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ORIGINAL INVESTIGATION

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Invasion by *Toxoplasma gondii* protects human-derived HL-60 cells from actinomycin D-induced apoptosis

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Abstract Intracellular microorganisms have to rely on the integrity of their host cells to persist. We, therefore, investigated the effect of infections with different *Toxoplasma gondii* strains on apoptosis of human-derived HL-60 cells at the single cell level. Infection with either mouse-avirulent (NTE strain) or virulent parasites (RH strain) did not induce apoptosis of HL-60 cells as compared to uninfected controls. In contrast, treatment with actinomycin D (act D) led to apoptosis in 15–25% of the cells. However, concomitant infection with *T. gondii* clearly abrogated act D-induced apoptosis. This was especially apparent in those host cells that were actually infected; in these parasite-positive cells the rate of apoptosis decreased by 82.8±4.3% (mean±SEM, $P=0.017$, Student's *t*-test) and 91.7±3.4% ($P=0.024$) after infection with either the NTE or the RH strain, respectively. Inhibition of host cell apoptosis was similarly observed in cells which had been invaded by UV-irradiated, non-replicating parasites ($P=0.001$, Student's *t*-test). However, incubation with heat-killed parasites or *T. gondii* lysates did not abrogate act D-induced apoptosis. In conclusion, inhibition of apoptosis by living, but not necessarily replicating *T. gondii* may facilitate parasite survival and persistence within its host cell.

Key words *Toxoplasma gondii* · Apoptosis · Actinomycin D · HL-60 · Intracellular survival

Introduction

Programmed cell death, i.e., apoptosis plays an essential role during embryonic development, cell turnover in healthy tissues, selection of the immune system and removal of functionally impaired cells [3, 37]. In addition, induction of apoptosis is thought to represent a strategy of pathogen-infected cells by which the multiplication of viable microorganisms may be avoided or reduced [36]. Alternatively, pathogen-induced apoptosis of immune cells may efficiently suppress the host's immune response [19]. Several intracellular pathogens, including bacteria and some viruses have indeed been shown to increase the rate of apoptosis of their host cells [2, 17, 24, 33, 38]. However, less is known about the effect of more complex eukaryotic parasites on programmed cell death of their host cells. An increase of apoptosis has been reported for mononuclear cells from patients with clinical malaria [34], as well as for $CD4^+$ T lymphocytes from mice infected with *Toxoplasma gondii* [18] or *Trypanosoma cruzi* [20].

However, intracellular microorganisms rely on the integrity of their host cells and this may be pivotal especially for those pathogens that cause persistent infections. Downregulation of host cell apoptosis might be one mechanism for supporting persistence of intracellular pathogens and has indeed been shown for several viruses [2, 12, 14, 29] or bacteria [8].

T. gondii, an obligate intracellular protozoan parasite, infects a broad spectrum of warm-blooded hosts, including up to 25% of the world's human population [28], and leads to lifelong persistence of the parasite predominantly within the brain and muscle tissue but also within other organs [7]. Toxoplasmosis in immunocompetent hosts is mostly asymptomatic, but acquisition of the parasite in utero may lead to abortion or overt clinical disease in newborns and children. Furthermore, *T. gondii* is a major opportunistic pathogen in immunocompromised patients, i.e., those with AIDS or those under immunosuppressive therapy, in which persisting parasites may reactivate and may lead to life-threatening disease [27]. *T. gondii* is able to actively invade essentially any cell type and may replicate within a broad range of nucleated host cells [16]. Interestingly, it has been shown recently that *T. gondii* rendered several murine cell types resistant to different inducers of programmed cell death [25], a mechanism which may facilitate intracellular survival and persistence of the para-

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site. In the present study, we extended these approaches and investigated the effect of infection with virulent and avirulent *T. gondii* strains on apoptosis of the human cell line HL-60 at the single cell level.

