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Theileria parva-Transformed T Cells Show Enhanced Resistance to Fas/Fas Ligand-Induced Apoptosis

Peter Kienzi, Pascal Schneider, and Dirk A. E. Dobbelaere

Lymphocyte homeostasis is regulated by mechanisms that control lymphocyte proliferation and apoptosis. Activation-induced cell death is mediated by the expression of death ligands and receptors, which, when triggered, activate an apoptotic cascade. Bovine T cells transformed by the intracellular parasite Theileria parva proliferate in an uncontrolled manner and undergo clonal expansion. They constitutively express the death receptor Fas and its ligand, FasL, but do not undergo apoptosis. Upon elimination of the parasite from the host cell by treatment with a theilericidal drug, cells become increasingly sensitive to Fas/FasL-induced apoptosis. In normal T cells, the sensitivity to death receptor killing is regulated by specific inhibitor proteins. We found that anti-apoptotic proteins such as cellular (c)-FLIP, which functions as a catalytically inactive form of caspase-8, and X-chromosome-linked inhibitor of apoptosis protein (IAP) as well as c-IAP, which can block downstream executioner caspases, are constitutively expressed in T. parva-transformed T cells. Expression of these proteins is rapidly down-regulated upon parasite elimination. Antiapoptotic proteins of the Bel-2 family such as Bcl-2 and Bcl-xL are also expressed but, in contrast to c-FLIP, c-IAP, and X-chromosome-linked IAP, do not appear to be tightly regulated by the presence of the parasite. Finally, we show that, in contrast to the situation in tumor cells, the phosphoinositide 3-kinase/Akt pathway is not essential for c-FLIP expression. Our findings indicate that by inducing the expression of antiapoptotic proteins, T. parva allows the host cell to escape destruction by homeostatic mechanisms that would normally be activated to limit the continuous expansion of a T cell population. The Journal of Immunology, 2003, 171: 1224–1231.

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4 Abbreviations used in this paper: P13-K, phosphoinositide 3-kinase; AICD, activation-induced cell death; FasL, Fas ligand; IAP, inhibitor of apoptosis protein; c-, cellular; rhs, recombinant human soluble; PARP, poly(ADP-ribose) polymerase; VAD-fmk, valylalanylaspartic acid fluoromethyl ketone; XIAP, X-chromosome-linked IAP; ACAD, activated T cell autonomous death.
bind to and inhibit caspases such as caspase-3, -6, -7, or -9. More recently, a family of inhibitors called FLIP (FLICE-inhibitory proteins) has been described that blocks caspase-8 activation at the level of the death receptor (27, 28). Whereas T cells are initially resistant to Fas/FasL-induced apoptosis, with time they become increasingly sensitive, and it has been shown that reduced resistance to apoptosis is paralleled by gradually waning levels of FLIP (reviewed in Ref. 27).

Continuous proliferation and survival of the T. parva-infected T cell is dependent on the presence of the parasite in the host cell cytoplasm. Addition of the naphthoquinone derivative BW720c to cultures of T. parva-transformed cells specifically kills the parasite (29). BW720c does not affect the expression of housekeeping genes (5, 30, 31), shows no toxicity for the host cell, and does not interfere with the capacity of T cells to become activated and proliferate in response to mitogens (32). When the parasite is eliminated, cells lose their activated phenotype, cease to proliferate after 3–4 days, and finally undergo apoptosis (10, 33). Despite the fact that T. parva-infected T cells show many characteristics of activated T cells (reviewed in Ref. 1) (30), they apparently escape AICD. To what extent molecular pathways that govern AICD are activated in T. parva-infected T cells and how parasitized cells acquire protection against this powerful homeostatic process have not yet been investigated. In the present study, we explored the reversibility of Thelliera-induced transformation to tackle these questions. We investigated Fas and FasL expression and compared the sensitivity of T. parva-transformed T cells to Fas/FasL-mediated apoptosis with that of BW720c-cured cells. In addition, we monitored the potential involvement of members of the different antiapoptotic protein families.

