European Journal of Immunology

Apoptotic lymphocytes treated with IgG from *Trypanosoma cruzi* infection increase TNF-α secretion and reduce parasite replication in macrophages

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Phagocytic removal of apoptotic lymphocytes exacerbates replication of *Trypanosoma cruzi* in macrophages. We investigated the presence of Ab against apoptotic lymphocytes in *T. cruzi* infection and the role of these Ab in parasite replication. Both control and chagasic serum contained IgG Ab that opsonized apoptotic lymphocytes. Treatment of apoptotic lymphocytes with purified IgG from chagasic, but not control serum, reduced *T. cruzi* replication in macrophages. The protective effect of chagasic IgG depended on Fcγ receptors, as demonstrated by the requirement for the intact Fc portion of IgG, and the effect could be abrogated by treating macrophages with an anti-CD16/CD32 Fab fragment. Chagasic IgG displayed increased reactivity against a subset of apoptotic cell Ag, as measured by flow cytometry and immunoblot analyses. Apoptotic lymphocytes treated with chagasic IgG, but not control IgG, increased production of TNF-α, while decreasing production of TGF-β1 by infected macrophages. Increased control of parasite replication required TNF-α production. Previous immunization with apoptotic cells or injection of apoptotic cells opsonized with chagasic IgG reduced parasitemia in infected mice. These results indicate that Ab raised against apoptotic cells could play a protective role in control of *T. cruzi* replication by macrophages.

Key words: Ab · Apoptosis · Fc receptors · Phagocytosis · *Trypanosoma cruzi*

Introduction

Infection with *Trypanosoma cruzi*, the causative agent of Chagas' disease, induces Ab against both parasite Ag [1–4] and self molecules [5–7]. Ab play a protective role in *T. cruzi* infection [1–5, 7], but the mechanisms involved are not completely understood.

Infection with *T. cruzi* induces lymphocyte apoptosis [8–12], and the phagocytic clearance of apoptotic lymphocytes drives replication of *T. cruzi* in macrophages [13]. Parasite replication results from a biochemical cascade that originates from binding of apoptotic cells

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to $\alpha_V\beta_3$ integrin, followed by production of PGE_2 and TGF-β1, induction of ornithine decarboxylase and increased production of the polyamine putrescine [13]. *T. cruzi* is unable to synthesize putrescine and depends on uptake of exogenous putrescine for intracellular growth [14]. Phagocytosis of apoptotic cells induces production of TGF-β1, but not proinflammatory cytokines [15]. However, phagocytosis of apoptotic cells is enhanced by serum opsonins, including Ab [16], and Ab to apoptotic cells can lead to secretion of proinflammatory cytokines [15, 17].

Immunization with apoptotic cells induces production of autoantibodies [18]. However, it is unclear whether such autoantibodies play an immunoregulatory role. In the present study, we sought to determine whether infection with *T. cruzi* elicited production of Ab against apoptotic lymphocytes. We found that apoptotic lymphocytes treated with chagasic IgG induced a proinflammatory cytokine response that reduced parasite replication in macrophages. These results characterized a potential mechanism of immune protection against parasite infection mediated by Ab against apoptotic cells.

Results

IgG from *T. cruzi* infection reduced parasite replication driven by apoptotic cells

Clearance of apoptotic lymphocytes drives *T. cruzi* replication in macrophages [13]. Apoptotic lymphocytes were treated with either control or chagasic serum, and added to *T. cruzi* infected macrophages in medium containing Nutridoma. Intramacrophagic parasite replication was measured by release of viable extracellular trypomastigotes (Fig. 1A). Compared to treatment with control serum, previous treatment of lymphocytes with chagasic serum reduced parasite replication in macrophages (Fig. 1A). We purified IgG from control and chagasic serum by protein G affinity chromatography, and purified IgM by mannan-binding protein affinity chromatography. We then investigated the effects of purified IgG and IgM on parasite replication in macrophages. Apoptotic lymphocytes coated with chagasic IgG, but not with chagasic IgM, reduced parasite replication in macrophages (Fig. 1B). By contrast, treatments with either control IgG or IgM did not change parasite replication compared to macrophages cultured in the presence of apoptotic cells alone.