Materials and methods

Maintenance and isolation of *T. gondii*

Tachyzoites of the mouse-avirulent *T. gondii* strain NTE [13] and the mouse-virulent strain RH [30] were co-cultured with L929 fibroblasts as host cells in RPMI 1640 medium containing 2 mM L-glutamine, 2 mg/ml $NaHCO_3$ and supplemented with 1% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (all reagents from Biochrom, Berlin, Germany). Following spontaneous host cell lysis, tachyzoites were isolated by centrifugation at 35 *g* for 5 min to pellet contaminating host cells. The supernatant was centrifuged at 1,350 *g* for 10 min and tachyzoites were resuspended in RPMI 1640 medium (as above) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Preparation of inactivated *T. gondii* and antigenic lysates

To inhibit replication of *T. gondii* within HL-60 cells, 1×10^7 parasites/ml cell culture medium were irradiated with UV light of 254 nm for 1 min (energy = 240 Joule) at a distance of 20 cm [9]. Heat-killed parasites were obtained by incubating 1×10^7 freshly isolated tachyzoites/ml for 15 min at 60 °C [32].

Antigenic lysates of *T. gondii* were prepared from NTE tachyzoites which were co-cultured with human foreskin fibroblasts (HFF) as host cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 110 µg/ml sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. After lysis of HFF, contaminating host cells were removed using a 3-µm Nucleopore polycarbonate filter (Costar, Fernwald, Germany). Parasites were washed with Dulbecco's phosphate-buffered saline (PBS, pH 7.4; Biochrom) and were then disrupted by three cycles of freezing in liquid nitrogen and rapid thawing. Thereafter, *T. gondii* were sonicated twice on ice for 10 min (30% cycle, cell disruptor B15, Branson Ultrasonics, Danbury, Conn.). This preparation was centrifuged at 20,000 *g* for 20 min at 4 °C and the supernatant (referred to as PBS-soluble extract) was filtered through a 0.2-µm filter and stored at –80 °C. The pellet was resuspended in 1% sodium deoxycholate (NaDOC; Merck, Darmstadt, Germany) and was sonicated and centrifuged as described above. The supernatant was sterile filtered and stored at –80 °C (referred to as NaDOC-soluble extract).

Protein concentrations of the extracts were determined by the BCA Protein Assay (Pierce, Rockford, Ill.) according to the manufacturers instructions using bovine serum albumin (BSA) as a standard.

Induction of apoptosis and *T. gondii* infection

Human promyelocytic leukemia (HL-60) cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. For immunofluorescence experiments, 2×10^5 HL-60 cells/well were seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark) containing 13-mm round glass coverslips. Twenty-four hours before infection with *T. gondii*, HL-60 cells were grown in culture medium (as above) supplemented with 5 nM phorbol 12-myristate 13-acetate (PMA; Sigma, Deisenhofen, Germany) to render adherent cells. Prior to infection, adherent HL-60 cells were washed three times to remove PMA and were then infected with freshly isolated tachyzoites at a parasite to host ratio of 4:1–30:1. Alternatively, UV-irradiated or heat-inactivated parasites were added at a parasite to host ratio of 30:1 to the HL-60 cells or these were incubated with 5–50 µg/ml *T. gondii* antigenic lysates. At 30–60 min after addition of parasites or parasite antigens, apoptosis of HL-60 cells was induced by treatment with 0.5 µg/ml act D (Calbiochem, La Jolla, Calif.) and cell cultures were then incubated for additional 8 h at 37 °C and 5% CO_2 in saturated humidity.

Immunofluorescence staining and microscopy

At 9 h after addition of *T. gondii*, infected and uninfected control cells were stained as described previously [21]. Briefly, cells were fixed with 4% paraformaldehyde (Merck) in PBS, pH 7.4 for 30 min at room temperature, washed with PBS and quenched with 50 mM NH_4Cl in PBS for 10 min. After washing, cells were permeabilized for 1 h with 0.1 mg/ml saponin (Sigma) in PBS containing 1% BSA (Sigma). The coverslips were then sequentially incubated with (i) polyclonal rabbit anti-*Toxoplasma* serum in PBS with saponin and BSA for 1 h, and (ii) 15 µg/ml fluorescein (DTAF)-conjugated donkey $F(ab')_2$ fragment anti-rabbit IgG (Dianova, Hamburg, Germany) in the same buffer for 1 h. For morphological detection of apoptosis, cells were stained with 50 ng/ml Hoechst 33258 (Sigma) in PBS for 1 h. To visualize the total cell population, coverslips were incubated with 5 µg/ml propidium iodide in PBS for 5 min. Finally, coverslips were mounted with Mowiol (Calbiochem, San Diego, Calif.) and were examined by conventional fluorescence microscopy.

