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# *Toxoplasma gondii* inhibits granzyme B-mediated apoptosis by the inhibition of granzyme B function in host cells

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ABSTRACT

Host defense to the apicomplexan parasite *Toxoplasma gondii* is critically dependent on $CD8^+$ T cells, whose effector functions include the induction of apoptosis in target cells following the secretion of granzyme proteases. Here we demonstrate that *T. gondii* induces resistance of host cells to apoptosis induced by recombinant granzyme B. Granzyme B induction of caspase-independent cytochrome c release was blocked in *T. gondii*-infected cells. Prevention of apoptosis could not be attributed to altered expression of the Bcl-2 family of apoptotic regulatory proteins, but was instead associated with reduced granzyme B-mediated, caspase-independent cleavage of procaspase 3 to the p20 form in *T. gondii*-infected cells, as well as reduced granzyme B-mediated cleavage of the artificial granzyme B substrate, GranToxiLux. The reduction in granzyme B proteolytic function in *T. gondii*-infected cells could not be attributed to altered granzyme B uptake or reduced trafficking of granzyme B to the cytosol, implying a *T. gondii*-mediated inhibition of granzyme B activity. Apoptosis and GranToxiLux cleavage were similarly inhibited in *T. gondii*-infected cells exposed to the natural killer-like cell line YT-1. The endogenous granzyme B inhibitor PI-9 was not up-regulated in infected cells. We believe these findings represent the first demonstration of granzyme B inhibition by a cellular pathogen and indicate a new modality for host cell protection by *T. gondii* that may contribute to parasite immune evasion.

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## 1. Introduction

*Toxoplasma gondii* is a ubiquitous apicomplexan parasite that infects an estimated one-third of the global population (Montoya and Liesenfeld, 2004; Kim and Weiss, 2008). In acute stages of infection, the parasite expands via the rapid proliferation of tachyzoite forms. Immunocompetent hosts can mount a T cell-mediated defense that limits this expansion, allowing the differentiation of tachyzoites to slower-growing bradyzoites which form intracellular cysts that persist for the life of the host. While infections are normally asymptomatic, immune deficiency of the host can result in reactivated disease in which latent bradyzoites transform to proliferating tachyzoites (Joynson and Wreghitt, 2001), implying that continuous T cell surveillance is required to limit tachyzoite emergence in encysted tissues. Indeed, studies of chronically infected brain have revealed the presence of persistent $CD8^+$ T cells recognising parasite-encoded antigen (Schluter et al., 2002; Lutjen et al., 2006), and have shown that whilst these antigen-specific cells do not associate with cysts, they cluster in the vicinity of isolated parasites that may be derived from cyst rupture (Schaeffer et al., 2009).

$CD8^+$ T cells, as well as natural killer (NK) cells, contribute to host defense against intracellular pathogens in large part via the induction of cell death in infected target cells. This function is accomplished primarily via the release of cytotoxic granule contents including perforin, which disrupts the membrane of target cells and granzymes, a family of death-inducing serine proteases that enter target cells in a perforin-dependent manner and are essential for the optimal function of cytotoxic lymphocytes in vivo (Bolitho et al., 2007; Chowdhury and Lieberman, 2008). Cytotoxic cells can also induce death by the activation of death receptors such as Fas on target cells. *Toxoplasma gondii*-infected cells have been shown to be resistant to apoptosis mediated by Fas (Vutova et al., 2007; Hippe et al., 2008), as well as by irradiation and various chemical inducers (Sinai et al., 2004; Carmen et al., 2006; Kim and Denkers, 2006). However, it remains undetermined whether the prevention of host cell apoptosis is of physiological significance in the pathogenesis of toxoplasmosis. Since $CD8^+$ cells play a vital role both in the control of acute *T. gondii* infection and in the maintenance of latency (Suzuki and Remington, 1988; Brown and Mcleod, 1990; Suzuki and Remington, 1990; Parker et al., 1991; Gazzinelli et al., 1992; Khan et al., 1999), we decided to examine the effect of the parasite on granzyme-induced apoptosis.

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The role of the perforin/granzyme pathway in toxoplasmosis is still uncertain. In the acute phase of *T. gondii* infection, successful host defense does not require perforin (Khan et al. 2006; Denkers et al., 1997), although the perforin-mediated cytotoxic action of NK cells appears to be a significant process at this stage (Persson et al., 2009). In chronically infected mice of the susceptible C57BL/6 strain, which fail to maintain latency and eventually succumb to toxoplasmic encephalitis, the absence of perforin increases brain cyst burden and accelerates mortality (Denkers et al., 1997). In contrast, in resistant BALB/c mice, perforin-deficient T cells are able to maintain latency, although this may be due to a compensatory up-regulation of IFN-$\gamma$ production (Wang et al., 2004). A recent study demonstrated that chronically infected BALB/c mice in fact contain CD8$^+$ T cells that are able to clear established cysts from the brain in a perforin-dependent manner (Suzuki et al., 2010), providing a potential explanation for the earlier observation of a perforin role in chronically infected C57BL/6 mice (Denkers et al., 1997).

