The importance of apoptosis for immune regulation in Chagas disease

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Host cell apoptosis plays an important immune regulatory role in parasitic infections. Infection of mice with Trypanosoma cruzi*, the causative agent of Chagas disease, induces lymphocyte apoptosis. In addition, phagocytosis of apoptotic cells stimulates the growth of* T. cruzi *inside host macrophages. In spite of progress made in this area, the importance of apoptosis in the pathogenesis of Chagas disease remains unclear. Here we review the evidence of apoptosis in mice and humans infected with* T. cruzi*. We also discuss the mechanisms by which apoptosis can influence underlying host responses and tissue damage during Chagas disease progression.*

Key words: apoptosis - lymphocytes - phagocytosis - cytokines - *Trypanosoma cruzi*

Programmed cell death by apoptosis is a biological response relevant for development, tissue renewal and homeostasis of the immune system. Defects in the regulation of apoptosis can lead to disease (Rudin & Thompson 1997). Apoptosis can also be induced in unicellular parasites, including *Trypanosoma cruzi* (Ameisen et al. 1995, Piacenza et al. 2001). Apoptosis of a proportion of *Leishmania* parasites is required for successful establishment of infection in the vertebrate host (van Zandbergen et al. 2006). In addition, infective forms of *Leishmania spp.* and *T. cruzi* display extracellular phosphatidylserine, a marker of apoptotic cells that is involved in host macrophage inactivation and increased parasite replication (de Freitas Balanco 2001, Damatta et al. 2007). Upon infection with parasites, host leukocyte apoptosis plays an important immune regulatory role (DosReis et al. 2007). In spite of progress made in this area, the importance of apoptosis for the pathogenesis of Chagas disease remains unclear. Here we review the evidence of apoptosis in mice and humans infected with *T. cruzi*. We also discuss the mechanisms by which apoptosis can influence host responses and tissue damage underlying Chagas disease.

Host cell apoptosis in *T. cruzi* infection and the immunosuppressive effect of apoptotic cells

Infection of fibroblasts and cardiomyocytes with *T. cruzi* either delays or induces host cell apoptosis, depending on the experimental system employed (de Souza et al. 2003, Petersen et al. 2006). In addition, infection with *T. cruzi* leads to T and B- lymphocyte apoptosis (Lopes et al. 1995a, Martins et al. 1998, Zuniga et al. 2002, de Meis et al. 2006), which can impact immune responses.

To investigate apoptosis, we infect mice with chemically induced metacyclic trypomastigotes. By all tested parameters, this model is identical to infection induced by insect-derived trypomastigotes (Lopes et al. 1995a). Restimulation of $CD4^+$ T cells from mice infected with *T. cruzi* results in in vitro formation of large cell clusters where most lymphocytes die. The cells that are excluded from the clusters remain viable, suggesting a fratricide mechanism of activation-induced cell death (AICD). Cell death occurs by apoptosis, as demonstrated by transmission electron microscopy and by nucleosome sized DNA fragmentation (Lopes et al. 1995b). Data gathered from independent experiments reveal a linear correlation between the extent of suppression of T-cell responses and the amplitude of AICD (Lopes & DosReis 1996). These results suggest that the immune suppression underlying acute infection with *T. cruzi* (Harel-Bellan et al. 1983) can be ascribed to AICD. The Fas apoptotic pathway plays an important role in the regulation of immune responses (Krammer et al. 2007). AICD in *T. cruzi* infection is mediated, at least in part, by the Fas pathway, since it is blocked by neutralizing anti-Fas ligand (FasL) antibodies and is absent in FasL deficient *gld* mice (Lopes et al. 1999). Studies employing more virulent isolates suggest that suppression of T-cell responses can also result from apoptosis induced by excess nitric oxide production (Martins et al. 1998).