Materials and Methods

Cell culture

TpM(803) T cells are CD4+ CD8+ αβ TCR+ T cells of bovine origin, transformed by T. parva; the characteristics of this cloned cell line have been described elsewhere (33). Cells were cultured at 37°C in Leibovitz (L-15 medium (Life Technologies, Paisley, U.K.) containing 10% (v/v) heat-inactivated FCS, 20 mM HEPES (pH 7.1), 2 mM t-glutamine, and 1× antibiotic/antimycotic solution (Life Technologies). To eliminate the parasite, BW720c (Bugarvaquone; Pitman-Moore, Mundelein, IL) was added at 50 ng/ml to the culture medium.

Ligands and Abs

Trimerized recombinant FasL (SuperFasL) was purchased from Apotech (Epalinges, Switzerland). The recombinant human soluble FasL fused to a linker peptide (26 aa) and a FLAG tag at the N terminus (rhsFasL; Alexis, San Diego, CA) was used together with an enhancer Ab for multimerization (M2 mouse mAb anti-FLAG; Sigma-Aldrich, Buchs, Switzerland). The mAb directed against FasL was purchased from Transduction Laboratories (BD Biosciences, Heidelberg, Germany). mAbs raised against human caspase-8 (AM46) and caspase-9 (AM47) were obtained from Calbiochem-Novabiochem (San Diego, CA). Ab specific for Fas (sc7151), Bcl-x(L/S) (sc1041), Bax (sc493), and cellular (c-) IAP-1 (sc7943) and mAb for caspase-2 (sc7382), which detect the corresponding proteins in multiple samples such as mouse, rat, and human, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An Ab directed against human FLIP (1-Flice; 67071N) was obtained from BD Pharmingen (Heidelberg, Germany). A mAb directed against human XIAP (M044-3) was purchased from Biogenex (Sunnyvale, CA) diluted 1/50 or affinity-purified Alexa-Fluor 488 goat anti-mouse IgG (H + L); Molecular Probes, Leiden, The Netherlands) diluted 1/1500 in PBS containing 10% heat-inactivated FCS. Nuclei were stained with Hoechst 33258 dye (diluted 1/2000 in PBS) for 1 min.

Results

Caspases are activated in T cells cured of the parasite

It has previously been reported that the proliferation of T. parva-infected T cells ceases when the parasite is removed from the host cell cytoplasm by treatment with BW720c and that cells displaying the morphological features of apoptosis appear after ~4 days in cultures of BW720c-cured cells (10, 33). We first monitored whether caspases become activated upon killing of the parasite. The caspase inhibitor VAD-fmk binds specifically to caspases that have become activated by proteolytic cleavage. Flow cytometric analysis using FITC-labeled VAD-fmk showed that activated caspases can be detected in a distinct population of T cells treated with BW720c for 3 days (Fig. 1A).

Caspases are normally present in the cell as proenzymes that are processed into activated fragments in response to an apoptotic stimulus. To investigate the processing of procaspase-8 and -9, immunoblot analysis was made of protein extracts prepared from preincubated in the absence or presence of recombinant Fc-FasL, followed by incubation with 1 μg of rhFasL, in a total volume of 25 μl of PBS, containing 10% heat-inactivated FCS and 0.02% sodium azide. FasL binding was detected by incubation with the anti-FLAG mAb M2 (0.16 mg/ml) and a FITC-conjugated anti-mouse IgG (diluted 1/25; Jackson Immunoresearch Laboratories, West Grove, PA). Staining with the combination Alexa Fluor V-488 (Boehringer Mannheim, Mannheim, Germany) and propidium iodide was performed according to the manufacturer’s instructions. All cells were analyzed in PBS containing 0.02% sodium azide using a FACScan (BD Biosciences). Alternatively, cells were seeded in 96-well microtiter plates and treated as described above. FITC-VAD-fmk (final concentration 5 μM) Promega) was added directly to the cell suspension (4 × 10^6 cells/ml), and cells were incubated at 37°C for 20 min, washed with PBS, and analyzed in PBS containing 0.02% sodium azide using a FACScan (BD Biosciences).
**FIGURE 1.** Caspase activity can be detected in T cells upon elimination of the parasite. A, T. parva-transformed cells were cultured for 3 days in the absence or presence of the theilericidal drug BW720c. To monitor caspase activity, cells were incubated with FITC-VAD-fmk for 20 min, fixed in paraformaldehyde and analyzed by flow cytometry. Shaded profile represents T. parva-infected cells; open profile represents BW720c-cured cells. B, Immunoblot analysis of protein extracts prepared from T. parva-transformed T cells and cured T cells cultured in the presence of BW720c for up to 3 days. Caspase-8 and -9 activation is reflected by the proteolytic processing of the respective proenzymes (indicated as Pro-Casp 8 and 9). The size of the cleavage products is indicated. FL1-H, Fluorescence.