To investigate the role of Fc gamma-receptors (FcγR) in the response elicited by IgG-coated lymphocytes, we generated control and chagasic $F(ab')_2$ fragments. Lymphocytes treated with $F(ab')_2$ from either control or chagasic IgG failed to decrease parasite replication, compared with intact chagasic IgG (Fig. 2A). These results indicated that the Fc portion of chagasic IgG was required for protection against parasite replication. To generate an antagonist ligand of FcγR, we prepared monovalent Fab fragments from anti-CD16/CD32 mAb 2.4G2, and from a control mAb. Apoptotic lymphocytes were pretreated with control or chagasic IgG, and incubated with *T. cruzi* infected macrophages in the presence of either control or anti-CD16/CD32 Fab. Addition of anti-CD16/CD32 Fab, but not control Fab, reverted the protective effect of chagasic IgG, leading to increased parasite replication in macrophages (Fig. 2B). These results indicated that macrophage FcγR were involved in the protective effects elicited by chagasic IgG.

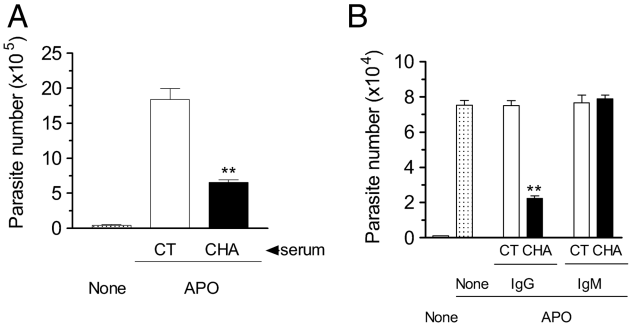

Figure 1. Treatment of apoptotic cells with Ab from *T. cruzi* infection reduces parasite replication in macrophages. (A) Macrophages were infected with *T. cruzi* and cultured alone (None), or with four-fold excess dead T cells (APO) previously treated with either control (CT) or chagasic (CHA) serum. Parasite replication was measured after 7 days by assessing the number of viable trypomastigotes produced. (B) Opsonization with chagasic IgG, but not chagasic IgM, reduces parasite replication in macrophages. Macrophages were infected with *T. cruzi* and cultured alone (none), or with fourfold excess dead T cells (APO), either untreated (dotted bars), or pretreated with either IgG or IgM purified from either control (open bars; CT) or chagasic serum (closed bars; CHA). Parasite replication was measured as in (A). Data show mean+SEM ($n = 3$) and are representative of two independent experiments. $^{**}p<0.01$ compared with CT.

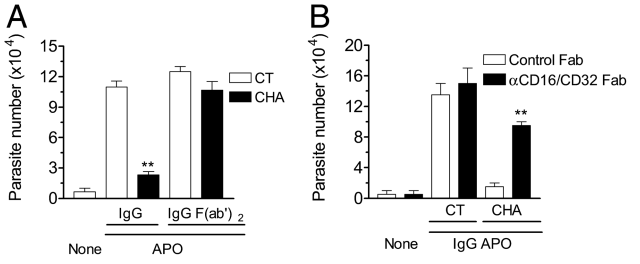

Figure 2. The protective effect of opsonization with chagasic IgG requires FcγR. (A) The effect of chagasic IgG requires an intact Fc portion. Macrophages were infected with *T. cruzi* and cultured alone (None), or with four-fold excess dead T cells (APO) pretreated with either intact IgG or IgG $F(ab')_2$ purified from either control (open bars; CT) or chagasic serum (closed bars; CHA). (B) The effect of chagasic IgG can be prevented by an anti-CD16/CD32 Fab fragment. Macrophages were infected with *T. cruzi* and cultured alone (none), or with four-fold excess dead T cells pretreated with intact IgG (IgG APO) purified from either control (CT) or chagasic (CHA) serum. Cultures received a control Fab fragment prepared from rat IgG_{2b} (open bars), or anti-CD16/CD32 Fab (closed bars). Parasite replication was measured as in Fig. 1A. Data show mean+SEM ($n = 3$) and are representative of two independent experiments. $^{**}p<0.01$ compared with CT.

Reactivity of IgG against lymphocytes

We investigated the ability of control or chagasic IgG to opsonize apoptotic lymphocytes. Coating with either control or chagasic IgG increased the percentages of phagocytosing macrophages and

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the number of phagocytosed lymphocytes after 3-h incubation (Fig. 3). No significant difference was found between control and chagasic IgG regarding opsonization. These results were confirmed by immunofluorescence studies. Both control and chagasic IgG stained apoptotic lymphocytes (data not shown). These results indicated that both control and chagasic IgG opsonized apoptotic lymphocytes for phagocytosis by macrophages.

