Inhibition of host cell apoptosis by *Toxoplasma gondii* is accompanied by reduced activation of the caspase cascade and alterations of poly(ADP-ribose) polymerase expression

Stefan Goebel, Uwe Gross and Carsten G. K. Lüder*

Department of Bacteriology, Georg-August-University Göttingen, Kreuzbergring 57, D-37075 Göttingen, Germany
*Author for correspondence (e-mail: clueder@gwdg.de)

Accepted 2 July 2001
Journal of Cell Science 114, 3495-3505 (2001) © The Company of Biologists Ltd

SUMMARY

The obligate intracellular protozoan parasite *Toxoplasma gondii* has been shown to protect different cell types from apoptosis induced by a variety of pro-apoptotic treatments. However, the precise cell biological mechanisms of this inhibition remained unknown. As shown in this study, apoptosis in human-derived HL-60 and U937 cells induced by treatment with actinomycin D or TNF-α in combination with cycloheximide, respectively, was indeed dose-dependently downregulated by prior infection with *T. gondii*, as determined by DNA fragmentation assays. Cleavage of caspase 3 and caspase 9 after treatment with pro-apoptotic stimuli was considerably diminished by *T. gondii*. Furthermore, release of mitochondrial cytochrome c during apoptosis in HL-60 cells was prevented by intracellular parasites and this was correlated with the absence of DNA strand breaks on the single cell level. Inhibition of cytochrome c release coincided with a twofold upregulation of Mcl-1 protein levels in HL-60 and U937 cells, while Bcl-2 expression did not increase after infection. Parasitic interference with the caspase cascade led to a reduced proteolytic cleavage of the nuclear target molecule protein kinase Cδ. In parallel, poly(ADP-ribose) polymerase protein levels were prominently downregulated by *T. gondii*, irrespective of whether HL-60 and U937 cells had been treated with pro-apototic stimuli or left untreated. However, poly(ADP-ribose) polymerase mRNA levels remained unchanged after infection as determined by RT-PCR analyses. These observations suggest that *T. gondii* has evolved different mechanisms that may contribute to downregulation of host cell apoptosis, namely inhibition of cytochrome c release and subsequent caspase activation as well as downregulation of poly(ADP-ribose) polymerase protein levels.

Key words: Apoptosis, *Toxoplasma gondii*, Poly(ADP-ribose) polymerase, Caspase, Mitochondria, Mcl-1

INTRODUCTION

Besides its essential roles during development and homoeostasis of multicellular organisms, programmed cell death (i.e. apoptosis) plays a critical role in the regulation of host responses during infection with viruses, bacteria and parasites (Williams, 1994; Liles, 1997). Furthermore, modulation of apoptosis by microorganisms has now been widely recognized (Liles, 1997; Barry and McFadden, 1998; Everett and McFadden, 1999). Depending on the pathogen under investigation, increased apoptosis may assist dissemination of intracellular pathogens (Hardwick, 1998; Griffin and Hardwick, 1999) or induce immunosuppression (Gougeon et al., 1996; Toure-Balde et al., 1996; Khan et al., 1996). In many cases, however, apoptosis may help to eradicate pathogens from the host (Laochumroonvorapong, 1996; Teodoro and Branton, 1997, Fratazzi et al., 1997). Consequently, several viruses, bacteria and parasites have evolved mechanisms to inhibit host cell apoptosis (Liles, 1997), a strategy that may support intracellular survival and persistence of the pathogen.

The obligate intracellular protozoan parasite *Toxoplasma gondii* is ubiquitously distributed and infects a wide range of warm-blooded hosts, including up to 30% of the human population worldwide (Holliman and Greig, 1997). Infection of immunocompetent humans is most often asymptomatic, but leads to lifelong persistence of the parasite (Gross et al., 1996). However, *T. gondii* may lead to life-threatening disease in fetuses or newborns from primarily infected mothers or after reactivation of dormant parasites in immunocompromised patients (i.e. those with AIDS or those under immunosuppressive therapy).

We (Goebel et al., 1999) and others (Nash et al., 1998) have recently reported that *T. gondii* downregulates apoptosis that has been induced by multiple stimuli in human and murine cell lines, respectively. An increased expression of heat shock protein 65 (HSP65) by peritoneal macrophages after infection has been correlated with reduced apoptosis of these cells (Hisaeda et al., 1997), and A1, an anti-apoptotic member of the Bcl-2 family, is upregulated in exudate cells during *T. gondii*-induced inflammation in the peritoneum (Orlofsky et al., 1999). However, detailed analyses of those mechanisms by

which *T. gondii* interferes with apoptosis-inducing signalling pathways of the host cell have not yet been described.

Apoptosis may be initiated through receptor engagement by external stimuli, such as TNF-α or FasL or by internal stimuli such as chemotherapeutic agents, irradiation, and serum or growth factor deprivation (Vaux and Strasser, 1996). These signals lead via different pathways to the activation of a family of cysteine proteases with specificity for aspartic acid residues, referred to as caspases (Thornberry and Lazebnik, 1998). Mitochondria play a critical role in the transduction of upstream pathways into the apoptotic effector cascade (Green and Reed, 1998). Release of cytochrome c from mitochondria into the cytosol and subsequent activation of the Apaf-1/procaspase 9-complex c have been shown to activate downstream caspases (Li et al., 1997; Pan et al., 1998). Furthermore, many proteins of the Bcl-2 family with either anti-apoptotic (e.g. Bcl-2, Bcl-x_L or Mcl-1) or pro-apoptotic function (e.g. Bax, Bak or Bik) reside in the outer mitochondrial membrane (Adams and Cory, 1998). Activation of the caspase cascade finally leads to cleavage of a variety of target proteins with structural or regulatory function, including poly(ADP-ribose) polymerase (PARP), nuclear lamins, protein kinase C (PKC) and others, leading to disassembly of the cell.

Mechanisms of microbial interference with host cell apoptosis have been characterized for a variety of viruses and some bacteria (Liles 1997; Fan et al., 1998) and several anti-apoptotic genes have been identified (Barry and McFadden, 1998). However, little is known about the mechanisms by which more complex eukaryotic parasites interfere with apoptosis-inducing signalling. Here, we show that the anti-apoptotic activity of *T. gondii* was accompanied by interference with mitochondrial cytochrome c release and subsequent downregulation of caspase activation. Furthermore, the protein level of PARP was prominently downregulated by the parasite. This suggests that *T. gondii* has evolved different mechanisms that may contribute to the inhibition of host cell apoptosis.

MATERIALS AND METHODS

Host cells and parasites

Human promyelocytic leukemia cells (HL-60; American Type Culture Collection, Rockville, MD) and human histiocytic lymphoma cells (U937; European Collection of Cell Cultures, Salisbury, UK) were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 2 mg/ml $NaHCO_3$ and supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents from Biochrom, Berlin, Germany).

Tachyzoites of the mouse-avirulent *T. gondii* strain NTE (Gross et al., 1991) were propagated in L929 fibroblasts as host cells. Tachyzoites were harvested after initiation of host cell lysis. For infection of HL-60 and U937 cells, tachyzoites were isolated from L929 cocultures as described (Goebel et al., 1999). Briefly, contaminating host cells were pelleted by centrifugation at 35 ***g*** for 5 minutes. The supernatant was then centrifuged at 1350 ***g*** for 10 minutes and tachyzoites were resuspended in RPMI 1640 (as above).

Infection with *T. gondii* and induction of apoptosis

For DNA fragmentation assays and western blot analyses, 1×10^6 HL-60 or U937 cells per well were cultured in 6-well tissue culture plates (Costar, Bodenheim, Germany), for RT-PCR analyses, 5×10^6 HL-60 or U937 cells were cultured in 90 mm tissue culture dishes (Nunc, Roskilde, Denmark), and for immunofluorescence microscopy, 2×10^5 HL-60 cells per well were seeded in 24-well plates (Costar) containing 13 mm round glass coverslips. Before infection with *T. gondii* and induction of apoptosis, HL-60 cells were treated for 24 hours with 5 nM phorbol 12-myristate 13-actetate (PMA; Sigma, Deisenhofen, Germany) to render cells adherent, and were then washed three times to remove PMA. HL-60 and U937 cells were infected with *T. gondii* at parasite to host cell ratios of 10:1 or 30:1; addition of parasites at these ratios routinely yielded infection rates of 10-15% and 40-50%, respectively, as revealed by microscopic analysis of HL-60 cells after immunofluorescence staining. Thirty minutes post infection (p.i.), apoptosis was induced in infected and uninfected HL-60 and U937 cells by treatment with 5 μg/ml actinomycin D (actD; Calbiochem, Bad Soden, Germany) or 40 ng/ml TNF-α (Boehringer, Mannheim, Germany) in combination with 2 μg/ml cycloheximide (Sigma), respectively. Treated and untreated control cells were maintained for 8 hours at 37°C and 5% CO_2 in saturated humidity.