These studies do not clarify the ability of the perforin/granzyme pathway to mediate host defense against tachyzoite-infected cells, either in acute toxoplasmosis or in recrudescent infection following cyst rupture. *Toxoplasma gondii*-infected animals generate cytotoxic T cells (CTLs) that recognise and kill tachyzoite-infected targets in vitro (Subauste et al., 1991). However, it is not clear whether this cell death represents granzyme-mediated apoptosis or a necrotic or egress response to high local concentrations of perforin, which can result in granzyme-independent cytotoxicity (Waterhouse et al., 2006b). CD8$^+$ T cells can trigger *T. gondii* egress in vitro via perforin in the absence of caspase function, suggesting that this egress is independent of granzyme-induced apoptosis (Persson et al., 2007). In addition, NK cell-derived perforin may elicit egress in vivo (Persson et al., 2009). These findings may account for an earlier observation that treatment of infected cells with CTLs results in cell lysis without the formation of apoptotic DNA fragments (Nash et al., 1998). Since perforin-mediated immune function displays granzyme-dependence in vivo (Chowdhury and Lieberman, 2008), observations of granzyme-independent perforin action in vitro may not be relevant to host defense to *T. gondii*. The question of whether, in the absence of perforin-elicited lysis or egress, *T. gondii* can modulate granzyme-dependent cell death is still unanswered.

Granzyme B (GrB) is the most extensively characterised member of the granzyme family. GrB possesses caspase-like proteolytic activity and shares multiple substrates with caspases, including lamin B, tubulin and poly ADP-ribose polymerase. Human GrB, unlike the mouse enzyme, cleaves additional caspase substrates, including inhibitor of caspase-activated DNase and the BH3 protein Bid (Chowdhury and Lieberman, 2008). The Bid cleavage product, tBid, induces oligomerisation of the pro-apoptotic Bcl-2 family members members Bax/Bak and consequent activation of the mitochondrial apoptosis pathway (Lalier et al., 2007). While some studies have shown a dependence of GrB-mediated apoptosis on Bid (Sutton et al., 2000), in other settings GrB can activate the mitochondrial apoptotic pathway in a Bid-independent, Bcl-2-insensitive manner (Goping et al., 2008). Furthermore, GrB can also directly cleave caspase 3 to a p20 form, which can then generate the active p17 form by autocatalysis, most likely as a result of mitochondrial release of Smac/Diablo and consequent relief of IAP-mediated caspase inhibition (Goping et al., 2003). This direct contribution of GrB to caspase 3 activation may account for the independence of GrB-induced apoptosis from caspase 9 (Pardo et al., 2008), which is normally required for apoptosome formation and caspase 3 activation in mitochondria-mediated apoptosis. Finally, while certain apoptotic manifestations in target cells are caspase-dependent, GrB can induce target cell death in a caspase-independent manner (Trapani et al., 1998).

*Toxoplasma gondii* can protect host mitochondria from apoptotic insults (Sinai et al., 2004; Carmen et al., 2006; Hippe et al., 2008). A recent study has shown that the parasite can prevent Bax/Bak activation downstream of BH3 protein signalling (Hippe et al., 2009). It is unclear whether this protective function of the parasite would suffice to counter the multiple apoptotic mechanisms triggered by GrB. We have found that *T. gondii* can protect host cells from GrB-induced apoptosis, and that, remarkably, this protection involves the abrogation of GrB activity in infected cells.

## 2. Materials and methods

### 2.1. Cell lines and parasites

YT-1 cells (a kind gift from Dr. Z. Nagy, University of Texas El Paso, USA) and the T-leukaemic cell line Jurkat were maintained in RPMI medium supplemented with 10% FCS. HeLa cells were maintained in DMEM supplemented with 10% FCS (HyClone). The RH strain of *T. gondii* was maintained in human foreskin fibroblasts. Some experiments employed strains, derived from RH, that express yellow fluorescent protein (YFP) (a kind gift of B. Striepen, University of Georgia, USA) (Gubbels et al., 2003) or mCherry (a kind gift of M.-J. Gubbels, Boston University, USA).

### 2.2. Recombinant GrB

GrB was prepared from yeast because commercially available GrB derived from bacteria is not glycosylated and is not taken up efficiently by mammalian cells (Giesubel et al., 2006). The plasmid pPIC9-GrB, consisting of the yeast expression vector pPIC9 (Invitrogen) inserted with mature human GrB was a kind gift of Dr. W. Wels (Chemotherapeutisches Forschungsinstitut, Frankfurt am Main, Germany) and was used to prepare recombinant GrB as previously described (Giesubel et al., 2006). Briefly, the yeast *Pichia pastoris*, strain GS115 (Invitrogen), was transformed with the plasmid and positive clones were selected as per the manufacturer's protocol. GrB-expressing clones were verified by Western blot using the monoclonal antibody 2C5 (Santa Cruz). High-expression clones were grown in medium containing 2% methanol to induce expression of GrB. After 5 days, the cells were centrifuged at 7500 *g* and the supernatants were passed through a nickel column (GE Healthcare). GrB was eluted using 250 mM imidazole, pH 8.0. Purity was verified by Coomassie Blue staining and immunoblot, which identified a unique 37 kD species (Supplementary Fig. S1). GrB was dialysed against PBS, pH 7.4 and stored at $-80$ °C until use. Activity was assessed in colorimetric assays containing 200 μM of the synthetic GrB substrate N-acetyl-Ile-Glu-Thr-Asp-nitroaniline in reaction buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) in a total volume of 100 μl per sample. Substrate cleavage was determined at an absorbance of 490 nm. The activity was comparable with that of a commercially available GrB prepared from *Escherichia coli* (Supplementary Fig. S2).

### 2.3. Delivery of recombinant GrB

Target cells were treated with 60 nM of GrB in the presence of sublytic doses of the endosomolytic agents listeriolysin O (LLO) (ProSpec, USA) or streptolysin O (SLO) (Sigma). Sublytic doses were determined in pilot experiments and were defined as doses resulting in less than 10% cell lysis as measured by flow cytometry after staining with propidium iodide (PI). Alternatively, Influx Pinocytic loading reagent (Molecular Probes, USA) was used according to the supplier's instructions to direct GrB to the cytosolic compartment by osmotic lysis of pinosomes.

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