**FIGURE 2.** Fas and FasL are expressed by T. parva-transformed (tr.) T cells and BW720c-cured T cells. A, Immunoblot analysis of T. parva-transformed T cells (labeled 0) and cells from which the parasite was eliminated by BW720c treatment for 1, 2, or 3 days (d). Protein extracts were analyzed by immunoblot analysis, using an Ab directed against Fas. B, Flow cytometric analysis of Fas expression. T. parva-transformed T cells and T cells cured of the parasite by treatment with BW720c for 3 days were first incubated with FLAG-tagged rhsFasL, followed by M2-anti-FLAG mAb and FITC-labeled anti-mouse IgG. Binding was monitored by flow cytometry. The dotted line shows the specific binding of rhsFasL to T. parva-transformed cells; the gray profile represents background fluorescence in the absence of rhsFasL. As control, cells were incubated with a 10-fold excess of Fc-FasL before the addition of FLAG-tagged rhsFasL; this blocked the available binding sites for Flag-tagged rhsFasL (black line), thus confirming the specificity. FL1-H, Fluorescence. C, Immunofluorescence microscopy of T. parva-transformed T cells and cells from which the parasite was eliminated by BW720c treatment for up to 3 days. Cells were stained using an Ab directed against FasL (white). Nuclei were visualized by counterstaining with 4,6-diamidino-2-phenylindole (Hoechst 33258; gray).
parva-transformed T cells exposed to SuperFasL for 16 h expressed phosphatidylserine, a marker for apoptosis, on their surface, and cells displayed all the classical morphological features of apoptosis (not shown). These findings suggest that the expression of Fas and FasL alone does not suffice to induce apoptosis in T. parva-transformed T cells, whereas a strong trigger, delivered by the artificial oligomerization of Fas, can initiate cell death.

T cells cured of the parasite become increasingly sensitive to Fas-induced apoptosis

We next investigated whether cells from which the parasite has been eliminated differ from T. parva-infected cells in their sensitivity to Fas-mediated apoptosis. For this purpose, T. parva-infected T cells and cells cured by treatment with BW720c for 3 days were incubated with increasing doses of soluble, epitope-tagged FasL, which binds to Fas without activating it. Fas oligomerization and activation were triggered by Ab-mediated cross-linking of epitope-tagged FasL, and caspase activation was monitored by flow cytometry 4 h after induction. The results show that elimination of the parasite leads to a pronounced increase in sensitivity to Fas-mediated caspase activation (Fig. 3). Additional experiments were also conducted to determine how soon after parasite elimination the cells became sensitive to Fas/FasL-induced apoptosis. In cells treated with BW720c for only 1 day, Fas-dependent caspase activation could be detected within 6 h after induction (Fig. 4A). In cells treated for 3 days, a robust induction of caspase activity could be measured within 3 h of Fas triggering. Under these conditions, T. parva-transformed T cells did not respond to Fas stimulation.

To further demonstrate the enhanced sensitivity of cured cells to Fas-mediated apoptosis, we subjected cells that had been treated with BW720c for 4 days to Fas stimulation and monitored the specific cleavage of PARP, which occurs as one of the last steps in the apoptotic cascade. Using Abs that specifically detect p85, a cleavage product of PARP, advanced apoptosis could be demonstrated in cured cells within 2 h of Fas triggering, whereas only little PARP activation could be detected in T. parva-transformed cells (Fig. 4B). Although BW720c is strictly parasite specific, we also tested its effects on a control transformed T cell line. Neither the proliferation rate nor the sensitivity of Jurkat T cells to Fas-induced apoptosis was affected by culturing the cells for 3 days in the presence of BW720c (data not shown). Taken together, these findings indicate that although Fas and Fasl, are expressed on T. parva-infected T cells, they display a high level of resistance against Fas-mediated apoptosis relative to cured cells and that protection appears to be tightly associated with the presence of the parasite in the host cell cytoplasm.

