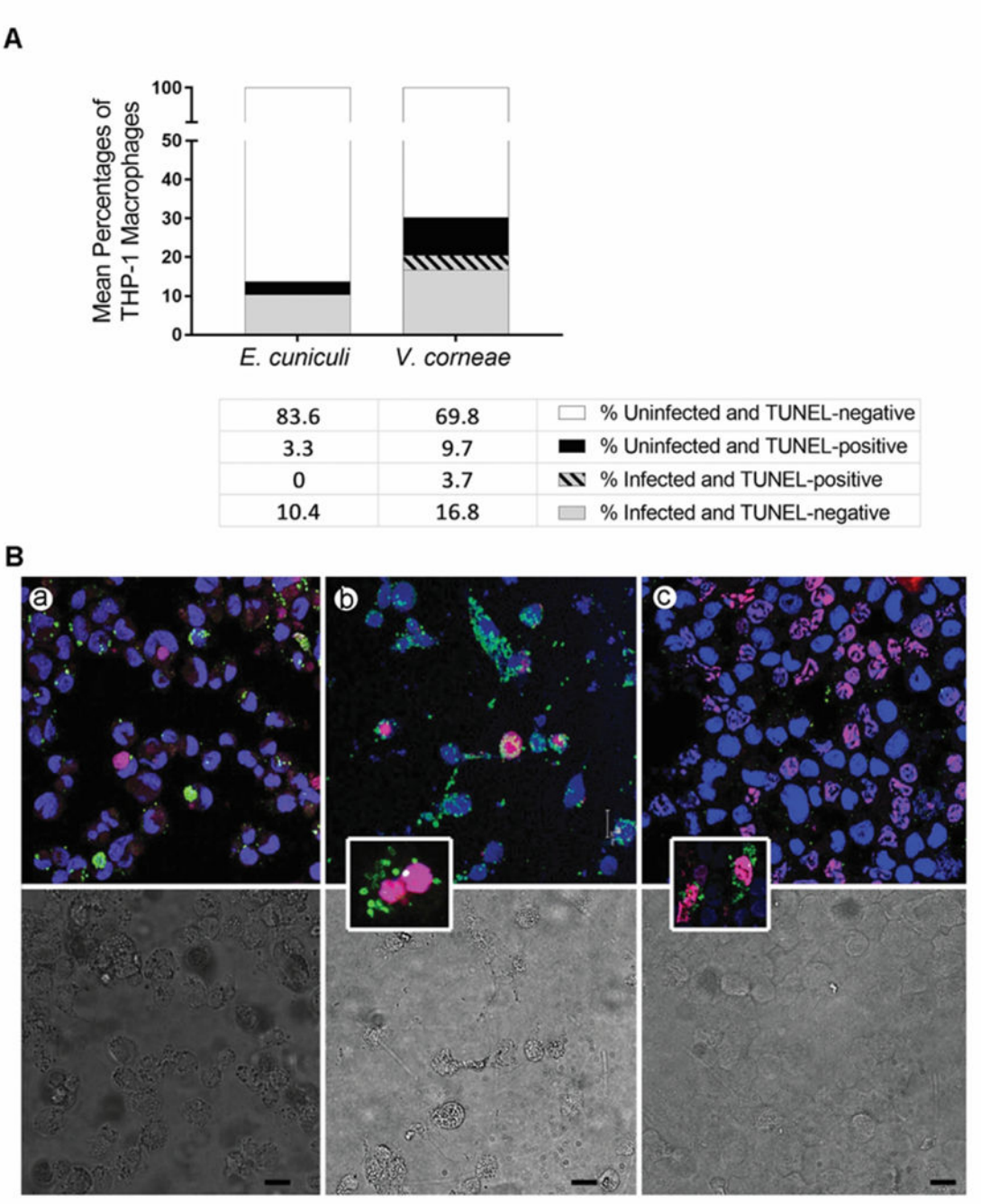

Figure 3. TUNEL staining and concurrent detection of microsporidia-infected THP-1 macrophages.

THP-1 macrophages were incubated with live *E. cuniculi*, live *V. corneae*, or dead *E. cuniculi,* and induced for apoptosis with staurosporine as described in Fig. 1. Cultures were subjected to fluorometric TUNEL-TMR red assay as well as immunofluorescence staining for microsporidia. **A.** Percentages of microsporidia-infected or non-infected THP-1 macrophages that were TUNEL-positive or TUNEL-negative four days after inoculation of cultures were counted from 4–5 fields of 10 culture wells of each treatment and plotted. **B.**

Representative images demonstrate Fluorometric TUNEL-TMR red assay staining (magenta - red) to detect TUNEL-positive cells, indirect IFA staining to detect microsporidia (green), and Topro-3 far red (blue in figure) to stain host cell nuclei of THP-1 cells infected with *E. cuniculi* (a), THP-1 cells infected with *V. corneae* (b), and THP-1 cells exposed to dead *E. cuniculi* spores (c). The lower panel shows the corresponding differential interference contrast (DIC) images. The insert in image b. demonstrates a double-nucleated host cell typical for *V. corneae* infection in which both host nuclei stain for TUNEL. The insert in image c. demonstrates TUNEL-positive cells containing dead *E. cuniculi* spore remnants. Magnification bars in a, b, c, and c insert = 20 μm and in b insert = 10 μm.