Infection with *T. cruzi* leads to polyclonal lymphocyte activation (Minoprio et al. 1989), which, by itself, promotes T-cell apoptosis (Welsh & McNally 1999). In addition, antigens released by *T. cruzi,* such as *trans*-sialidase and HSP70, induce lymphocyte apoptosis (Leguizamón et al. 1999, Marañón et al. 2000). Therefore, it is possible that the parasite exploits host cell apoptosis in order to evade the immune response. To investigate the impact of lymphocyte apoptosis on parasite replication, infected macrophages are cultured with T cells from infected mice. Apoptosis is induced in T cells and intracellular

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parasite load is assessed in macrophages (Nunes et al. 1998). Induction of $CD4^+$ T cell apoptosis by in vitro TCR co-aggregation or Fas stimulation amplifies *T. cruzi* growth, and a neutralizing anti-FasL mAb blocks both AICD and parasite growth (Nunes et al. 1998). Separation of T cells and macrophages by a cell-impermeable membrane prevents parasite growth (Nunes et al. 1998), suggesting that phagocytosis of dead cells is required for parasite survival.

Apoptosis leads to rapid and silent removal of dead cells by neighbouring cells and professional phagocytes (Ravichandran & Lorenz 2007). Dead cell clearance is followed by the immunosuppressive effects of apoptotic cells, which impair secretion of IL-12 (Voll et al. 1997) and induce secretion of TGF-β by engulfing macrophages (Fadok et al. 1998). Phagocytes express an array of innate receptors to recognize, tether and engulf apoptotic cells, most of which are directed to exposed phosphatidylserine molecules (Ravichandran & Lorenz 2007). One such receptor is the integrin $\alpha_V\beta_3$, which binds phosphatidylserine through the opsonin milk fat globule-EGF factor 8 (MFG-E8) or lactadherin, a glycoprotein secreted by macrophages (Hanayama et al. 2002). Transglutaminase 2 binds to both MFG-E8 and $\alpha_V\beta_3$ and is required to stabilize the adhesion of apoptotic cells to phagocytes (Tóth et al. 2009). In addition, the integrin $\alpha_V\beta_3$ binds thrombospondin on the surface of apoptotic cells (Savill et al. 1992). Macrophages infected with *T. cruzi* support increased parasite replication when exposed to apoptotic lymphocytes (Nunes et al. 1998, Freire-de-Lima et al. 2000). The clue for this biological effect comes from the observation that *T. cruzi* is unable to synthesize the essential polyamine putrescine and depends on the uptake of exogenous putrescine for intracellular growth (Ariyanayagam et al. 2003). Binding of apoptotic lymphocytes to $\alpha_V\beta_3$ expressed by macrophages leads to PGE_2 and TGF-β production, followed by the induction of ornithine decarboxylase (ODC) and the synthesis of putrescine, which functions as a growth factor for intracellular forms of *T. cruzi* (Freire-de-Lima et al. 2000). This deleterious effect of apoptotic cells is abolished by inhibitors of prostaglandin synthesis and by neutralizing antibodies against TGF-β (Freire-de-Lima et al. 2000).

Consequences of apoptosis for development of new therapies against *T. cruzi*

These results suggest that lymphocyte apoptosis followed by phagocytic removal helps the establishment of *T. cruzi* infection. In agreement with this finding, injection of apoptotic cells increases parasitemia in vivo. On the other hand, injection of cyclooxygenase inhibitors aspirin and indomethacin, which block production of TGF-β and polyamines, reduces parasitemia (Freire-de-Lima et al. 2000). Treatment with cyclooxygenase inhibitors can lead to different outcomes, depending on the protocol employed (Celentano et al. 1995, Freire-de-Lima et al. 2000, Michelin et al. 2005, Hideko Tatakihara et al. 2008). The continuous use of cyclooxygenase inhibitors, particularly in resistant mouse strains, leads to higher mortality (Celentano et al. 1995, Hideko Tatakihara et al. 2008).