c-FLIP, c-IAP, and XIAP are expressed in a parasite-dependent manner in T. parva-transformed T cells

Fas stimulation involves the activation of the initiator caspase-8 and the subsequent activation of effector/executioner caspases such as caspase-3, -6, and -7. Caspase-8 activity is inhibited by FLIP and effector/executioner caspases can be blocked by the binding of IAPs such as c-IAP1/2 and XIAP. In primary T cells, high levels of FLIP are transiently expressed upon activation; down-regulation of FLIP expression coincides with the onset of sensitivity to Fas/FasL-mediated apoptosis. We examined whether FLIP and other IAP proteins are expressed in T. parva-infected T cells. Immunoblot analysis shows that c-FLIP and XIAP are expressed in T. parva-infected cells (Fig. 5A). Levels of both proteins start to decrease, however, within 1 day of BW720c treatment; after 3 days, no protein could be detected. Importantly, in transformed Jurkat T-cells, treatment for 3 days with BW720c did not affect the steady state levels of c-FLIP and X-IAP expression (Fig. 5A, lower panel), indicating that the drug per se does not block the expression of these antia apoptotic proteins. Together, our findings strongly indicate that in T. parva-transformed T-cells expression of antia apoptotic proteins is tightly linked to the parasitized state of the cell.

When analyzed by immunofluorescence microscopy, cytoplasmic c-IAP expression could readily be demonstrated in T. parva-infected cells; whereas only weak or no signals could be detected in BW720c-cured cells (Fig. 5B). The low level of c-IAP expression that can be detected in control Jurkat T-cells, in contrast, was not affected by culturing the cells for 3 days in the presence of BW720c (data not shown). Bcl-2 family members regulate apoptosis induced through the mitochondrial pathway. Interestingly, in cured cells, members of the Bcl-2 family are still expressed at similar levels as in T. parva-transformed T cells, independently of whether they belong to the prosurvival (e.g., Bcl-2 and Bcl-xL) or proapoptotic (e.g., Bax and Bcl-xS) family of Bcl-2 proteins.

Finally, it has recently been reported that the PI3-K/Akt pathway regulates c-FLIP expression in tumor cells (35). T. parva-transformed T cells show many properties of tumor cells (1), and we have demonstrated that the PI3-K/Akt pathway is constitutively activated (6). We therefore tested whether blocking PI3-K by treatment of the cells with the inhibitor LY294002 could down-regulate c-FLIP expression, as has been shown to be the case in tumor cells (35). Fig. 5C shows that treatment with LY294002 for up to 3 days did not adversely affect the expression of c-FLIP, c-IAP, or XIAP. On the contrary, treatment with LY294002 resulted in a clear increase in the levels of c-IAP and also in a modest increase in XIAP. Levels of procaspase-8 remained constant, indicating that

**FIGURE 3.** Increased sensitivity to Fas-induced apoptosis in BW720c-cured cells (BW). Upon elimination of the parasite, T cells become more sensitive to Fas-induced apoptosis. T. parva-transformed (Tp-transf.) T cells (gray profile) and cells cured of the parasite by treatment with BW720c for 3 days (open profile) were incubated with increasing doses of FLAG-tagged recombinant soluble Fasl. Fas activation was induced by cross-linking Fasl using an anti-FLAG mAb. Caspase activation was monitored 4 h after induction, using FITC-VAD-fmk and flow cytometry. The percent of T. parva-transformed cells or BW720c-cured cells that were positive for FITC-VAD-Fmk are indicated on the right. FL1-H, Fluorescence.
caspase-8 does not become activated. Taken together, these data demonstrate that c-FLIP, which interferes with apoptotic signaling through death receptors, and c-IAP or XIAP, which block the activation of downstream effector/executioner caspases, are all up-regulated in a parasite-dependent manner in _T. parva_-transformed T cells.

**Discussion**

T cells transformed by the intracellular parasite _T. parva_ express both the death receptor Fas and its ligand, a combination that is potentially lethal to the cell. Parasite-transformed cells appear to be protected, however, through the concomitant expression of several antiapoptotic proteins known to block apoptotic signaling at the level of initiator as well as effector/executioner caspases. This way, parasite-transformed T cells maintain a delicate balance between cell death and cellular proliferation.