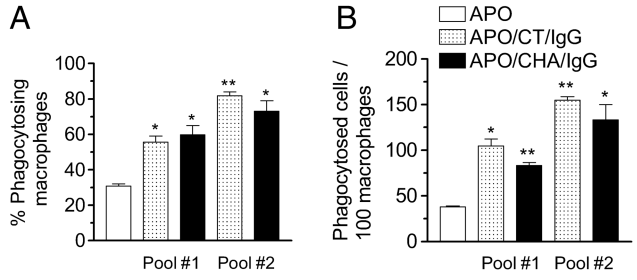

Figure 3. Opsonization of apoptotic cells by control and chagasic IgG. Macrophages were infected with *T. cruzi* and cultured with four-fold excess dead T cells, either alone (open bars; APO), or previously treated with either control (dotted bars; CT) or chagasic IgG (closed bars; CHA). The percentage of phagocytosing macrophages (A), and the number of cells ingested/100 macrophages (B) were determined after 3 h. Data show mean+SEM ($n = 3$) and are representative of two independent experiments. *$p<0.05$ and **$p<0.01$ compared with the absence of IgG.

To perform a more refined comparison between the reactivities of control and chagasic IgG, we did flow cytometry analysis. Both control and chagasic IgG labeled apoptotic lymphocytes. However, chagasic IgG stained an increased number of apoptotic cells, albeit with lower fluorescence intensity (Fig. 4A). This increased reactivity was reproduced with additional pools of IgG. We also applied an immunoblot technique for global analysis of the IgG repertoire recognizing polypeptides derived from apoptotic lymphocytes. Both control and chagasic IgG reacted with most self peptides in a quite conserved manner (Fig. 4B). However, selected specificities from chagasic IgG showed enhanced reactivity toward lymphocyte Ag, compared to control IgG (specifities shown as arrows in Fig. 4B; analysis shown in Fig. 4C; specificities 403, 901 and 1055). Together, these results indicated that chagasic IgG displayed enhanced reactivity against a subset of apoptotic cell Ag.

Purified chagasic IgG modulated cytokine production and reduced parasite replication in macrophages

Replication of *T. cruzi* driven by apoptotic cells requires TGF-β1 production by macrophages [13]. We therefore investigated whether reduced parasite replication was associated with modulation of TGF-β1 secretion by infected macrophages. Compared with lymphocytes alone, lymphocytes treated with