DNA fragmentation assay

The DNA fragmentation assay was performed as described (Eldadah et al., 1996). Briefly, 2×10^6 HL-60 or U937 cells were lysed in 2 ml of 7 M guanidine hydrochloride. Genomic DNA was isolated using the Wizard® Plus Minipreps DNA Purification Kit as recommended by the manufacturer (Promega, Madison, WI). After elution from the Wizard® minicolumns, contaminating RNA was digested by incubation with 1 μg RNAse A (Sigma) for 30 minutes at 37°C. DNA was then electrophoretically separated in a 1.5% agarose gel and visualized by ethidium bromide staining. Results were quantified by densitometric analyses of bands corresponding to fragmentated DNA on a BioDoc II digital imaging system (Biometra, Göttingen, Germany).

Western blot analyses

The total cellular content of caspases 3, 8 and 9, PARP, nPKCδ, Bcl-2, Mcl-1 and actin was analysed by western blotting. Equal amounts of *T. gondii*-infected HL-60 and U937 cells or uninfected controls were washed in PBS (pH 7.4; Biochrom) and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium desoxycholate (NaDOC), 0.1% sodium dodecylsulfate (SDS), 10 μg/ml leupeptin, 1 μg/ml each of aprotinin and pepstatin A, and 1 mM each of PMSF and sodium orthovanadate. After 30 minutes on ice, the samples were centrifuged at 13,000 ***g*** for 15 minutes and equal amounts of supernatant were resolved in 10% (PARP) or 15% (all proteins except PARP) polyacrylamide gels by standard SDS-PAGE under reducing conditions. After semi-dry transfer to nitrocellulose, equal loading of each lane was confirmed by ponceau S staining (Sigma) and membranes were blocked with 5% nonfat dried milk, 0.2% Tween 20, 100 mM NaCl in 10 mM Tris-HCl, pH 7.4. Membranes were then incubated for 90 minutes with rabbit anti-caspase 3 polyclonal antibody (pAb; No. 65906E; 1:1500), mouse anti-caspase 8 pAb (No. 66231A; 1:250), rabbit anti-Mcl-1 pAb (No. 13656E; 1:1000), mouse anti-PARP mAb (clone C2-10; 1:2000; all Ab from Pharmingen, Hamburg, Germany), rabbit anti-caspase 9 pAb (H-83; 1:100), rabbit anti-PKCδ pAb (C-20; 1:2000; both from Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Bcl-2 mAb (clone 100; 3 μg/ml), or mouse anti-actin mAb (clone JLA20; 3 μg/ml; both from Calbiochem). Bound Ab were visualized with 0.04 μg/ml HRPO-conjugated $F(ab')_2$ fragment goat anti-rabbit or anti-mouse IgG (Dianova, Hamburg, Germany), or with 0.05 μg/ml HRPO-conjugated goat anti-mouse IgM (Calbiochem) and ECL chemiluminescence detection as recommended by the manufacturer (Amersham Pharmacia Biotech). Signals were densitometrically quantified on a BioDoc II imaging system (Biometra).

To determine the subcellular distribution of cytochrome c, digitonin-soluble and -insoluble fractions of infected and uninfected HL-60 cells were prepared as described (Single et al., 1998). This method has been shown to separate cytosolic fractions from heavy

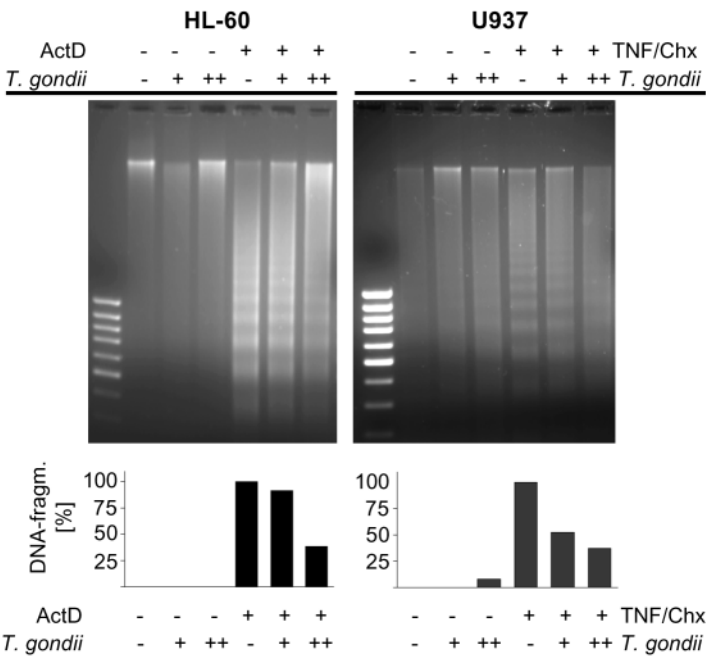

Fig. 1. *T. gondii*-infected HL-60 and U937 cells are partially protected from fragmentation of genomic DNA induced by pro-apoptotic stimuli. Human-derived cells were infected at parasite to host ratios of 10:1 (+) and 30:1 (++) or were left uninfected. After 30 minutes, HL-60 and U937 cells were treated with 5 μg/ml actinomycin D or 40 ng/ml TNF-α in combination with 2 μg/ml cycloheximide, respectively. Eight hours after infection, genomic DNA was analysed by agarose gel electrophoresis and ethidium bromide staining. DNA molecular weight markers (100 bp ladder) were separated in parallel. Signal intensities of bands corresponding to fragmentated genomic DNA were quantified by densitometry. Bars represent the relative intensities of representative bands, the intensities in uninfected HL-60 and U937 cells after treatment with pro-apoptotic stimuli were defined as 100%.

organelles including mitochondria (Single et al., 1998). Briefly, 4×10^6 cells were resuspended in 125 μl PBS and mixed with an equal volume of 150 μg/ml digitonin (Fluka, Deisenhofen, Germany) in 0.5 M sucrose. After 30 seconds on ice, mitochondria were pelleted by centrifugation for 60 seconds at 14,000 ***g***. The supernatants were saved as digitonin-soluble fractions, and pellets were lysed as described above for preparation of total cell lysates. After centrifugation at 13,000 ***g*** for 15 minutes, equal amounts of supernatants (digitonin-insoluble fractions) were separated together with the digitonin-soluble fractions by SDS-PAGE. Cytochrome c was detected by immunoblotting as described above using mouse anti-cytochrome c mAb (clone 7H8.2C12; 2 μg/ml; Pharmingen) and HRPO-conjugated $F(ab')_2$ fragment goat-anti mouse IgG (as above). In parallel, mitochondrial contamination of digitonin-soluble extracts or extraction of control proteins from the mitochondria by digitonin lysis was controlled using a cytochrome c oxidase (COX)-specific mAb (subunit IV, clone 10G8-D12-C12; 2 μg/ml; Molecular Probes, Leiden, The Netherlands).

Semi-quantitative RT-PCR analyses

Total cellular RNA was isolated from *T. gondii*-infected HL-60 or U937 cells and uninfected controls using the RNeasy® Mini Kit as recommended by the manufacturer (Qiagen, Hilden, Germany). Reverse transcription and PCR were carried out by the OneStep RT-PCR protocol (Qiagen) using the following oligonucleotide primers: PARP forward 5′-AAGCCCTAAAGGCTCAGAAC-3′; PARP reverse 5′-TTGGGTGTCTGTGTCTTGAC-3′; β-actin forward 5′-GTGG-GGCGCCCCAGGCACCA-3′; β-actin reverse 5′-CTCCTTAATGT-CACGCACGATTTC-3′. To exclude amplification of contaminating genomic DNA, control reactions were performed by inhibition of reverse transcriptase activity. PCR products were electrophoresed on a 1.5% agarose gel and amplified DNA visualized by ethidium bromide staining. Results were quantified by densitometry using an digital imaging system (Biometra).

Immunofluorescence staining and confocal microscopy

The distribution of cytochrome c in *T. gondii*-infected and uninfected cells was morphologically analysed by triple immunofluorescence staining and confocal microscopy. Eight hours after induction of apoptosis with 5 μg/ml actinomycin D, infected and uninfected HL-60 cells were washed in PBS and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS, pH 7.4 for 30 minutes at room temperature. After washing, cells were quenched for 10 minutes in 50 mM NH_4Cl in PBS and permeabilized for 1 hour with 0.1 mg/ml saponin (Sigma, Deisenhofen, Germany) in PBS containing 1% bovine serum albumin (Sigma). Cells were then simultaneously incubated with mouse anti-cytochrome c mAb (clone 6H2.B4; 10 μg/ml; Pharmingen) and rabbit anti-*Toxoplasma* serum diluted in PBS with saponin and BSA. After 1 hour at room temperature, coverslips were washed and were then incubated for 1 hour in 1.4 μg/ml Cy3-conjugated $F(ab')_2$ fragments donkey anti-mouse IgG and 6.5 μg/ml Cy5-conjugated $F(ab')_2$ fragments donkey anti-rabbit IgG (secondary Ab from Dianova, Hamburg, Germany). After washing, apoptotic cells were visualized using the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). Coverslips were mounted with Mowiol (Calbiochem, Schwalbach, Germany) and were examined by confocal laser scanning microscopy using Leica TCS SP2. For selected cells, 10 optical sections at intervals of 0.5 μm were generally recorded. To analyse the intracellular distribution of cytochrome c quantitatively, the optical sections from a selected cell preparation were superimposed, and the fluorescence intensity profile of the cytochrome c-labelling in apoptotic and non-apoptotic cells was determined.