When the parasite is eliminated from the host cell cytoplasm, expression of antiapoptotic proteins is down-regulated and cured cells become increasingly sensitive to Fas-mediated apoptosis. The protection of parasitized cells appears to depend on a threshold mechanism and is not absolute. This is reflected by the fact that crossing the threshold level of protection by artificially over-stimulating Fas with SuperFasL results in apoptosis of _T. parva_-infected T cells.

In previous studies, we have shown that the transcription factor NF-κB is constitutively activated in a parasite-dependent manner (3, 4). NF-κB plays a dual, apparently contradictory, role in the regulation of apoptosis (reviewed in Refs. 36 and 37). Its antiapoptotic function is well documented and has been linked to the fact that the expression of several antiapoptotic proteins, including c-FLIP (38–40), c-IAP1/2 (41–44), and XIAP (45, 46), is regulated, at least in part, by this transcription factor. In contrast, NF-κB also appears to be involved in regulating the expression of both the _fas_ (17, 18) and _fasl_ (19–21) genes, thus potentially priming the T cell for AICD. The complex role of NF-κB in apoptotic regulation is further highlighted by the finding that c-FLIP, XIAP, and c-IAP can themselves, in turn, contribute to the regulation of NF-κB (47–49), potentially providing regulatory feedback loops. To what extent such feedback loops participate in regulating NF-κB in _T. parva_-infected cells is not known, but the fact that
NF-κB is activated in a constitutive rather than transient manner may be a critical factor in guaranteeing protection against Fas-mediated apoptosis. Increased resistance to Fas-induced apoptosis might also contribute to the pathogenesis of East Coast fever. Theileria-transformed cells have been shown to induce a pronounced autologous mixed lymphocyte reaction in vitro (50, 51), a process that, in vivo, is thought to contribute to the accumulation of large numbers of uninfected lymphoblastoid cells in the animal’s lymphoid tissues (52, 53). As it can be expected that Fas and FasL are up-regulated as a consequence of this activation, enhanced resistance against Fas-induced apoptosis might impart a selective advantage to parasitized cells over uninfected cells, thus contributing to the clonal expansion and spread of the infected cells through the body. East Coast fever is characterized by an initial phase of lymphoproliferation, which is usually followed by massive lympholysis involving uninfected as well as T. parva-infected cells (53). At this stage, the molecular basis for this lytic response is not known, but it will be of interest to determine to what extent Fas/FasL expression plays a role in this process.

Recent reports point toward an important role for c-FLIP in the control of AICD (54). It is thought that upon antigenic stimulation of primary T cells, c-FLIP is first up-regulated and then down-regulated. Down-regulation requires IL-2, which may explain the role of IL-2 in sensitizing T cells to cell death (55, 56). A survey of different cell lines has shown that T. parva-transformed cells express only low levels of (31) or no (57, 58) IL-2. This might be explained by the fact that signaling through the TCR does not occur in T. parva-transformed T cells (2). It could be argued that suppressed levels of IL-2 would help protect T. parva-transformed T cells against AICD. In contrast, it has been shown that long term propagation of T. parva-infected T cells in the presence of IL-2 enhances proliferation without inducing cell death (59).

Several lines of evidence link FLIP to tumor progression. FLIP expression correlates with resistance to Fas-induced apoptosis in cell lines derived from B cell lymphomas and is also linked to the escape of tumors from T cell immunity (see review in Ref. 27 and references therein). Our findings indicate that this might also apply to T. parva-transformed T cells. Despite the many similarities, differences between established tumor cell lines and T. parva-infected cells do exist. Studies on a number of tumor cell lines have shown that c-FLIP expression is dependent on the activity of the PI3-K/Akt pathway (35) and is down-regulated by inhibitors of PI3-K. We and others (6, 7) have recently demonstrated that the PI3-K/Akt pathway is activated in T. parva-transformed T cells. Culturing cells in the presence of the PI3-K inhibitor LY294002 resulted in a rapid arrest of proliferation rather than apoptosis (6, 60). Furthermore, in contrast to observations made in tumor cells (35), we observed that LY294002 treatment over a period of 3 days did not result in down-regulation of c-FLIP, c-IAP, or XIAP expression. Instead, a modest increase in the protein levels of c-FLIP did not result in down-regulation of c-FLIP, c-IAP, or XIAP expression. Instead, a modest increase in the protein levels of c-FLIP did not result in down-regulation of c-FLIP, c-IAP, or XIAP expression. Instead, a modest increase in the protein levels of c-FLIP did not result in down-regulation of c-FLIP, c-IAP, or XIAP expression. Instead, a modest increase in the protein levels of c-FLIP did not result in down-regulation of c-FLIP, c-IAP, or XIAP expression. Instead, a modest increase in the protein levels of c-FLIP did not result in down-regulation of c-FLIP, c-IAP, or XIAP expression.