One important question is how to target cell death for the modulation of immune responses during *T. cruzi* infection. During *T. cruzi* infection, apoptosis can be induced through at least three distinct pathways: (i) extrinsic soluble or membrane attached ligands for death receptors, such as Fas, (ii) granzymes released by cytotoxic cells and (iii) the intrinsic mitochondrial pathway. The key event in cell death signalling is the activation of caspases and the cleavage of target substrates. In order to understand whether the mechanisms of apoptosis involve the extrinsic or intrinsic pathways, we used inhibitors of caspase-8 and caspase-9 (Silva et al. 2005, de Meis et al. 2008). One criticism is that the specificity of these inhibitors occurs only at low doses (Pereira & Song 2008). For understanding the role of individual caspases, a combination of strategies such as the use of genetically modified mice and the injection of caspase inhibitors reinforce each other (Silva et al. 2005). These experiments indicate that T cells from infected mice undergo apoptosis through the extrinsic pathway (Silva et al. 2005). Two distinct approaches are used to block apoptosis in *T. cruzi* infection: in vivo injection of neutralizing antibody to FasL (Guillermo et al. 2007) or treatment with the general caspase inhibitor zVAD (Silva et al. 2007). Treatment with zVAD or anti-FasL reduces apoptosis and improves type-1 immune responses. T cells produce more IFN-γ and macrophages show increased control of intracellular infection. Moreover, both strategies reduce parasitemia, possibly by sustaining protective immune responses (Guillermo et al. 2007, Silva et al. 2007). A possible consequence of blocking apoptosis is to increase the number of inflammatory cells in target tissues. On the other hand, inhibition of tissue cell death may improve tissue function (Guillermo et al. 2009). In *T. cruzi* infection, treatment with anti-FasL, but not zVAD, increases inflammatory infiltrates in the hearts. It is unknown whether this effect helps to control infection or enhances tissue dysfunction (Guillermo et al. 2009).

Apoptosis and cardiac inflammation in Chagas disease

Apoptosis can be identified in parasites, cardiomyocytes and in inflammatory cells in heart tissues from dogs and mice acutely infected with *T. cruzi* (Zhang et al. 1999, de Souza et al. 2003). In addition, apoptosis can be identified in cardiomyocytes and inflammatory cells in heart tissue from chagasic patients, a finding associated with heart failure (Tostes et al. 2005). An immune regulatory role for apoptosis is indicated by the findings of low peripheral blood mononuclear cell proliferative responses to *T. cruzi* antigens, increased levels of Fas and FasL expression, and increased apoptosis in association with heart failure in Chagas disease patients (Rodrigues et al. 2008). In addition, another study suggests that the expression of FasL and Fas regulates the extent of cardiac inflammation and cardiomyocyte destruction in *T. cruzi* infection (de Oliveira et al. 2007). Taken together, these studies suggest a causal link between apoptosis and heart damage, but the mechanisms involved are unclear.

The immunosuppressive effects of apoptotic cell removal suggest that it plays a role in the resolution of inflammation. However, apoptosis can promote inflammation and autoimmunity, depending on the inflammatory context of apoptotic cell removal. Concomitant exposure to apoptotic cells and a Toll-like receptor (TLR) ligand, such as bacterial lipopolysaccharide (LPS), leads to proinflammatory cytokine secretion by phagocytes (Lucas et al. 2003). In addition, immunization with apoptotic cardiomyocytes and LPS results in autoimmune myocarditis (Eriksson et al. 2003). In this regard, it is noteworthy that *T. cruzi* expresses unmethylated CpG motifs in DNA, which are ligands for TLR9 (Bartholomeu et al. 2008). One hypothesis suggests that phagocytosis of apoptotic parasites and cardiomyocytes by immature dendritic cells in the presence of TLR ligands leads to dendritic cell maturation and priming of T cells against parasite and self cardiac antigens (DosReis et al. 2005).

The identification of deleterious effects of apoptotic cells and their effect on *T. cruzi* replication provides a new conceptual framework for the pathogenesis and treatment of Chagas disease. However, several questions remain unsolved, including the role of apoptosis in cardiac inflammation and the therapeutic efficacy of blocking host cell apoptosis. The high incidence of apoptosis in abnormal T cells from mice deficient in Fas signalling, together with the general immune defects of mice carrying a T-cell specific deficiency in caspase-8 (Wu et al. 2004), make the evaluation of the role of apoptosis more difficult. Mice deficient in selected pathways of cell death, the use of RNA interference to block cell death and the development of more selective caspase inhibitors could be useful future approaches to determine the importance of apoptosis for the pathogenesis of Chagas disease.

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