TUNEL assay

For the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay, cells were similarly fixed and stained as described above. However, immunolabelled *T. gondii* were detected by a Cy3-conjugated donkey $F(ab')_2$ fragment anti-rabbit IgG as secondary antibody. Thereafter, apoptosis was detected by labelling DNA strand breaks with an in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) as described by the manufacturer. Briefly, the cells were permeabilized in 0.1% Triton X and 0.1% sodium citrate for 2 min on ice, and were then incubated with the TUNEL reaction mixture for 1 h at 37 °C in the dark. Coverslips were mounted with Mowiol and were examined by conventional fluorescence microscopy or confocal microscopy using a Leica TCS 4D.

Viability assay

To distinguish apoptosis and necrosis, the viability of the cells was tested by trypan blue exclusion for each apoptosis assay. In each experiment, an additional set of coverslips was subjected to staining with 0.1% (w/v) trypan blue (Biochrom) in PBS.

Statistical analysis

Results are expressed as mean values ± SEM of four or five independent experiments. The W-test of Wilk-Shapiro was used to test for normality. Significant differences between mean values were identified by Student's *t*-test using paired observations. To take account of multiple testings within each set of experiments, only *P* values of 0.05 divided by the number of tests were considered significant (Bonferroni correction) [31].

Results

Reduction of act D-induced apoptosis by *T. gondii* at the single cell level

Preliminary experiments indicated that infection of HL-60 cell cultures with *T. gondii* reduced the in vitro-induced apoptosis of these cells (data not shown). Since HL-60