RESULTS

Inhibition of in vitro-induced apoptosis in human-derived cell lines by *T. gondii*

Human-derived HL-60 and U937 cells were used to analyse the effects of *T. gondii* on apoptosis of its host cell. Both cell lines are well characterized in vitro models to investigate programmed cell death, since high levels of apoptosis are inducible by treatment with actinomycin D (Martin et al., 1990) or TNF-α in combination with cycloheximide (Vanags et al., 1996), respectively. Furthermore, cells of the monocytic/granulocytic lineage may represent important hematopoetic host cells of *T. gondii* (Channon et al., 2000). Our previous studies have shown that *T. gondii* downregulates in vitro-induced apoptosis in HL-60 cells (Goebel et al., 1999). In this study, apoptosis in monocyte-like U937 cells induced by treatment with TNF-α and cycloheximide was similarly decreased by concomitant infection with *T. gondii* as shown by the DNA fragmentation assay (Fig. 1). Furthermore, addition of increasing numbers of *T. gondii* tachyzoites dose-dependently downregulated DNA-fragmentation in both HL-

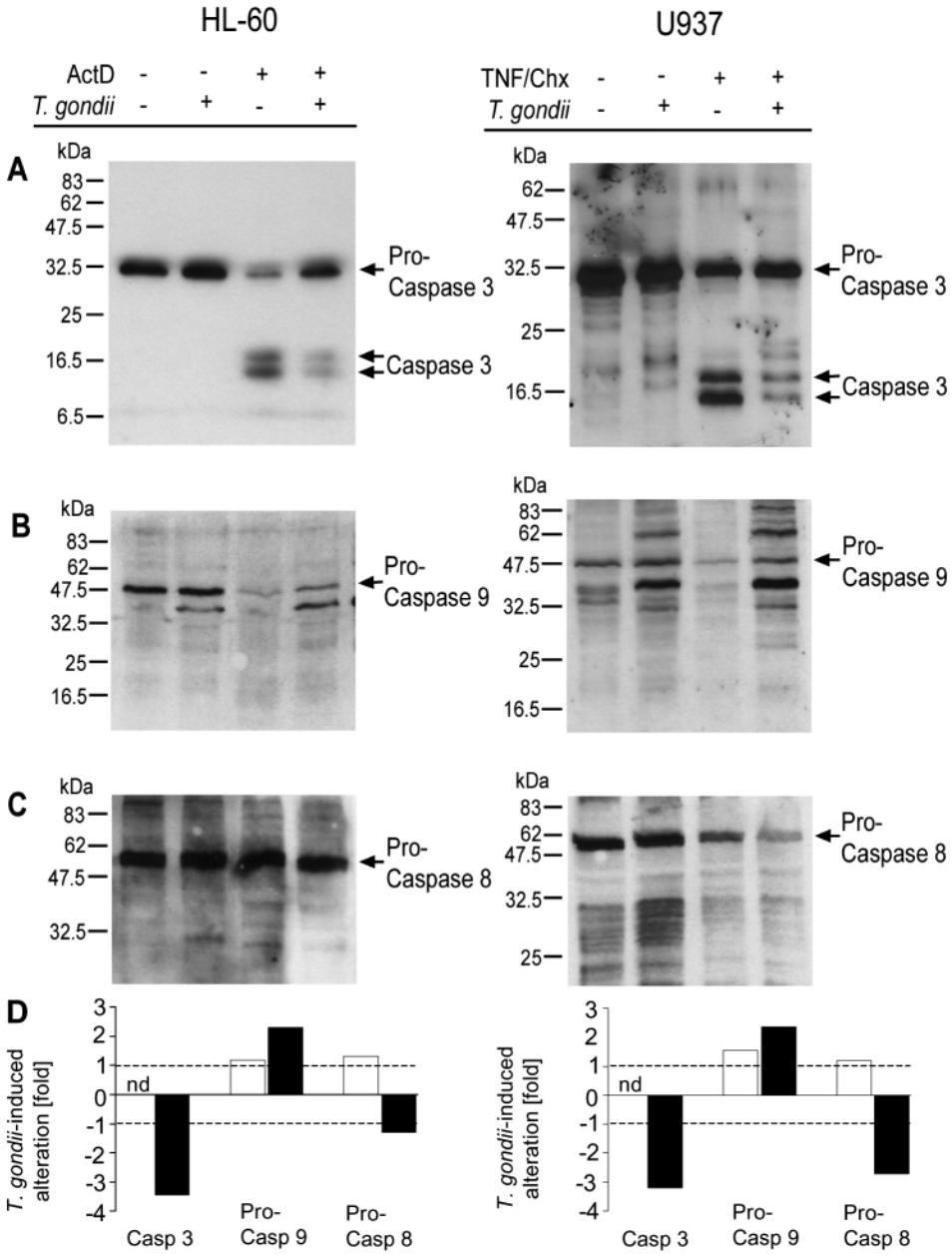

Fig. 2. *T. gondii* downregulates activation of caspases 3 and 9 after induction of apoptosis in human-derived cell lines. HL-60 and U937 cells were infected at a parasite to host ratio of 30:1 and were 30 minutes later treated with actinomycin D or TNF-α in combination with cycloheximide as indicated. Eight hours after infection, antigenic extracts were prepared from equal numbers of cells and were separated by standard SDS-PAGE. After transfer to nitrocellulose membranes, caspase 3 (A), caspase 9 (B) and caspase 8 (C) were visualized by immunostaining using enhanced chemiluminescence detection. *T. gondii*-induced alterations of caspase activation were quantified densitometrically by determining the levels of the active subunits (caspase 3) or the levels of the inactive proforms (caspases 8 and 9) (D). Bars represent the relative changes in caspase levels after parasitic infection of untreated cells (open bars) or those treated with pro-apoptotic stimuli (closed bars) compared with levels in uninfected cells. Horizontal dashed lines indicate an unchanged protein level after parasitic infection compared with levels in uninfected controls.

60 and U937 cells treated with pro-apoptotic stimuli. Densitometric analyses revealed that the appearance of fragmented DNA in these cells decreased by 60-65% after addition of *T. gondii* at a parasite to host cell ratio of 30:1, compared with that seen in uninfected controls (Fig. 1). By contrast, fragmented genomic DNA was undetectable in untreated HL-60 and U937 cells and was not or only slightly enhanced after infection of these cells with increasing parasite to host ratios.

T. gondii inhibits proteolytic cleavage of caspases 3 and 9 during apoptosis

Since activation of the transcription factor NFκB has been shown to suppress apoptosis in response to a variety of apoptosis-inducing agents (Wang et al., 1996; Wang et al., 1998), we first analysed the activation of NFκB in *T. gondii*-infected and uninfected HL-60 cells by EMSA. After induction of apoptosis with actinomycin D, NFκB-specific binding activity of nuclear extracts was completely abrogated in *T. gondii*-infected cells, compared with that in untreated control cells; however, this was similarly observed in uninfected as well as *T. gondii*-infected HL-60 cells (data not shown). This indicates that *T. gondii* does not inhibit apoptosis in human-derived promyelocytic cells by increasing NFκB activation.

Since proteolytic activation of caspases in a cascade-like fashion is a central component in the execution of apoptosis (Thornberry and Lazebnik, 1998), we next analysed expression and cleavage of several key caspases in *T. gondii*-infected and uninfected cells by immunoblotting. Caspase 3 is one of the critical downstream members of the caspase family and is thought to be an essential effector of cell death (Thornberry and Lazebnik, 1998). Induction of apoptosis in HL-60 and U937 cells by treatment with actinomycin D or TNF-α in combination with cycloheximide induced prominent activation of caspase 3 in non-infected cells, as indicated by the increase in active cleavage products and the decrease in inactive proform (Fig. 2A). After concomitant infection with *T. gondii*, however, proteolytic cleavage of the procaspase 3 into the active subunits was clearly diminished in both cell lines. Quantitative analyses by densitometry showed that the appearance of the active caspase 3 after induction of apoptosis decreased 3.4- or 3.2-fold in *T.gondii*-infected compared with

that seen in uninfected HL-60 and U937 cells, respectively, thus indicating interference of the parasite with activation of the caspase cascade (Fig. 2D). Cleavage of the effector caspase 3 may be mediated by activation of upstream initiator caspases 8 and 9, which are associated with apoptosis induced by death receptor engagement or apoptosis-inducing cytotoxic agents, respectively (Thornberry and Lazebnik, 1998). Therefore, we asked which of these caspases might be affected by *T. gondii*. The amount of inactive procaspase 9 diminished after induction of apoptosis in HL-60 and U937 cells; however, this decrease was inhibited 2.3-fold in both cell lines after concomitant infection with *T. gondii* (Fig. 2B,D). Although the antibody used for these analyses only recognized the proform but not the active subunits of caspase 9, these data suggested that proteolytic cleavage of caspase 9 during apoptosis in HL-60 and U937 cells is downregulated by *T. gondii*. No differences were observed in the appearance of the inactive procaspase 8 after treatment of infected and uninfected HL-60 cells with actinomycin D, which rules out an involvement of caspase 8 in actinomycin D-induced apoptosis in HL-60 cells (Fig. 2C). Furthermore, *T. gondii* only slightly altered the protein level of procaspase 8 in untretaed as well as treated HL-60 cells (Fig. 2D). By contrast, the amount of inactive caspase 8 considerably decreased in U937 cells treated with TNF-α in combination with cycloheximide, compared with that in untreated control cells (Fig. 2C). However, this decrease was even more prominent in parasite-infected cells than in uninfected ones (Fig. 2D), suggesting that *T. gondii* does not abrogate proteolytic cleavage of caspase 8 after receptor engagement by TNF-α. Taken together, these results indicated that *T. gondii* partially inhibits cleavage of caspase 3 and 9, but not caspase 8 after induction of apoptosis in HL-60 and U937 cells.