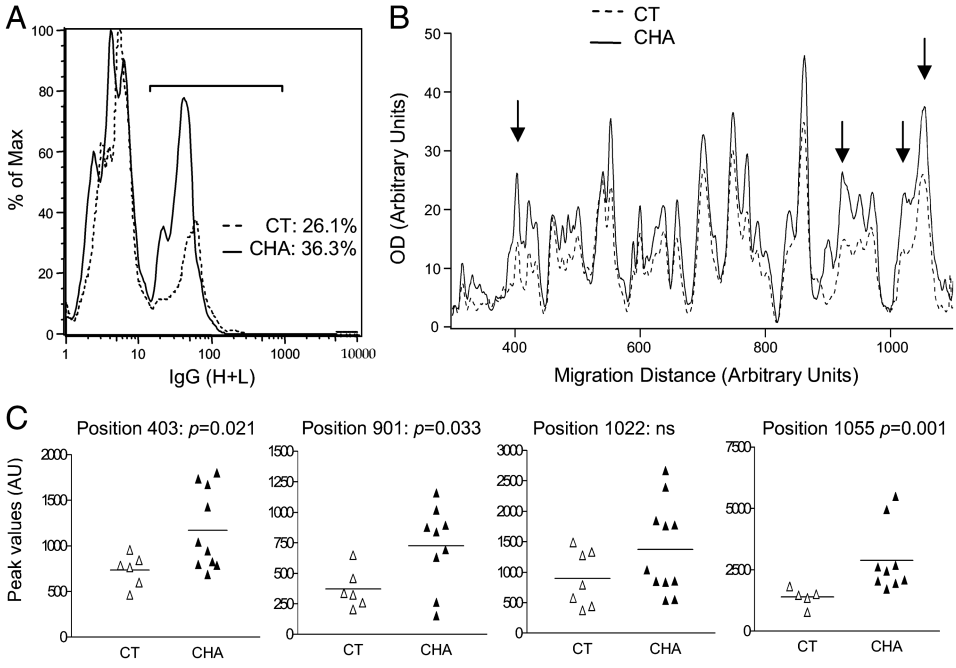

Figure 4. Increased reactivity of chagasic IgG against apoptotic cells. (A) Flow cytometryic analysis for the presence of surface IgG. Apoptotic EL-4 cells were incubated with control (CT) or chagasic (CHA) IgG, and the reaction was detected by a PE-labeled $F(ab')_2$ goat anti-mouse IgG (H+L), and gated on Annexin V-positive cells. Note the increased reactivity of chagasic IgG for determinants expressed at low density on apoptotic cells. (B, C) Repertoire analysis. (B) Pools of control (CT) and chagasic (CHA) IgG were tested on apoptotic cell extract by immunoblot. The densitometric profiles represent the mean of several IgG pools. Arrows indicate selected specificities where chagasic IgG reacted stronger than control IgG. (C) Immunoreactivity of individual IgG pools at the same positions specified in (B). The densitometric values of control and chagasic samples were compared by Mann–Whitney test, and the p values for each specified position are indicated at the top (ns: not significant). Data in (A) are representative of two independent experiments; data in (B) and (C) integrate 5–11 independent experiments.

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control IgG failed to reduce parasite replication (Fig. 5A) and the amount of TGF-β1 secreted by macrophages (Fig. 5B). However, lymphocytes treated with chagasic IgG reduced both parasite replication (Fig. 5A) and secretion of TGF-β1 by infected macrophages (Fig. 5B).

Secretion of TNF-α is involved in microbicidal effects induced by the clearance of apoptotic neutrophils [19]. We therefore investigated secretion of TNF-α by infected macrophages. Compared with lymphocytes alone, or with lymphocytes treated with control IgG, lymphocytes treated with chagasic IgG induced increased secretion of TNF-α by macrophages (Fig. 6A). Addition of a neutralizing mAb against mouse TNF-α prevented the protective effect of chagasic IgG, and led to increased parasite replication (Fig. 6B). An isotype control had no effect (Fig. 6B). Addition of anti-TNF-α had no effect on parasite replication driven by apoptotic cells alone, or by apoptotic cells that had been pretreated with control IgG (Fig. 6B). These results indicated that engulfment of apoptotic cells in the presence of chagasic IgG elicited proinflammatory cytokine secretion in macrophages, and that TNF-α secretion was required for control of parasite replication mediated by chagasic IgG.

We investigated the requirement of endosomal acidification for induction of TNF-α secretion by chagasic IgG. To this end, macrophages were infected and cultured with IgG-coated lymphocytes in the presence of increasing doses of chloroquine. Chloroquine blocked TNF-α secretion in a dose-dependent manner (Fig. 6C). These results suggested that endosomal acidification is required for the proinflammatory effect of chagasic IgG.

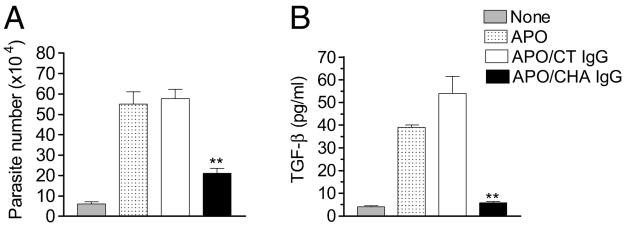

Figure 5. Treatment of apoptotic lymphocytes with chagasic IgG controls parasite replication and inhibits production of TGF-β1 by macrophages. Macrophages were infected with *T. cruzi* and cultured alone (none), or with four-fold excess dead T cells, either alone (dotted bars; APO), or pretreated with either control IgG (open bars; APO/CT IgG) or chagasic IgG (closed bars; APO/CHA IgG). (A) Parasite replication was measured as in Fig. 1A. (B) Production of TGF-β1 was measured in similar cultures after 48 h. Data show mean+SEM ($n = 3$) and are representative of two independent experiments. $^{**}p<0.01$ compared with CT IgG.

Effects on parasitemia *in vivo*

We investigated the effects of opsonized apoptotic lymphocytes in *T. cruzi* infection *in vivo*. A single injection of apoptotic lymphocytes opsonized with chagasic IgG markedly reduced parasitemia, compared with lymphocytes opsonized with control IgG (Fig. 7A). We also investigated the effect of previous immunization with apoptotic cells. Mice that were previously immunized with apoptotic lymphocytes developed lower parasitemias, compared with animals treated with adjuvant alone (Fig. 7B). Taken together, these results suggested that opsonization of apoptotic lymphocytes with IgG could play a protective role during infection *in vivo*.

Discussion

The uptake of apoptotic cells drives replication of *T. cruzi* in macrophages [13]. However, our results have demonstrated that IgG Ab elicited by *T. cruzi* infection react against apoptotic lymphocytes and protect against increased parasite replication in macrophages through a mechanism dependent on TNF-α and FcγR.