Upon elimination of the parasite, c-FLIP, c-IAP, and XIAP were down-regulated within 1–2 days of BW720c treatment, whereas the expression of Fas and FasL remained constant for at least 3 days. The down-regulation correlated with the appearance of activated caspases. The rapid disappearance of c-FLIP might point toward a short half-life of the protein. Moreover, it has been shown that c-FLIP mRNA is unstable, given that inhibitors that act either on transcription or translation rapidly abolish c-FLIP expression (61).

In addition to Fas, AICD can also be triggered through TNF receptors. We have found in binding studies that receptors for TNF-α are expressed on the surface of T. parva-infected cells (P. Kuenzi, unpublished observation). These receptors are functional because they are capable of enhancing IκB-α degradation upon stimulation with recombinant TNF. As has been demonstrated before (62), however, TNF-receptor triggering did not induce apoptosis and a logical explanation would be that parasite-dependent up-regulation of antiapoptotic proteins also provides protection against TNF-induced apoptosis.

Whereas c-FLIP, c-IAP, and XIAP were down-regulated within 1–2 days of BW720c treatment, levels of Bcl-2 family proteins remained constant, even after 3 days of treatment. Several observations support the notion that in activated T cells Bcl-2 controls a death pathway that is distinct from that induced by Fas (63, 64) because Bcl-2 overexpression does not protect activated T cells from Fas-driven death. Bcl-2 protects T cells from activated T cell autonomous death (ACAD), a form of T cell death in response to activation by foreign Ags, which is distinguishable from that driven by death receptors (see review in Ref. 13). ACAD is typically accompanied by a pronounced decrease in Bcl-2 levels. The fact that Bcl-2 is not down-regulated indicates that ACAD is probably not involved in the apoptotic death of BW720c-cured cells.

In addition to ACAD, Bcl-2 also counters other apoptotic pathways. Effector caspases can be induced by both caspase-8 and -9. Caspase-9 is predominantly triggered through the mitochondrial pathway, which is induced by cellular stress and lymphokine/cytokine withdrawal. This process involves the activity of proapoptotic BH3-only proteins (65) which, in turn, require the presence of proapoptotic Bax and Bak to exert their proapoptotic function (66). Antiapoptotic Bcl-2 family members protect cells against apoptosis by antagonizing proapoptotic Bcl-2 proteins. Thus, whereas c-FLIP may protect T. parva-transformed T cells against Fas and TNF-R-mediated apoptosis, Bcl-2 may contribute to protection against ACAD, lymphokine/cytokine withdrawal, and other forms of cellular stress.

Viruses have evolved a range of efficient strategies to avoid destruction by delaying death of the infected cell (67). In fact, the search for apoptosis-regulatory proteins first led to the discovery of v-FLIP in γ-herpesviruses, which in turn led to the identification of c-FLIP (27). There is increasing evidence that, at one stage in evolution, intracellular eukaryotic parasites joined the ranks of pathogens that developed strategies to prevent death of the cell they seek refuge in (68, 69). Our findings indicate that the parasite T. parva also engages apoptosis-regulating proteins such as FLIP, IAP, Bcl-2, and Bcl-xL to mediate resistance against apoptosis. In contrast to viruses that directly appropriate host cell genes, this is achieved indirectly by selectively scavenging those host cell signaling pathways such as NF-κB that control the expression of these genes.

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References

T. parva-INFECTED T CELLS RESIST Fas-INDUCED APOPTOSIS