PARP protein levels and cleavage of nuclear target proteins are downregulated by *T. gondii*

Activation of effector caspases during apoptosis induces proteolysis of a variety of cytosolic and nuclear target proteins leading to those cellular changes that are associated with apoptosis (Thornberry and Lazebnik, 1998). We therefore next investigated whether interference of *T. gondii* with activation of the caspase cascade alters cleavage of two nuclear target proteins, PARP and PKCδ. Treatment of HL-60 and U937 cells with actinomycin D and TNF-α plus cycloheximide, respectively, induced cleavage of the 116 kDa full length PARP into the 85 kDa subunit (Fig. 3A). Interestingly, expression of either full length PARP in untreated cells or the 85 kDa PARP subunit in those cells treated with pro-apoptotic stimuli was prominently inhibited after infection with *T. gondii*. It should be noted, however, that residual 85 kDa PARP fragment was still detectable in *T. gondii*-infected HL-60 and U937 cells after treatment with apoptosis inducers, whereas the 116 kDa full length molecule was almost not detectable after infection of untreated cells with *T. gondii*. This suggests that treatment of HL-60 and U937 cells with pro-apoptotic stimuli increased the steady state level of PARP proteins, compared with that in untreated parasite-infected cells. Alternatively, downregulation of PARP by *T. gondii* may be more prominent in untreated cells than in those treated with pro-apoptotic stimuli.

To elucidate possible mechanisms of the decreased PARP protein levels after infection, the amount of PARP-specific mRNA was determined by semi-quantitative RT-PCR (Fig. 4). PARP transcript levels normalized against β-actin mRNA decreased after treatment of HL-60 cells with actinomycin D, compared with levels in untreated control cells; however, this was similarly observed in *T. gondii*-infected and uninfected control cells (Fig. 4A,B). Likewise, the relative PARP transcript levels did not change in untreated HL-60 cells after parasitic infection. In U937 cells, the relative amont of PARP transcripts was neither altered after treatment with TNF-α/cycloheximide nor after infection with *T. gondii* (Fig. 4A,B). Nevertheless, since control western blots confirmed downregulation of the PARP protein levels in infected compared with uninfected HL-60 and U937 cells (Fig. 4C),

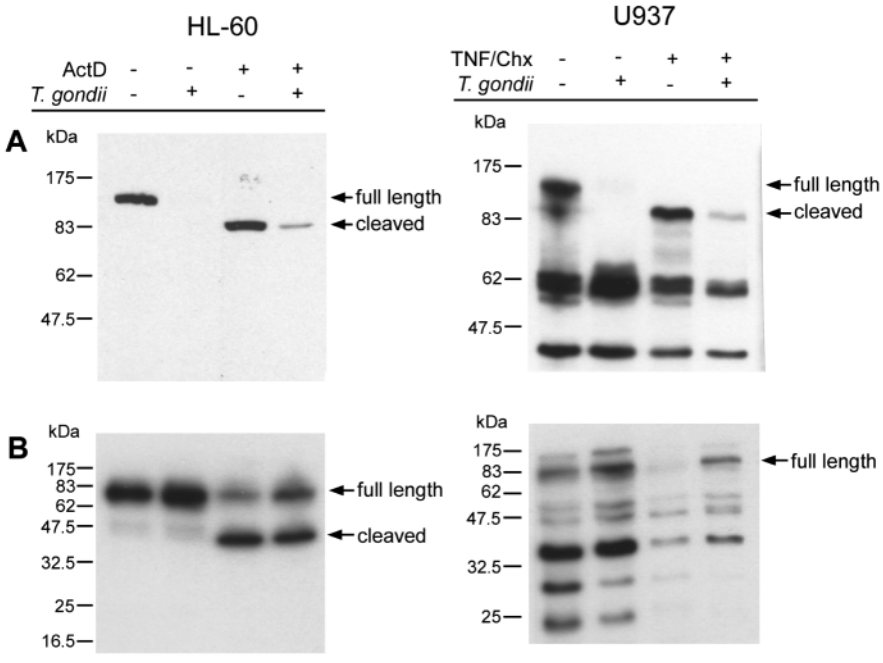

Fig. 3. Expression of PARP as well as cleavage of nuclear target proteins during apoptosis in human-derived cell lines are decreased by *T. gondii*. After infection of HL-60 and U937 cells at a parasite to host ratio of 30:1, infected and uninfected control cells were treated with 5 μg/ml actinomycin D or 40 ng/ml TNF-α in combination with 2 μg/ml cycloheximide as indicated. Eight hours after infection, antigenic lysates were prepared and separated by SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes and PARP (A) and PKCδ (B) were detected by immunostaining using enhanced chemiluminescence detection.

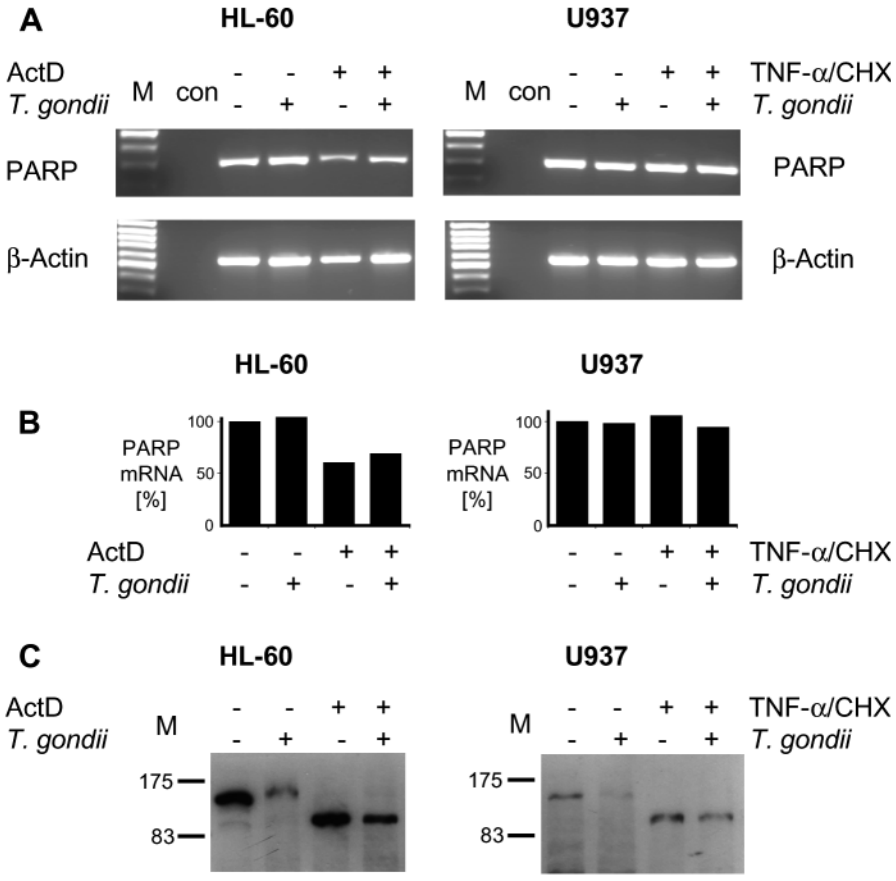

Fig. 4. RT-PCR analyses of PARP transcript levels in *T. gondii*-infected and uninfected human-derived cell lines. After infection of HL-60 and U937 cells at a parasite to host ratio of 30:1, infected and uninfected control cells were treated with 5 μg/ml actinomycin D or 40 ng/ml TNF-α plus 2 μg/ml cycloheximide or left untreated. Eight hours after infection, total RNA was isolated and PARP and β-actin transcripts were reverse transcribed and amplified using an one-step RT-PCR protocol. Amplified mRNAs, a negative control without RNA (con), and a 100 bp ladder (M) were separated by agarose gel electrophoresis and visualized by ethidium bromide staining (A). Band intensities were quantified by densitometry. Bars represent the relative amount of amplified PARP mRNA normalized against β-actin in the same sample (PARP/β-actin ×100), the amount of amplified PARP mRNA from untreated, uninfected control cells were defined as 100% (B). As a control, levels of PARP protein were determined in parallel by immunoblotting as described in Fig. 4 legend (C).

these results indicate that inhibition of PARP by *T. gondii* is post-transcriptionally regulated.