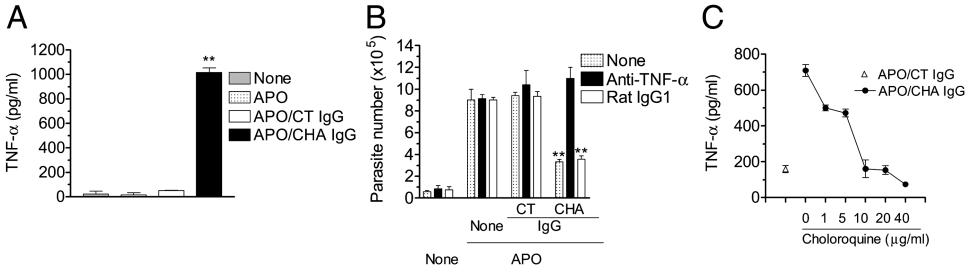

Figure 6. Treatment of apoptotic lymphocytes with chagasic IgG controls parasite replication, which is mediated by macrophage-derived TNF-α. (A) TNF-α production. Macrophages were infected with *T. cruzi* and cultured alone (none), or with four-fold excess dead T cells, either alone (dotted bars; APO), or pretreated with either control IgG (open bars; APO/CT IgG) or chagasic IgG (closed bars; APO/CHA IgG). Production of TNF-α was measured after 48 h. Data show mean+SEM ($n = 3$) and are representative of two independent experiments. $^{**}p<0.01$ compared with CT IgG. (B) Neutralization of TNF-α abolishes the protective effect of chagasic IgG. Infected macrophages were cultured alone, or with dead T cells (APO), either alone (None) or pretreated with control IgG (CT/IgG) or chagasic IgG (CHA/IgG). Cultures received medium alone (dotted bars), a neutralizing anti-TNF-α mAb (closed bars), or a rat IgG1 isotype control (open bars). Parasite replication was measured as in Fig. 1A. Data show mean+SEM ($n = 3$) and are representative of two independent experiments. $^{**}p<0.01$ compared with anti-TNF-α. (C) Effect of treatment with chloroquine on TNF-α production. Macrophages were infected and treated with APO/CT IgG or APO/CHA IgG, as in Fig. 6A, in the absence or in the presence of the indicated doses of chloroquine. Data show mean+SEM ($n = 3$) and are representative of two independent experiments. Data obtained from APO/CHA IgG in the presence of chloroquine at 10 μg/mL or higher gave non-significant statistical differences compared to APO/CT IgG.

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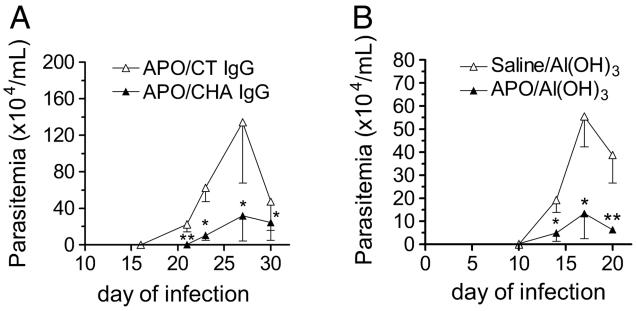

Figure 7. Effects of opsonization on parasitemia *in vivo*. (A) Treatment of apoptotic cells with chagasic IgG. BALB/c mice ($n = 6$) were infected with *T. cruzi* and, after 7 days, were injected with apoptotic lymphocytes opsonized with either control (CT) or chagasic (CHA) IgG. Parasitemia was measured as indicated. (B) Immunization with apoptotic lymphocytes. BALB/c mice were immunized with apoptotic lymphocytes in $Al(OH)_3$ and, after 14 days, were infected with *T. cruzi*. Parasitemia was measured as indicated. Data show mean and SEM ($n = 6$ in A; $n = 4$ in B) and are representative of two independent experiments. $^*p<0.05$, and $^{**}p<0.01$ compared to controls.

Macrophages can ingest either IgG-coated particles or apoptotic cells in the course of immune responses. These two phagocytic responses are biochemically distinct. Ingestion of apoptotic cells, but not IgG-coated particles, requires the ABC1 transporter and redistribution of membrane phosphatidylserine on both macrophage and dying cell [20]. On the other hand, ingestion of IgG-coated particles, but not apoptotic cells, activates Syk tyrosine kinase and is regulated by LTB_4 [21]. Phagocytosis mediated by FcγR is often proinflammatory and leads to secretion of TNF-α [15, 22], while engulfment of apoptotic cells induces the secretion of the immunosuppressive cytokine TGF-β1, and is associated with resolution of inflammation [15, 23].