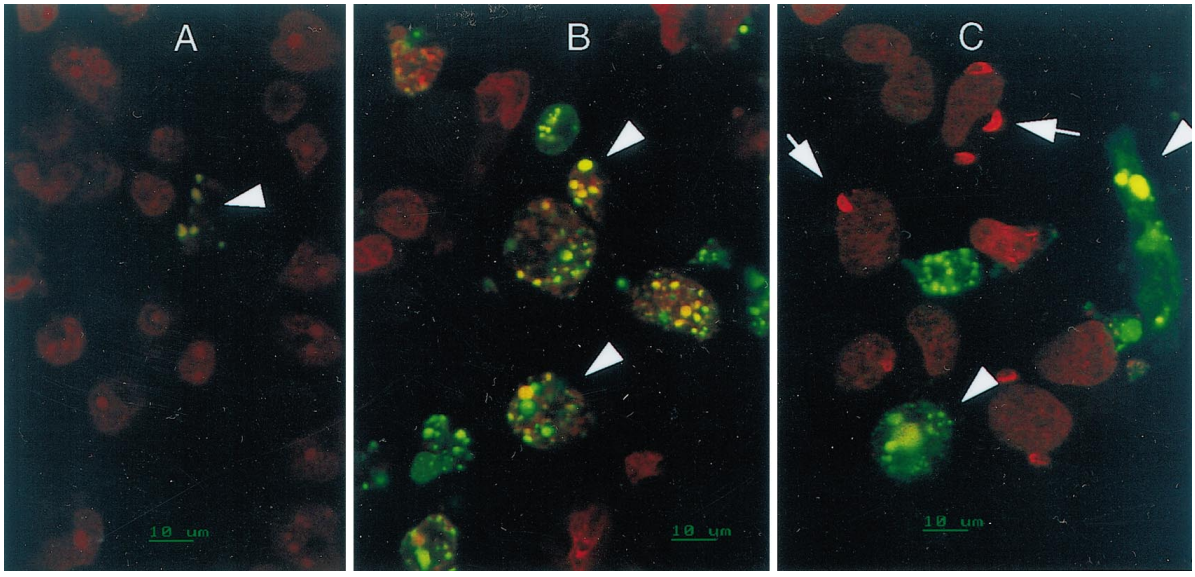

Fig. 1A–C Resistance of *Toxoplasma gondii*-positive HL-60 host cells to act D-induced apoptosis. Single- and double-stranded DNA breaks (*green fluorescence*; indicated by *arrowheads*) in HL-60 cells were visualized by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). *T. gondii* tachyzoites of the NTE strain were simultaneously detected by immunofluorescence staining as described in Materials and methods (*red fluorescence*; indicated by *arrows*) and the total cell population by staining the host cell nuclei with propidium iodide. In **A** non-infected HL-60 cells were cultivated in the absence of act D; in **B** treatment of HL-60 cells with 0.5 µg/ml act D for 8 h induced single- and double-stranded DNA breaks, indicating apoptosis of these cells; and in **C** HL-60 cells were infected with *T. gondii* at a parasite to host ratio of 20:1 and 1 h later treated with act D for 8 h

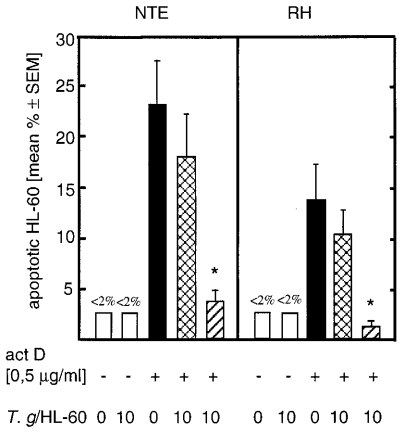

Fig. 2 Percentages of apoptotic HL-60 cells after infection with *T. gondii* and treatment with act D. HL-60 cultures were infected with tachyzoites of the mouse-avirulent strain NTE or the high-virulent strain RH at a parasite to host ratio of 10:1 or were left non-infected as indicated. After 30 min, HL-60 cells were cultivated in the presence of 0.5 µg/ml act D for 8 h or were left untreated (*open bars*). Cell cultures were stained with Hoechst 33258 to visualize nuclear DNA condensation as a typical feature of apoptosis and *T. gondii* were simultaneously detected by immunofluorescence staining. The percentages of apoptotic cells were separately determined for parasite-negative (*cross-hatched bars*) as well as parasite-positive (*hatched bars*) cells of an infected HL-60 culture and were compared with uninfected control cultures (*black bars*). At least 500 cells of each sample were examined by fluorescence microscopy. Data represent means ± SEM from four independent experiments. Mean percentages were compared by Student's *t*-test, *bars* marked with an *asterisk* are significantly different in comparison to act D-treated, non-infected control cultures

cell cultures infected with *T. gondii* contained both parasite-positive as well as parasite-negative cells, we asked whether act D-induced apoptosis is equally down-regulated irrespective of the state of infection of the individual cell. Therefore, the TUNEL test or Hoechst 33258 staining was used to visualize apoptotic cells, and *T. gondii* was simultaneously detected by immunofluorescence staining. In the absence of act D, only single cells underwent apoptosis as judged by labelling DNA strand breaks using the TUNEL assay, and this was similarly observed in non-infected cultures (Fig. 1A) as well as in *T. gondii*-infected cell cultures (data not shown). After treatment with 0.5 µg/ml act D for 8 h, the number of apoptotic cells increased dramatically; however, this increase was considerably lower in cultures concomitantly infected with *T. gondii* at a parasite to host ratio of 20:1 (rate of infection 47.7%) as compared to non-infected HL-60 cultures (Fig. 1C, B, respectively). Furthermore, a clear correlation between *T. gondii* infection and inhibition of apoptosis was observed, since nearly all parasite-positive cells showed no characteristics of apoptosis, while most apoptotic cells were parasite negative (Fig. 1C). This indicated that parasite-positive host cells were indeed more resistant to induction of apoptosis than parasite-negative cells.