Since these experiments did not unravel whether interference of *T. gondii* with the caspase cascade results in a decreased proteolysis of target proteins, we determined cleavage of the nuclear target protein PKCδ. As shown in Fig. 3B, treatment of uninfected HL-60 cells with actinomycin D induced prominent cleavage of the holoenzyme into its subunits. Although the PKCδ subunit was still clearly detectable after concomitant infection with *T. gondii*, the increase of the holoenzyme indicated that apoptosis-associated cleavage of PKCδ was reduced in parasite-infected cells. Similarly, the level of full length PKCδ was clearly higher in *T. gondii*-infected U937 cells after treatment with TNF-α plus cycloheximide than in uninfected controls (Fig. 3B). However, no cleavage subunits of PKCδ could be detected in infected and uninfected U937 cells and this was similarly observed with TNF-α/cycloheximide-treated as well as untreated control cells.

Inhibition of mitochondrial cytochrome c release by *T. gondii*

To further characterize those upstream signalling pathways by which *T. gondii* may downregulate activation of the caspase cascade, we next analysed release of cytochrome c from the mitochondria into the cytosol, a process that regulates caspase activation by multiple apoptosis inducers (Green and Reed, 1998; Li et al., 1997). Therefore, HL-60 were fractionated into digitonin-soluble and -insoluble fractions containing cytosolic and mitochondrial proteins, respectively (Single et al., 1998), which were subsequently analysed by immunoblotting. In untreated cells (i.e. those cultured without actinomycin D), cytochrome c was almost exclusively detected in the digitonin-insoluble fraction and was not or only faintly visible in digitonin-soluble fractions, suggesting a mitochondrial localization (Fig. 5A). Furthermore, contamination of the digitonin-soluble extracts with mitochondria, as well as extraction of mitochondrial proteins by the digitonin lysis was excluded since the control protein COX was detected in the digitonin-insoluble fraction only (Fig. 5B). Treatment of uninfected HL-60 cells with actinomycin D induced release of cytochrome c from the mitochondria into the cytosol, as indicated by the prominent decrease of cytochrome c in the digitonin-insoluble fraction and a simultaneous increase in the digitonin-soluble fraction (Fig. 5A). However, in parasite-infected cells, the actinomycin D-induced cytochrome c redistribution was considerably diminished compared with that in uninfected control cells, indicating that *T. gondii* inhibits mitochondrial cytochrome c-release during apoptosis.

These results were further confirmed on the single cell level by triple immunofluorescence staining and confocal microscopy (Fig. 6). Treatment of uninfected HL-60 cultures with actinomycin D induced apoptosis in the majority of cells, as determined by a positive nick end labelling with fluorescein-conjugated dUTP (Fig. 6A). Within these cells, apoptosis was associated with a more diffuse staining pattern of cytochrome c consistent with a cytoplasmic distribution of cytochrome c (Fig. 6A, arrowheads), whereas non-apoptotic cells showed a granular distribution indicating a mitochondrial localization of cytochrome c (Fig. 6A, thick arrows). Furthermore,

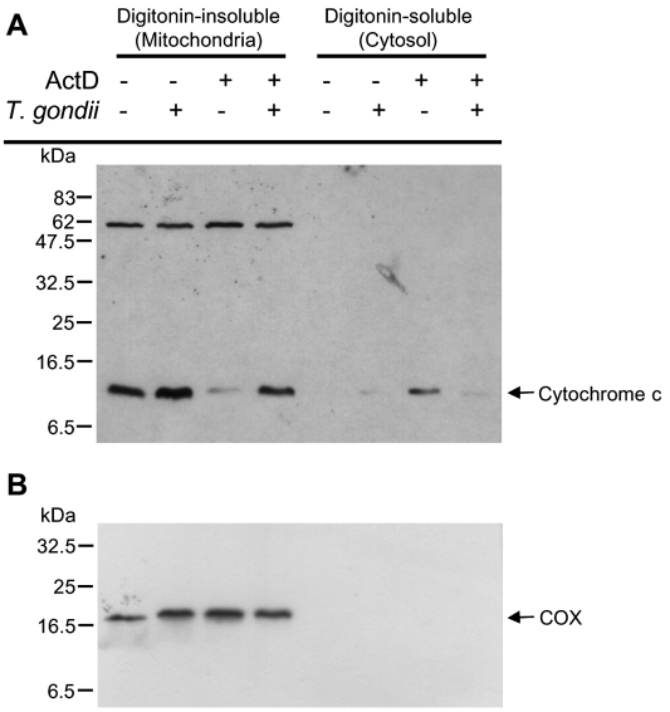

Fig. 5. Apoptosis-associated cytochrome c-release from mitochondria into the cytosol of human-derived HL-60 cells is inhibited by *T. gondii*. HL-60 cells were infected at a parasite to host ratio of 30:1 and were then treated with 5 μg/ml actinomycin D for 8 hours. Equal numbers of cells per treatment were fractionated into digitonin-soluble and -insoluble extracts, containing cytosolic and mitochondrial proteins, respectively. Lysates were separated by SDS-PAGE and were then transferred to nitrocellulose membranes. Immobilized proteins were probed with a cytochrome c-specific (A) or a cytochrome c oxidase (COX) subunit IV-specific (B) monoclonal antibody and an appropriate secondary antibody using enhanced chemiluminescence detection. COX served as a marker for mitochondrial contamination of the digitonin-soluble extracts.

quantitative analyses of the cytochrome c labelling in superimposed optical sections from different cells showed a highly irregular fluorescence intensity profile in TUNEL-negative cells, whereas this was more homogenous in TUNEL-positive cells (Fig. 6B). These analyses also confirmed that the overall intensity of the cytochrome c labelling did not differ significantly between apoptotic and non-apoptotic HL-60 cells (Fig. 6A,B). Interestingly, after concomitant infection with *T. gondii*, the presence of intracellular parasites closely correlated with a granular distribution of cytochrome c, as well as the absence of DNA strand breaks (Fig. 6A,B, thin arrows), whereas most parasite-negative host cells were apoptotic and showed a diffuse distribution of cytochrome c. Together, these results suggest that blockade of mitochondrial cytochrome c release by *T. gondii* protects parasite-positive host cells from in vitro-induced apoptosis.

Increased Mcl-1 protein levels after *T. gondii*-infection

Several members of the Bcl-2 family have been shown to regulate programmed cell death by alterations of the apoptosis-promoting activity of mitochondria (Adams and Cory, 1998). Therefore, we next analysed expression of Bcl-2 and Mcl-1, two anti-apoptotic members of this protein family. Immunoblot analyses revealed that the protein levels of Bcl-2 were not considerably altered after infection of HL-60 and U937 cells with *T. gondii* compared with levels in uninfected control cells. This was similarly observed in untreated cells as well as in those that had been treated to undergo apoptosis (Fig. 7A,C). By contrast, expression of Mcl-1 was clearly inhibited by treatment of HL-60 and U937 cells with actinomycin D or TNF-α plus cycloheximide, respectively (Fig. 7B). However, the apoptosis-associated decrease of Mcl-1 was partially inhibited in *T. gondii*-infected compared with that in uninfected control cells (Fig. 7B). Densitometric analyses revealed that after infection with *T. gondii*, Mcl-1 levels increased more than twofold in actinomycin D-treated HL-60 or TNF-α/cycloheximide-treated U937 cells (Fig. 7C), indicating that *T. gondii* either upregulates Mcl-1 expression or increases Mcl-1 protein stability.

DISCUSSION

Inhibition of host cell apoptosis by *T. gondii* (Nash et al., 1998; Goebel et al., 1999) and other intracellular protozoan parasites (Moore and Matlashewski, 1994; Heussler et al., 1999) is thought to represent a crucial factor of the pathogen-host interaction. However, little is known about the cell biological mechanisms of such interferences. In the present study, we therefore analysed different components of the apoptotic machinery of human-derived cells after infection with *T. gondii*. Our results indicate that *T. gondii* has evolved at least two mechanisms that may contribute to the parasite's anti-apoptotic activity: (1) inhibition of cytochrome c release leading to decreased activation of the caspase cascade, a mechanism that may be partially mediated by increased Mcl-1 protein levels after infection; and (2) a prominent parasite-induced decrease in PARP protein levels. By contrast, a role for the transcription factor NFκB in mediating the anti-apoptotic activity of *T. gondii*, at least in human-derived granulocytic/monocytic cells, as described for other microorganisms or their gene products (Heussler et al., 1999; Spender et al., 1999; Manna and Aggarwal, 1999) can be ruled out, since infection with *T. gondii* did not enhance DNA binding activity of NFκB compared with that in uninfected controls (data not shown).

Consistent with our previous findings (Goebel et al., 1999), *T. gondii* dose-dependently downregulated host cell apoptosis in human-derived HL-60 and U937 cells treated with different pro-apoptotic stimuli. Nevertheless, it should be mentioned that even after addition of *T. gondii* at the highest parasite to host ratio, apoptosis was not completely eliminated. Since protection against apoptosis by *T. gondii* requires the presence of intracellular parasites (Goebel et al., 1999), this finding probably reflects the fact that, after addition of *T. gondii* at a parasite to host ratio of 30:1, infection rates do not exceed 50% (data not shown), the remaining parasite-negative host cells still being susceptible to induction of apoptosis.