Infection with *T. cruzi* increases lymphocyte apoptosis [8–12], and systemic exposure to apoptotic cells elicits production of autoantibodies reactive with apoptotic cells [18]. Therefore, we investigated whether infection by *T. cruzi* elicits autoantibodies against apoptotic lymphocytes. In addition, we assessed the functional consequence of binding of such Ab to host lymphocytes. Our results demonstrated the presence of IgG Ab reactive with apoptotic lymphocytes in the serum of either control mice or mice chronically infected with *T. cruzi*. More detailed studies employing flow cytometry and global repertoire analysis revealed that chagasic IgG displayed increased reactivity against a subset of apoptotic lymphocyte Ag. The molecular structures targeted by chagasic IgG were not identified in the present study. However, several epitopes could be involved, including oxidized phospholipids [16], cardiolipin [18] and nuclear Ag [18, 24, 25]. During apoptosis, nucleosomes become exposed at the cell surface [26]. Mice infected with *T. cruzi* develop anti-nuclear Ab [27], and the serum of chagasic patients contains Ab reactive with cardiolipin [28] and small nuclear ribonucleoproteins [29]. These results raise the possibility that *T. cruzi* infection increases the production of Ab against apoptotic cells.

Opsonization of apoptotic cells with chagasic IgG impacted on replication of *T. cruzi* in macrophages. While lymphocytes treated with control IgG exacerbated parasite replication in macrophages, lymphocytes treated with chagasic IgG reduced *T. cruzi* growth compared to controls. Coating with $F(ab')_2$ fragments from chagasic IgG failed to reduce parasite replication, indicating that the Fc portion of chagasic IgG was required. Furthermore, the protective effect of opsonized lymphocytes was abrogated by coculture with a monovalent anti-CD16/CD32 Fab fragment. These results suggested that FcγR are involved. Reduced parasite growth correlated with increased secretion of TNF-α. Neutralizing TNF-α activity abrogated the protective effect of chagasic IgG on parasite replication. Although cells coated with chagasic IgG reduced secretion of TGF-β1 by infected macrophages, the contribution of the amounts of TGF-β1 or other anti-inflammatory cytokines such as IL-10 were not directly evaluated. On the other hand, lymphocytes treated with chagasic IgM failed to reduce parasite load, and did not elicit TNF-α production by macrophages (not shown). IgM mediates clearance through deposition of C1q and iC3b on apoptotic cells [16, 30, 31]. Opsonization by C1q or iC3b leads to silent, non-inflammatory removal of dead cells [32–34]. However, we did not investigate the role of complement activation in our system.

The reason why opsonization with chagasic IgG leads to proinflammatory clearance of apoptotic lymphocytes remains to be determined. Identification of the apoptotic cell Ag targeted by chagasic IgG might help to resolve this issue. Nucleosomes become exposed at the surface of apoptotic cells [26]. Opsonization by lupus Ab leads to increased DC phagocytosis through FcγR, and increased presentation of nuclear Ag [35]. Furthermore, uptake of immune complexes containing both IgG and nuclear chromatin induces a proinflammatory response [36, 37]. Phagocyte activation is achieved through engagement of both FcγR and TLR [36, 37]. One possibility is that chagasic IgG reacts with nucleosomal structures on apoptotic lymphocytes, leading to simultaneous engagement of FcγR and TLR. In favor of this possibility, we found that TNF-α production elicited by IgG-coated lymphocytes could be blocked by chloroquine, an inhibitor of lysosome acidification and TLR9 signaling [38].

Lymphocyte apoptosis plays a deleterious role in *in vivo* immune responses against *T. cruzi* [10, 39, 40]. Our data showed that injection of apoptotic lymphocytes opsonized with chagasic IgG reduced parasitemia *in vivo*. Furthermore, previous immunization with apoptotic cells resulted in lower parasitemias following challenge with *T. cruzi*. These results suggested that both natural infection and immunization with adjuvants are able to turn Ab responses to apoptotic cells protective to the host. In summary, our results have demonstrated that apoptotic cells generated in the course of *T. cruzi* infection induce production of Ab directed against apoptotic lymphocytes. Opsonization leads to proinflammatory engulfment mediated by FcγR, helping to control intracellular parasite replication. Further investigation should help identifying the epitopes targeted by Ab, as well as their significance in pathogenesis of Chagas' disease.