Figure 2 summarizes the results of four independent experiments after infection with the mouse-avirulent *T. gondii* strain NTE as well as with the mouse-virulent strain RH. After addition of *T. gondii* at a parasite to host ratio of 10:1, 12.3±1.4% and 22.0±4.3% (mean±SEM) of HL-60 cells harbored intracellular NTE or RH tachyzoites, respectively. However, the percentages of apoptotic cells

in untreated HL-60 cultures did not increase after parasitic infection as compared to uninfected cultures. In contrast, the rate of apoptotic HL-60 increased to 15–25% after treatment with 0.5 μg/ml act D for 8 h, but this increase in the overall rate of act D-induced apoptosis was partially abolished in cultures that were concomitantly infected with tachyzoites of the NTE strain (18.5 ± 6.1% apoptotic cells) or the RH strain (8.8 ± 1.9% apoptotic cells, data not shown). Whereas only a slight reduction was observed in parasite-negative cells of an infected HL-60 culture, parasite-positive cells were significantly protected from act D-induced apoptosis as compared to uninfected HL-60 cultures (Fig. 2; Student's *t*-test, $P = 0.017$ for NTE and $P = 0.024$ for RH). In these cases, the percentage of apoptotic cells was reduced by 82.8 ± 4.3% and 91.7 ± 3.4% (mean ± SEM, $n = 4$) after infection with tachyzoites of the NTE strain and the RH strain, respectively. Similar results were obtained after infection with NTE tachyzoites at a higher parasite to host ratio of 20 : 1 (data not shown).

In parallel to all experiments, the viability of HL-60 cells was tested by trypan blue exclusion to distinguish apoptotic from necrotic cells. In non-infected HL-60 cultures, less than 5% of the host cells underwent necrosis, and similar findings were observed in untreated cultures as well as after addition of 0.5 μg/ml act D for 8 h. Furthermore, the percentage of necrotic HL-60 cells was not augmented 9 h after infection with *T. gondii* tachyzoites at parasite to host ratios of 4 : 1–20 : 1 (data not shown).

Effect of inactivated parasites and antigenic extracts on host cell apoptosis

To investigate whether vitality of the parasite is necessary for protection from act D-induced apoptosis, HL-60 cells were co-cultured with either untreated, UV-irradiated or heat-inactivated parasites or were incubated with different antigenic lysates of NTE tachyzoites. After addition of UV-irradiated *T. gondii* at a parasite to host ratio of 30 : 1, the infection rate of HL-60 cells was only slightly reduced (41.2 ± 9.9%; $n = 5$) as compared to HL-60 cells co-cultured with untreated control parasites (50.8 ± 8.1%). However, intracellular replication of UV-irradiated *T. gondii* was completely abrogated (data not shown). In contrast, after heat inactivation for 15 min at 60 °C, NTE tachyzoites completely lost their ability to invade host cells. As shown in Fig. 3, UV irradiation of *T. gondii* did not abrogate their apoptosis-inhibiting effect since parasite-positive HL-60 cells were similarly protected from act D-induced apoptosis than host cells invaded by untreated control parasites (Student's *t*-test, $P = 0.001$ in both cases as compared to uninfected controls). In contrast, the act D-induced apoptosis was not significantly reduced after co-culture of HL-60 cells with heat-inactivated parasites. Furthermore, incubation with 5–50 μg/ml of either PBS-soluble or NaDOC-soluble parasitic extract did not significantly inhibit apoptosis of HL-60 cells after treatment with act D (Fig. 3).