Activation of caspases represents a central step in the apoptosis signalling cascade and transduces regulatory upstream signals into the cell death-executing machinery. Our results clearly show that *T. gondii* interferes with this caspase

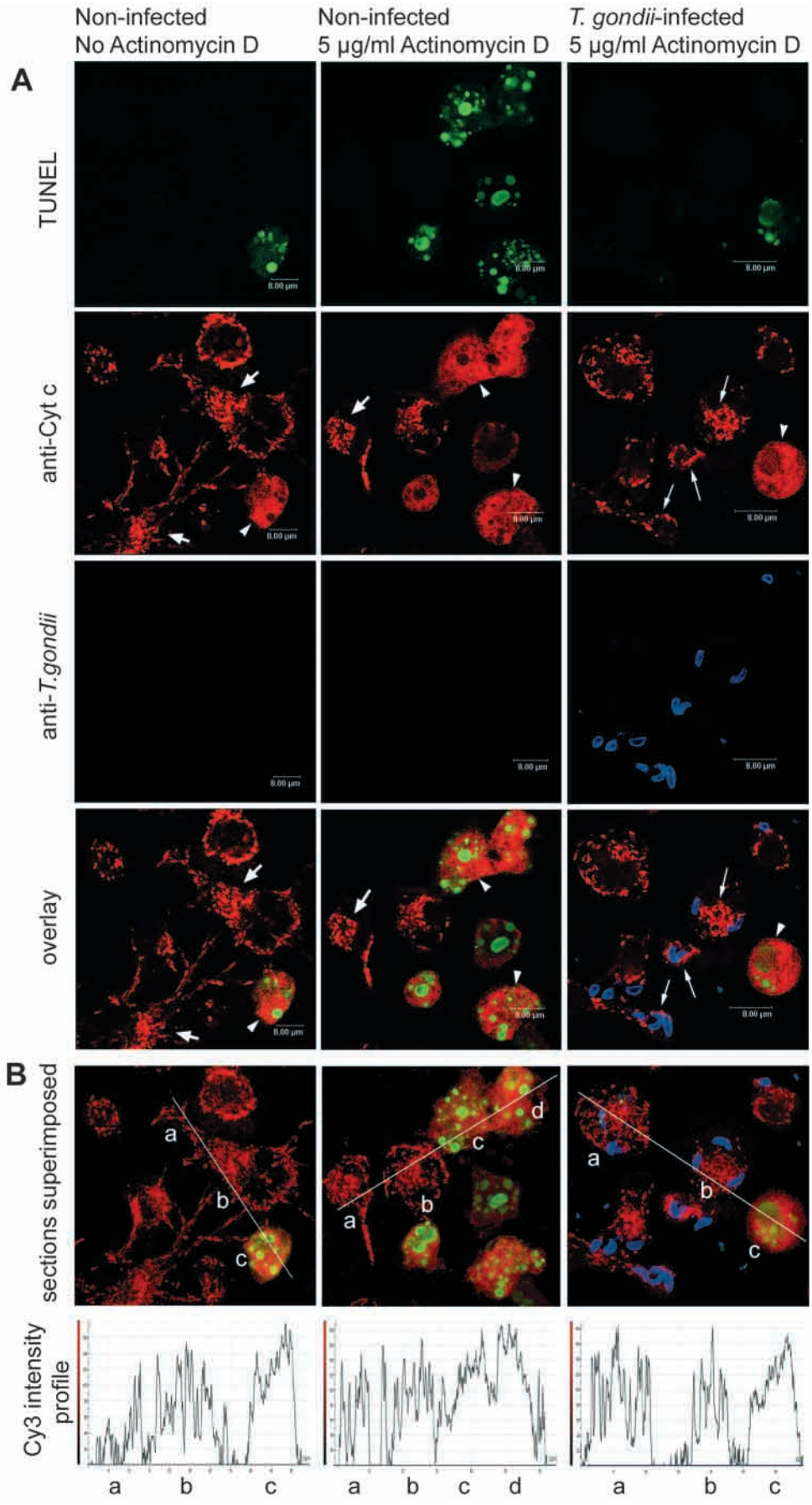

Fig. 6. Intracellular distribution of cytochrome c in apoptotic and non-apoptotic human-derived HL-60 cells after infection with *T. gondii*. Cells were infected at a parasite to host ratio of 30:1 and were then treated with 5 µg/ml actinomycin D for 8 hours. The subcellular distribution of cytochrome c was determined by triple immunofluorescence staining and confocal microscopy. After fixation and permeabilization, cell preparations were stained using fluorescein-labelled dUTP to visualize DNA strand breaks (green fluorescence), a cytochrome c-specific monoclonal antibody and Cy3-conjugated secondary antibody (red fluorescence), and a *Toxoplasma*-specific antiserum and Cy5-conjugated secondary antibody (blue fluorescence).(A) Single optical sections from representative cells of the indicated treatments are shown. In non-apoptotic cells (i.e. those without signs of DNA strand breaks), cytochrome c was granularly distributed indicating a mitochondrial localization (thick arrows), while this molecule was homogenously distributed in apoptotic cells indicating translocation into the cytoplasm (arrowheads). Parasite-positive cells showed no signs of apoptosis and this was correlated with a granular (i.e. mitochondrial) distribution of cytochrome c (thin arrows). (B) 10 optical sections for each cell preparation were taken at intervals of 0.5 µm and were superimposed. A fluorescence intensity profile of the cytochrome c labelling was determined for selected cells as indicated by the straight line in the overlay micrographs. The lowercase letters below the intensity profiles refer to the cells or parts thereof for which the subcellular distribution of cytochrome c has been determined.

cascade by downregulating activation of caspase 3 and 9. Interestingly, this was not only observed after treatment of HL-60 cells with actinomycin D but also after treatment of U937 cells with TNF-α. This confirms recent findings that the mitochondria/caspase 9 pathway is activated not only by internal pro-apoptotic stimuli but also during receptor-mediated induction of apoptosis (Kuwana et al., 1998; Budihardjo et al., 1999; Scaffidi et al., 1999), and amplifies receptor-mediated apoptosis at least under certain conditions (Scaffidi et al., 1999). In contrast to caspase 3 and 9, proteolytic cleavage of procaspase 8 after treatment of U937

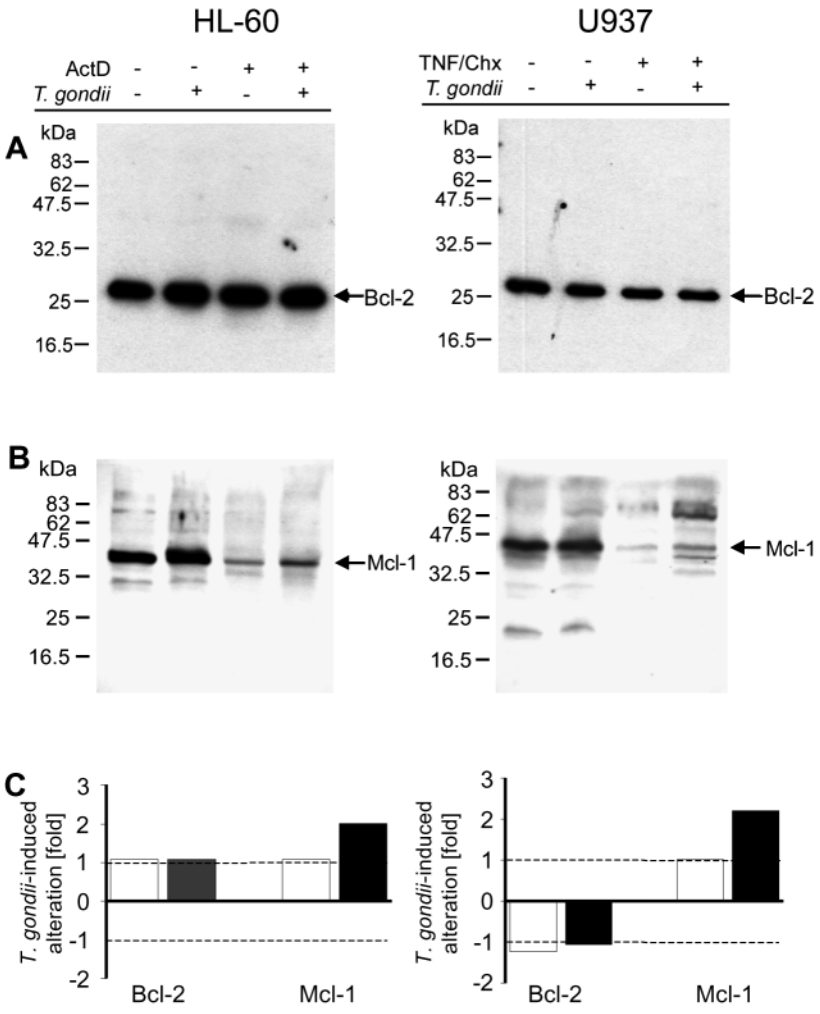

Fig. 7. *T. gondii* upregulates protein levels of Mcl-1, but not Bcl-2, in human-derived cell lines. Thirty minutes after infection at a parasite to host ratio of 30:1, HL-60 and U937 cells were treated for 8 hours with 5 μg/ml actinomycin D or 40 ng/ml TNF-α in combination with 2 μg/ml cycloheximide, respectively. Antigenic lysates were then separated by SDS-PAGE under reducing conditions, proteins transferred to nitrocellulose membranes and Bcl-2 (A) and Mcl-1 (B) were detected by immunostaining using enhanced chemiluminescence detection. *T. gondii*-induced alterations of Bcl-2 and Mcl-1 protein levels were quantified densitometrically (C). Bars represent the relative changes in protein levels after parasitic infection of untreated cells (open bars) or those treated with pro-apoptotic stimuli (closed bars) compared with levels in uninfected cells. Horizontal dashed lines indicate an unchanged protein level after parasitic infection compared with levels in uninfected controls.