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Materials and methods

Mice, parasite and infection

Male BALB/c mice aging 6–8 wk were from the Oswaldo Cruz Institute Animal Care facility, Fiocruz, Rio de Janeiro. All animal work was approved and conducted according to institutional guidelines. Chemically induced metacyclic forms of the *T. cruzi* clone Dm28c were used. Chemically induced and vector derived parasites cause similar infections in mice [41]. Mice were infected with 10^5 parasites/0.1 mL i.p. After 90 days of infection, mice were sacrificed; sera were collected, pooled and inactivated by heat treatment. Serum from age matched uninfected controls was also collected, pooled and inactivated.

Ab, Ab fragments and reagents

IgG and IgM were purified from serum using kits based on immobilized protein G and mannan-binding protein affinity columns, respectively, according to the manufacturer (ImmunoPure™ IgG and IgM purification kits, Pierce Biotechnology, Rockford, IL, USA). Anti-CD16/CD32 mAb 2.4G2 was from BD Biosciences (San Diego, CA, USA), and a rat IgG_{2b} mAb from BioSource Europe (Belgium). $F(ab')_2$ from purified IgG and monovalent Fab were produced and purified using kits according to the manufacturer (ImmunoPure™ $F(ab')_2$ Preparation kit and Fab kit, Pierce Biotechnology). Purity and size of purified Ab and their fragments were verified by ELISA assays and SDS-PAGE. For immunofluorescence assays, PE-labeled goat $F(ab')_2$ anti-mouse IgM, goat $F(ab')_2$ anti-mouse IgG and control $F(ab')_2$ (Southern Biotechnology, Birmingham, AL, USA) were employed. Neutralizing anti-TNF-α mAb (clone MP6-XT3), and isotype control rat IgG1 for tissue culture were from BD-Biosciences.

Infected macrophages and lymphocytes

Peritoneal washout cells (4×10^5) were adhered for 2 h in 48-well culture vessels (Nunc, Denmark) and washed, yielding resident macrophages (2.0×10^5/0.5 mL). Macrophages were infected for 18 h with *T. cruzi* at a 10:1 parasite/macrophage ratio in complete culture medium – 10% FBS at 37°C, 7% CO_2. Extracellular parasites were removed by washing. Culture medium consisted of DMEM, supplemented with 2 mM glutamine, 5×10^{-5} M 2-ME, 10 μg/mL gentamycin, sodium pyruvate, MEM nonessential aa, 10 mM HEPES buffer and 10% FBS (all from GIBCO-Invitrogen Corporation, Carlsbad, CA, USA). Normal splenocytes were treated with red-cell lysing buffer and passed through nylon wool columns for T-cell enrichment. Apoptosis was induced by heating at 43°C for 60 min followed by incubation at 37°C for 24 h (modified from [42]), both in complete culture medium containing 1% v/v Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN, USA), instead of FBS. Following this treatment, 84–89% of lymphocytes were apoptotic; 11–16% were necrotic, as judged by microscopic analysis of pyknotic nuclei and Trypan blue exclusion. Dead lymphocytes (10^7) were incubated (1 mL) in medium containing 1% v/v control mouse serum, chagasic mouse serum, or with either 1–10 μg/mL purified IgG or IgM or 10 μg/mL purified $F(ab')_2$ fragments for 60 min at 4°C. Following incubation, apoptotic cells were washed and added to infected macrophages (8×10^5 lymphocytes, or 4:1 lymphocyte:macrophage ratio). Culture with apoptotic cells was performed in the presence of Nutridoma, instead of FBS. No extracellular parasite was observed up to 3 days in culture. Infected macrophages were cultured with apoptotic cells in the presence of 50 μg/mL anti-CD16/CD32 Fab or a control Fab; or with 10 μg/mL of neutralizing anti-TNF-α mAb or rat IgG1. Cultures lasted either 2 days, for cytokine production, or 7 days, for assessment of parasite load. Parasite load was assessed by the number of motile trypomastigotes released into the extracellular medium of triplicate cultures [43]. The yield of trypomastigotes varied between experiments, but was reproducible within the same experiment.

Phagocytosis assay

Peritoneal cells were adhered to glass coverslips placed in 24-well plates (Corning, Corning, NY, USA) for 2 h at 37°C, to yield 5×10^5 macrophages. Dead lymphocytes were treated with 5 μg/mL purified IgG or IgM, washed and added to macrophages in serum-free medium for 3 h at 37°C. Coverslips were washed, fixed with methanol and stained with May-Grunwald Giemsa. Phagocytosis of apoptotic cells induces the formation of spacious phagosomes [44]. Therefore, phagocytosed cells were identified as surrounded by a large translucent vacuole. Results represent the mean and SEM of triplicate cultures.