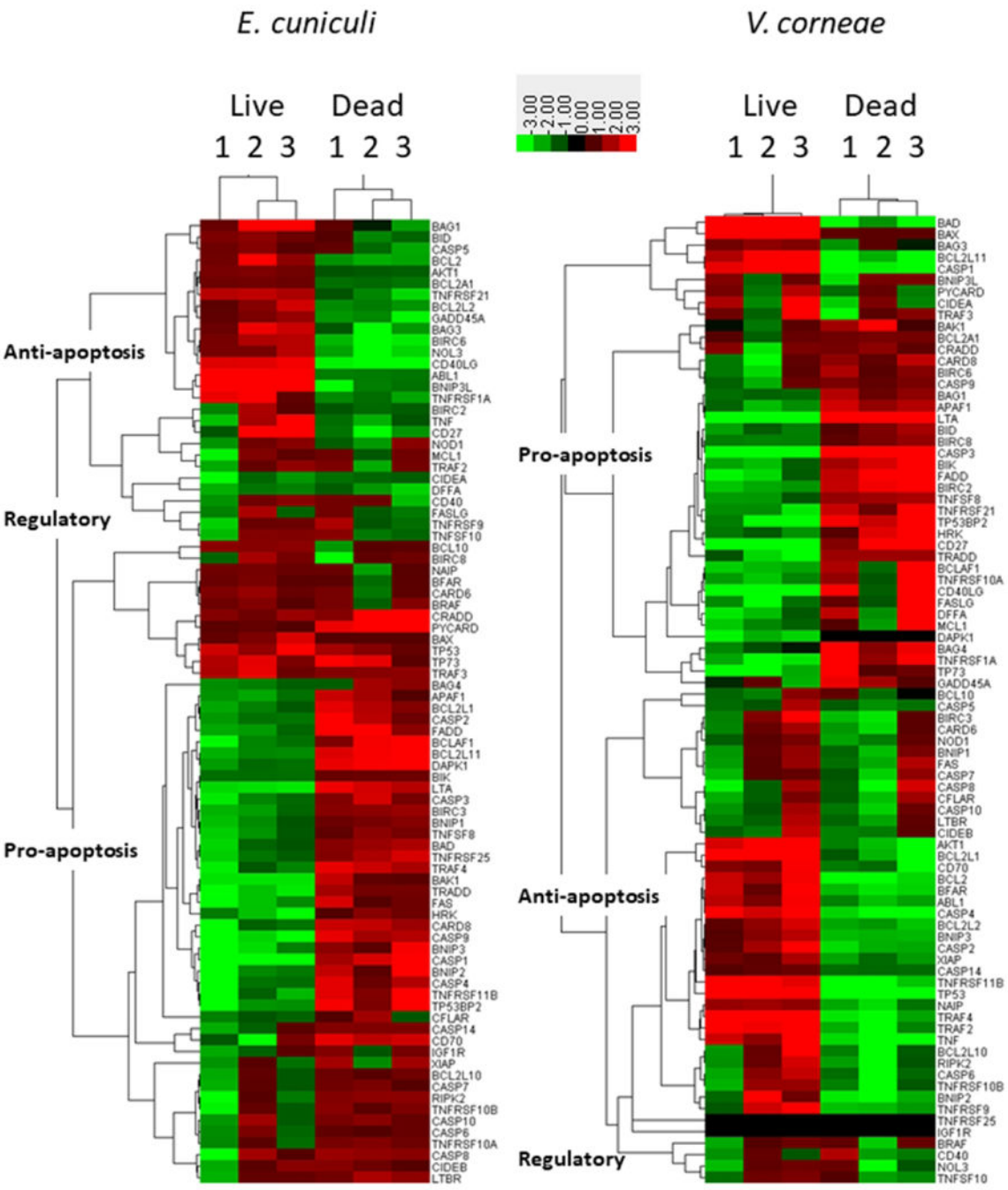

Figure 4. Heat maps of apoptosis-related gene expression in THP-1 macrophages.

THP-1 human macrophages in replicates of three were incubated with medium, live or dead *E. cuniculi* (left panel) or live or dead *V. corneae* (right panel) for four days. Staurosporine was then added and four hours later, cells were processed for measuring expression of the 84 apoptosis-related genes using the Apoptosis RT^2 Profiler PCR Array kit and as described in the Materials and Methods. Heat maps compared gene expression between microsporidia-treated macrophage and medium-treated macrophages. Results were analyzed by Cluster software using uncentered hierarchical clustering. Dendograms were prepared using

TreeView software, and categories of pro-, anti-, and regulatory apoptosis genes were designated as described in the Materials and Methods.