cells with TNF-α and cycloheximide was even enhanced after *T. gondii* infection, indicating that the parasite downregulates proteolysis of distinct caspases only, rather than generally alters caspase activation. Despite continuous activation of caspase 8, apoptosis in U937 cells was nevertheless downregulated after infection, indicating that induction of apoptosis under these conditions may mainly involve the mitochondria/caspase 9 pathway rather than direct activation of caspase 3. This is supported by Tafani et al., who show that apoptosis can indeed be prevented by inhibition of the mitochondrial pathway despite continuous activation of caspase 8 (Tafani et al., 2000). However, it should be mentioned that induction of apoptosis in U937 cells by TNF-α requires the presence of low concentrations of cycloheximide (Vanags et al., 1996), the contribution of which to the signalling pathway is unknown.

Activation of the caspase 9/caspase 3 pathway during apoptosis involves release of mitochondrial cytochrome c into the cytoplasm, which then leads to dATP-dependent formation of an Apaf1/caspase 9-complex and to activation of caspase 9 (Li et al., 1997; Pan et al., 1998). Results from both subcellular fractionation analyses and confocal microscopy indicated that *T. gondii* inhibits such mitochondrial cytochrome c release. On the single cell level, the presence of intracellular parasites positively correlated with a mitochondrial distribution of cytochrome c and the absence of DNA strand breaks as a characteristic feature of apoptosis. This confirms and extends our previous findings that protection of HL-60 cells from apoptosis requires the presence of viable, but not neccessarily replicating, intracellular parasites (Goebel et al., 1999). Thus, intracellular *T. gondii* might secrete a parasitic factor that mediates inhibition of mitochondrial cytochrome c release, leading to decreased activation of the caspase 9/caspase 3 pathway. Alternatively, active invasion by the parasite (Dobrowolski and Sibley, 1996) may irreversible modify the host cell physiology, which results in protection against induction of apoptosis. Downregulation of host cell apoptosis induced by different pro-apototic stimuli in human-derived cell lines via inhibition of cytochrome c release has been similarly described for *Chlamydia trachomatis* and was correlated with the presence of viable intracellular chlamydia (Fan et al., 1998).

Proteins of the Bcl-2 family are known to regulate mitochondria-associated induction of apoptosis (Adams and Cory, 1998). For example, Bcl-2 inhibits apoptosis by preventing the translocation of cytochrome c into the cytoplasm (Yang et al., 1997; Kluck et al., 1997), and a variety of virus-encoded Bcl-2 homologues have been shown to modulate host cell apoptosis after infection (Barry and McFadden, 1998). However, expression of Bcl-2 was not altered after infection by *T. gondii*. By contrast, parasitic infection increased the steady state levels of Mcl-1 protein (Kozopas et al., 1993), another anti-apoptotic member of the Bcl-2 protein family, after induction of apoptosis in HL-60 and U937 cells. In HL-60 cells, an increase of Mcl-1 has been associated with inhibition of apoptosis by downregulating mitochondrial cytochrome c release (Wang and Studzinski, 1997) and may thus contribute to the anti-apoptotic activity of *T. gondii*, at least in this cell type. Interestingly, upregulation of A1, a protein with similarity to Mcl-1 (Lin et al., 1993), has recently been described in exudate cells from *T. gondii*-infected mice (Orlofsky et al., 1999), indicating that additional

members of the Bcl-2 family may also be involved in *T. gondii*-induced inhibition of apoptosis. In addition, these results demonstrate that upregulation of proteins of the Bcl-2 family occurs not only after parasitic infection in vitro but also in *T. gondii*-infected mice in vivo. However, the exact contribution of Mcl-1 or similar proteins to the inhibition of host cell apoptosis by *T. gondii* awaits further clarification.

Execution of apoptosis involves caspase-mediated cleavage of several target proteins leading to disassembly of the cell. Reduced activation of the effector caspase 3 after infection with *T. gondii*, as described here, partially inhibited cleavage of PKCδ, indicating that parasitic interference with the caspase cascade is of functional relevance to proteolysis of target proteins. Interestingly, our initial attempts to determine the extent of PARP cleavage, a commonly used marker for apoptosis research (Duriez and Shah, 1997) revealed that *T. gondii* prominently downregulates the protein levels of PARP in HL-60 and U937 cells, irrespective of whether these cells have been treated with pro-apoptotic stimuli or left untreated. Since PARP mRNA levels were not altered by *T. gondii*, such downregulation may be achieved post-transcriptionally; however, the detailed mechanisms of this interference needs further investigations. Although these results hampered the determination of PARP cleavage in infected and uninfected cells after induction of apoptosis, our novel finding of decreased PARP protein levels after parasitic infection is nevertheless of major interest since it not only may contribute to *T. gondii*-induced inhibition of host cell apoptosis, but also may be of fundamental relevance for the host-parasite interaction. PARP is thought to be involved in a variety of cellular functions by catalyzing the transfer of ADP-ribose from NAD^+ to acceptor proteins and by its activity as a transcriptional coactivator (Jacobsen and Jacobsen, 1999; Meisterernst et al., 1997). Although the role of PARP during apoptosis is not completely understood (Le Rhun et al., 1998), recent evidence suggests that under conditions of excessive DNA damage PARP promotes cell death (Jacobsen and Jacobsen, 1999). Indeed, depletion or inhibition of PARP has been shown to protect different cell lines, including HL-60 and U937 cells, against apoptosis induced by a variety of pro-apoptotic stimuli (Nosseri et al., 1994; Tanaka et al., 1995; Shiokawa et al., 1997; Simbulan-Rosenthal et al., 1998; Pacini et al., 1999). Downregulation of PARP expression by *T. gondii* may thus contribute to parasite-induced inhibition of host cell apoptosis either by inhibiting excessive depletion of cellular NAD^+ and ATP (Simbulan-Rosenthal et al., 1998) or by preventing irreversible binding of the 24 kDa PARP fragment to DNA strand breaks (Duriez and Shah, 1997). Further experiments will clarify the exact contribution of reduced PARP expression on inhibition of apoptosis by *T. gondii* as well as on the parasite-host interaction in general.

In conclusion, *T. gondii* interferes with at least two different components of the apoptosis-inducing signalling cascade of its host cell. Evolution of different mechanisms to downregulate host cell apoptosis possibly explains how parasite-induced protection against a variety of apoptosis inducers may be achieved.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Gr 906/5-4; Lu 777/1-3).

REFERENCES

Adams, J. M. and Cory, S. (1998). The Bcl-2 protein family: Arbiters of cell survival. *Science* **281**, 1322-1326.

Barry, M. and McFadden, G. (1998). Apoptosis regulators from DNA viruses. *Curr. Opin. Immunol.* **10**, 422-430.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. *Ann. Rev. Cell Dev. Biol.* **15**, 269-290.

Channon, J. Y., Seguin, R. M. and Kasper, L. H. (2000). Differential infectivity and division of *Toxoplasma gondii* in human peripheral blood leukocytes. *Infect. Immun.* **68**, 4822-4826.

Dobrowolski, J. M. and Sibley, L. D. (1996). *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84**, 933-939.

Duriez, P. J. and Shah, G. M. (1997). Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem. Cell Biol.* **75**, 337-349.

Eldadah, B. A., Yakovlev, A. G. and Faden, A. I. (1996). A new approach for the electrophoretic detection of apoptosis. *Nucl. Acid Res.* **24**, 4092-4093.

Everett, H. and McFadden, G. (1999). Apoptosis: an innate immune response to virus infection. *Trends Microbiol.* **7**, 160-165.

Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G. A., Nance, D. M., Greenberg, A. H. and Zhong, G. (1998). Inhibition of apoptosis in chlamydia-infected cells: Blockade of mitochondrial cytochrome c release and caspase activation. *J. Exp. Med.* **187**, 487-496.

Fratazzi, C., Arbeit, R. D., Carini, C. and Remold, H. G. (1997). Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *J. Immunol.* **158**, 4320-4327.