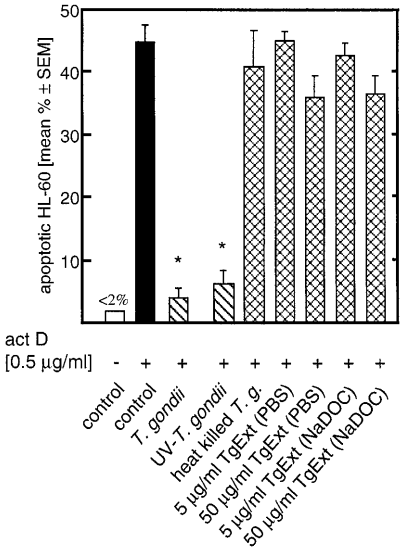

Fig. 3 Percentages of apoptotic HL-60 cells after incubation with different *T. gondii* preparations and treatment with act D. HL-60 cell cultures were inoculated with untreated, UV-irradiated and heat-killed NTE tachyzoites at a parasite to host ratio of 30 : 1 or were incubated with 5–50 μg/ml PBS-soluble or NaDOC-soluble *T. gondii* extract [TgExt (PBS) and TgExt (NaDOC), respectively]. After 30 min, HL-60 cells were treated with 0.5 μg/ml act D for 8 h or were left untreated (*open bar*). Cell cultures were stained with Hoechst 33258 to visualize nuclear DNA condensation as a typical feature of apoptosis and *T. gondii* were simultaneously detected by immunofluorescence staining. Percentages of apoptotic cells were determined for parasite-positive host cells (*hatched bars*) or for parasite-negative HL-60 (*cross-hatched bars*) in those cases in which *T. gondii* was unable to invade host cells and were compared with act D-treated HL-60 cultures in the absence of *T. gondii* (*black bar*). At least 500 cells of each sample were examined by fluorescence microscopy. Data represent means ± SEM from five independent experiments, *bars* marked by an *asterisk* are significantly different in comparison to act D-treated non-infected control cultures (Student's *t*-test) (*NaDOC* sodium deoxycholate)

Discussion

Inhibition of programmed cell death during tissue turnover as well as down-regulating immunologically induced apoptosis of infected host cells may represent an important strategy by which intracellular pathogens circumvent host defence mechanisms and ensure intracellular survival [19]. For *T. gondii*, it has been shown recently that infected murine cell cultures are resistant to a variety of physiological and non-physiological stimuli of apoptosis [25]. However, from that study it did not become clear whether non-apoptotic cells were actually parasite-positive or not, and whether this phenomenon applies to human cells as well. In the present study, we therefore extended these approaches and investigated the effect of parasitic infection on apoptosis of human-derived promyelocytic HL-60 cells at the single cell level. This cell line was selected as a well characterized in vitro model to investigate programmed cell death, since large scale apoptosis is inducible by inhibition of RNA or protein synthesis [22], and thus allows detection of apoptosis-inhibiting as well as apoptosis-in-

ducing [24] effects of microorganisms. The results of our study show that *T. gondii*-positive human-derived cells were highly protected from act D-induced apoptosis, while the percentage of apoptotic cells was only slightly reduced in parasite-negative cells from infected cultures as compared to uninfected HL-60 cultures. This indicates that intracellular *T. gondii* parasites directly interfere with those signal transduction pathways which lead to act D-induced apoptosis of HL-60 cells.

This is supported by our observation that antigenic extracts as well as heat-killed parasites which no longer invade host cells had no significant effect on act D-induced apoptosis. In contrast, UV-irradiated *T. gondii* [9] efficiently inhibited apoptosis of infected HL-60 cells, indicating that intracellular replication is not required for the anti-apoptotic effect of the parasite. Thus, down-regulation of act D-induced apoptosis is rather triggered during the active process of host cell invasion [5] and/or the modification of the host cell by living *T. gondii* [9, 16]. Resistance of *T. gondii*-infected murine cell cultures to in vitro-induced apoptosis was similarly abolished after killing intracellular parasites with either pyrimethamine or ciprofloxacin [25], which confirms the necessity of living parasites for inhibition of apoptosis.