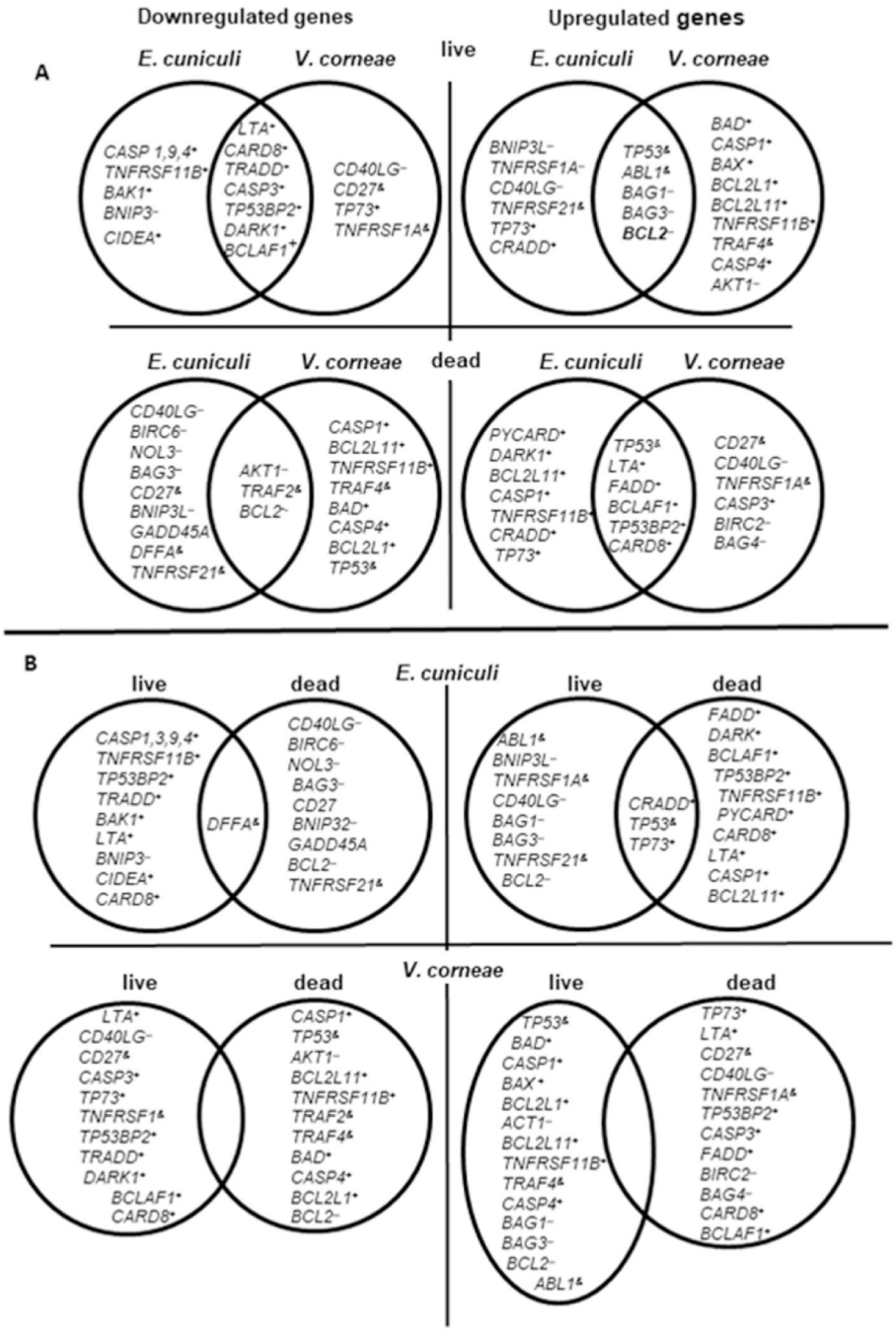

Figure 5. Venn diagrams indicating independent and overlapping apoptosis-related gene expression in THP-1 macrophages incubated with live and dead *E. cuniculi* and *V. corneae* spores.

Gene expression was measured in macrophages four days after incubation with microsporidia (or medium controls) and four hours after staurosporine treatment as described in Materials and Methods. Results were averaged from triplicate cultures. **A.** Comparisons between species of microsporidia affecting apoptosis-related gene expression in macrophages. **B.** Comparisons between live and dead spores for each microsporidia

species affecting apoptosis-related gene expression in macrophages. +, pro-apoptosis genes;$^{--}$, anti-apoptosis genes; $^{\&}$ regulatory genes.