Goebel, S., Lüder, C. G. K. and Gross, U. (1999). Invasion by *Toxoplasma gondii* protects human-derived HL-60 cells from actinomycin D-induced apoptosis. *Med. Microbiol. Immunol.* **187**, 221-226.

Gougeon, M.-L., Lecoeur, H., Dulioust, A., Enouf, M.-G., Crouvoisier, M., Goujard, C., Debord, T. and Montagnier, L. (1996). Programmed cell death in peripheral lymphocytes from HIV-infected persons. *J. Immunol.* **156**, 3509-3520.

Green, D. R. and Reed, J. C. (1998). Mitochondria and apoptosis. *Science* **281**, 1309-1312.

Griffin, D. E. and Hardwick, J. M. (1999). Perspective: virus infections and the death of neurons. *Trends Microbiol.* **7**, 155-160.

Gross, U., Müller, W. A., Knapp, S. and Heesemann, J. (1991). Identification of a virulence-associated antigen of *Toxoplasma gondii* by a mouse monoclonal antibody. *Infect. Immun.* **59**, 4511-4516.

Gross, U., Bohne, W., Soete, M. and Dubremetz, J. F. (1996). Developmental differentiation between tachyzoites and bradyzoites of *Toxoplasma gondii*. *Parasitol. Today* **12**, 30-33.

Hardwick, J. M. (1998). Viral interference with apoptosis. *Semin. Cell Dev. Biol.* **9**, 339-349.

Heussler, V. T., Machado, J. Jr, Fernandez, P. C., Botteron, C., Chen, C. G. Pearse, M. J. and Dobbeelaere, D. A. (1999). The intracellular parasite *Theileria parva* protects infected T cells from apoptosis. *Proc. Natl. Acad. Sci. USA* **96**, 7312-7317.

Hisaeda, H., Sakai, T., Ishikawa, H., Maekawa, Y., Yasutomo, K., Good, R. A. and Himeno, K. (1997). Heat shock protein 65 induced by γδ T cells prevents apoptosis of macrophages and contributes to host defense in mice infected with *Toxoplasma gondii*. *J. Immunol.* **159**, 2375-2381.

Holliman, R. E. and Greig, J. R. (1997). Toxoplasmosis in immunocompromised patients. *Curr. Opin. Infect. Dis.* **10**, 281-284.

Jacobson, M. K. and Jacobson, E. L. (1999). Discovering new ADP-ribose polymer cycles: protecting the genome and more. *TIBS* **24**, 415-417.

Khan, I., Matsuura, T. and Kasper, L. H. (1996). Activation-mediated CD4+ T cell unresponsiveness during acute *Toxoplasma gondii* infection in mice. *Int. Immunol.* **8**, 887-896.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. (1997). The release of cytochrome c mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132-1136.

Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P. and Craig, R. W. (1993). Mcl-1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to Bcl-2. *Proc. Natl. Acad. Sci. USA* **90**, 3516-3520.

Kuwana, T., Smith, J. J., Muzio, M., Dixit, V., Newmeyer, D. D. and Kornbluth, S. (1998). Apoptosis induction by caspase 8 is amplified

through the mitochondrial release of cytochrome c. *J. Biol. Chem.* **273**, 16589-16594.

Laochumroonvorapong, P., Paul, S., Elkon, K. B. and Kaplan, G. (1996). H_2O_2 induces monocyte apoptosis and reduces viability of *Mycobacterium avium-M. intracellulare* within cultured human monocytes. *Infect. Immun.* **64**, 452-459.

Le Rhun, Y., Kirkland, J. B. and Shah, G. M. (1998). Cellular responses to DNA damage in the absence of Poly(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.* **245**, 1-10.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S. and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489.

Liles, W. C. (1997). Apoptosis – role in infection and inflammation. *Curr. Opin. Infect. Dis.* **10**, 165-170.

Lin, E. Y., Orlofsky, A., Berger, M. S. and Prystowsky, M. B. (1993). Characterization of A1, a novel-hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J. Immunol.* **151**, 1979-1988.

Manna, S. K. and Aggarwal, B. B. (1999). Lipopolysaccharide inhibits TNF-induced apoptosis: role of Nuclear factor-κB activation and reactive oxygen intermediates. *J. Immunol.* **162**, 1510-1518.

Martin, S. J., Lennon, S. V., Bonham, A. M. and Cotter, T. G. (1990). Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J. Immunol.* **145**, 1859-1867.

Meisterernst, M., Stelzer, G. and Roeder, R. G. (1997). Poly(ADP-ribose) polymerase enhances activator-dependent transcription in vitro. *Proc. Natl. Acad. Sci. USA* **94**, 2261-2265.

Moore, K. J. and Matlashewski, G. (1994). Intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis. *J. Immunol.* **152**, 2930-2937.

Nash, P. B., Purner, M. B., Leon, R. P., Clarke, P., Duke, R. C. and Curiel, T. J. (1998). *Toxoplasma gondii*-infected cells are resistant to multiple inducers of apoptosis. *J. Immunol.* **160**, 1824-1830.

Nosseri, C., Coppola, S. and Ghibelli, L. (1994). Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp. Cell Res.* **212**, 367-373.

Orlofsky, A., Somogyi, R. D., Weiss, L. M. and Prystowsky, M. B. (1999). The murine antiapoptotic protein A1 is induced in inflammatory macrophages and constitutively expressed in neutrophils. *J. Immunol.* **163**, 412-419.

Pacini, A., Quattrone, A., Denegri, M., Fiorillo, C., Nediani, C., Ramon y Cajal, S. and Nassi, P. (1999). Transcriptional down-regulation of poly(ADP-ribose) polymerase gene expression by E1A binding to pRb proteins protects murine keratinocytes from radiation-induced apoptosis. *J. Biol. Chem.* **274**, 35107-35112.

Pan, G., Humke, E. W. and Dixit, V. M. (1998). Activation of caspases triggered by cytochrome c in vitro. *FEBS Lett.* **426**, 151-154.

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S. J., Krammer, P. H. and Peter, M. E. (1999). Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *J. Biol. Chem.* **274**, 22532-22538.

Shiokawa, D., Maruta, H. and Tanuma, S. (1997). Inhibitors of poly(ADP-ribose) polymerase suppress nuclear fragmentation and apoptotic-body formation during apoptosis in HL-60 cells. *FEBS Lett.* **413**, 99-103.

Simbulan-Rosenthal, C. M., Rosenthal, D. S., Iyer, S., Boulares, A. H. and Smulson, M. E. (1998). Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. *J. Biol. Chem.* **273**, 13703-13712.

Single, B., Leist, M. and Nicotera, P. (1998). Simultaneous release of adenylate kinase and cytochrome c in cell death. *Cell Death Differ.* **5**, 1001-1003.

Spender, L. C., Cannell, E. J., Hollyoake, M., Wensing, B., Gawn, J. M., Brimmell, M., Packham, G. and Farrell, P. J. (1999). Control of cell cycle entry and apoptosis in B lymphocytes infected by Epstein-Barr virus. *J. Virol.* **73**, 4678-4688.

Tanaka, Y., Yoshihara, K., Tohno, Y., Koyima, K., Kameoka, M. and Kamiya, T. (1995). Inhibition and down-regulation of poly(ADP-ribose) polymerase results in a marked resistance of HL-60 cells to various apoptosis-inducers. *Cell. Mol. Biol.* **41**, 771-781.

Tafani, M., Schneider, T. G., Pastorino, J. G. and Farber, J. L. (2000). Cytochrome c-dependent activation of caspase-3 by tumor necrosis factor requires induction of the mitochondrial permeability transition. *Am. J. Pathol.* **156**, 2122-2121.

Teodoro, J. G. and Branton, P. E. (1997). Regulation of apoptosis by viral gene products. *J. Virol.* **71**, 1739-1746.

Thornberry, N. A. and Lazebnik, Y. (1998). Caspases: Enemies within. *Science* **281**, 1312-1316.

Toure-Balde, A., Sarthou, J. L., Aribot, G., Michel, P., Trape, J. F., Rogier, C. and Roussilhon, C. (1996). *Plasmodium falciparum* induces apoptosis in human mononuclear cells. *Infect. Immun.* **64**, 744-750.

Vanags, D. M., Porn-Ares, M. I., Coppola, S., Burgess, D. H. and Orrenius, S. (1996). Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J. Biol. Chem.* **271**, 31075-31085.

Vaux, D. L. and Strasser, A. (1996). The molecular biology of apoptosis. *Proc. Natl. Acad. Sci. USA* **93**, 2239-2244.

Wang, C. Y., Mayo, M. W. and Baldwin, A. S. Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* **274**, 784-787.

Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V. and Baldwin, A. S. Jr. (1998). NF-κB antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281**, 1680-1683.

Wang, X. and Studzinski, G. P. (1997). Antiapoptotic action of 1,25-dihydroxyvitamin D3 is associated with increased mitochondrial MCL-1 and RAF-1 proteins and reduced release of cytochrome c. *Exp. Cell Res.* **235**, 210-217.

Williams, G. T. (1994). Programmed cell death: a fundamental protective response to pathogens. *Trends Microbiol.* **2**, 463-464.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I, Jones, D. P. and Wang, X. (1997). Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. *Science* **275**, 1129-1132.