The parasitic factor as well as the mechanisms by which *T. gondii* interferes with apoptosis-inducing cellular pathways are still unknown and need further investigations. A variety of viruses have been shown to down-regulate programmed cell death by encoding gene products which interfere with different apoptosis-inducing cellular pathways. For example, the cowpox crmA gene product inhibits apoptosis by inactivating IL-1β converting enzyme (ICE), while other viruses encode an homolog of the anti-apoptotic molecule bcl-2 or antagonize the major pro-apoptotic gene product p53 [35]. In *Chlamydia trachomatis*-infected host cells, in vitro-induced apoptosis was prevented by blockade of cytochrome *c* release from mitochondria and inhibition of caspase activation [10]. Furthermore, autocrine secretion of TNF-α by infected monocytes/macrophages seems to be partially responsible for the apoptosis-suppressive effect of *Mycobacteria* and *Leishmania* [8, 23]. At least in human cells, however, this cytokine is probably not involved in the *T. gondii*-induced inhibition of host cell apoptosis, since TNF-α secretion seems not to be increased after exposure of human-derived monocytic cells to tachyzoites [26]. In addition, infection of HL-60 cultures by *T. gondii* selectively protected parasite-positive, but not parasite-negative cells, indicating that parasite-induced cytokine secretion is unlikely to mediate suppression of apoptosis.

Strain-specific differences in the induction of apoptosis by *T. gondii* have been described by Hisaeda et al. [15] with the high-virulent strain RH strongly promoting apoptosis in mouse-derived peritoneal macrophages after infection. They suggested that this may be due to a low expression of heat shock protein 65 (HSP65), which possibly plays a protective role for the integrity of the host cell. In contrast, this protein was highly induced by the low-virulent Beverly strain and macrophages from mice infected with these parasites indeed did not undergo apoptosis. In our system, however, neither the low-virulent strain NTE [13] nor the high-virulent strain RH induced apoptosis, but both strains similarly inhibited act D-induced apoptosis in infected host cells. This suggests that the effect of *T. gondii* on the rate of programmed cell death of human-derived HL-60 cells does not differ strain specifically. Furthermore, the resistance of infected host cells against induction of apoptosis as reported herein is unlikely to be an HSP65-mediated process, since expression of HSP65 was either dependent on the presence of $\gamma\delta$ T cells in vivo or on the addition of exogenous IFN-γ and TNF-α in vitro [15].

Besides the induction of apoptosis by *T. gondii* in macrophages [15], Khan et al. [18] reported that acute toxoplasmosis in mice induces unresponsiveness of $CD4^+$ T cells to mitogenic or antigenic stimulation followed by increased apoptosis of these cells when cultured ex vivo for several days. Furthermore, activation-induced apoptosis of $CD4^+$ T lymphocytes has been described as a prominent feature of experimental Chagas' disease [20]. Induction of apoptosis in cells of the immune system by protozoan parasites may represent a mechanism to induce immune dysregulation during the acute phase of infection which consequently promotes persistence.

However, parasite-induced inhibition of programmed cell death may also represent a mechanism by which microorganisms evade the host's immune response. Killing of infected cells or cancer cells by cytotoxic T cells and by natural killer cells involves induction of apoptosis; down-regulation of such defence mechanisms by parasite-positive host cells may contribute to ongoing intracellular development. Furthermore, for long-term survival of intracellular parasites the integrity of the host cell is a major prerequisite. In infected individuals, *T. gondii* is thought to persist predominantly within neurons of the central nervous system [11]. Since neurons do not undergo apoptosis in healthy individuals, questions may arise about the importance of *T. gondii*-induced inhibition of host cell apoptosis. However, recent data suggest that neurons may die from apoptosis under pathological conditions [1], and it can therefore not be excluded that encystation of *T. gondii* within neurons would eventually lead to functional inactivation and apoptosis. Such a theoretically possible process would need to be inhibited by *T. gondii* to ensure intracellular persistence of tissue cysts within neurons. More importantly, data from patients receiving organ transplants [4] and from experimental models of toxoplasmosis [6] indicate that *T. gondii* also persists in organs besides the central nervous system. In such tissues, inhibition of host cell apoptosis may represent a mechanism that enables long-term survival of the parasite even in non-neuronal cells. The precise mechanism that trigger resistance against act D-induced apoptosis by *T. gondii* infection as well as the significance of this host-pathogen interaction for the course of toxoplasmosis, however, needs future clarification and is currently under investigation in our laboratory.

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