Immunofluorescence

Macrophages were incubated for 3 h at 37°C with dead lymphocytes previously treated with different sera to allow phagocytosis of dead cells. Coverslips were washed and fixed with 1% paraformaldehyde. Coverslips were treated with 1 μg PE-labeled control, anti-IgM and anti-IgG $F(ab')_2$, mounted and examined using a Zeiss confocal laser scan microscope.

Flow cytometry

Apoptotic EL-4 cells were obtained by overnight incubation with emetine (Sigma) at 2 μg/mL in complete medium without FBS [45]. Apoptotic EL-4 cells were washed, treated with anti-CD16/CD32 (Fc Block, 10 μg/mL), and incubated for 40 min on ice with purified IgG (100 μg/mL). Cells were washed and stained with

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PE-labeled $F(ab')_2$ goat anti-mouse IgG (H+L) (Southern Biotechnology) and FITC-labeled Annexin V (Pharmingen) for 30 min. Cells were analyzed on a BD X-Calibur flow cytometer. Annexin V positive cells were electronically gated, and the percentages of cells positive for IgG were determined.

Immunoblot and data analysis

Apoptotic EL-4 cells were obtained by treatment with emetine, and lysed in extraction buffer (2% SDS, 5% 2-mercaptoethanol and 62.5 mM Tris, pH 6.8) on ice, without protease inhibitors. The extract was sonicated, boiled for 10 min, centrifuged at $1000 \times g$, and then at $10\,000 \times g$. Aliquotes of the supernatant were stored at -20°C. IgG reactivities against apoptotic cell extract were investigated by a modified immunoblot technique [46, 47]. Briefly, apoptotic cell extracts (600 μg/mL) were subjected to SDS-PAGE in a Mighty Small II SE 250 electrophoresis apparatus (Hoefer Scientific Instruments), and proteins were transferred to a nitrocellulose membrane. Membranes were blocked overnight with PBS-0.2% Tween-20 (PBS-T) at room temperature, incubated with purified IgG samples (100 μg/mL) for 4 h, using the Cassette Miniblot System (Immunetics, Cambridge, MA, USA). Alkaline phosphatase conjugated secondary goat anti-mouse IgG Ab (Southern Biotechnology) were added for 90 min. After washing, immunoreactivities were revealed with nitroblue-tetrazolium/bromo-chloro-indolyl-phosphate (NBT/BCIP) (Promega, Madison, WI, USA), and analyzed by densitometry. Total blotted proteins were then stained using colloidal gold (Bio-Rad) and subjected to a second densitometry to score the protein profile. Data analysis was performed on an iMac computer using the Igor software (Wavemetrics, Lake Oswego, OR, USA) and macros written specifically for this purpose. The immunoblot and protein scans were superimposed and rescaled to correct migration irregularities, and to allow comparisons of IgG immunoreactivities. Adjusted profiles were divided into sections, each one representing an IgG reactivity. Section reactivities were quantified as the average density within a respective section, and individual numerical values of reactivities expressed as peak values.

Cytokine production

Supernatants from cultures of infected macrophages plus lymphocytes were collected after 2 days, cleared by centrifugation and immediately assayed for TGF-β1, and TNF-α content, by sandwich ELISA, according to the manufacturer (BD Biosciences for TGF-β1; R&D Systems, Minneapolis, MN, USA, for TNF-α).

In vivo assays

For *in vivo* treatment with opsonized apoptotic cells, apoptotic cells produced as above, were incubated with either control or chagasic IgG (10 μg/10^6 cells/mL), washed, and a total of 8×10^6 cells were injected i.v. in BALB/c mice at day 7 of infection. Mice were infected with 10^5 *T. cruzi* Dm28c metacyclic trypomastigotes i.p. For previous immunization with apoptotic cells, BALB/c mice received s.c. injections of a total 2.5 mg $Al(OH)_3$ emulsified with either saline or 10^7 apoptotic T lymphocytes. After 14 days, mice were infected i.p. with *T. cruzi*, and parasitemia was evaluated at the indicated days. Blood was obtained by tail vein puncture, and viable parasites were counted in a Neubauer chamber.

Statistical analysis

Data were analyzed by Student' *t*-test for independent samples, using SigmaPlot™ for Windows. Data from assessment of parasitemia were first normalized by a logarithmic transformation before applying the *t*-test. Data from densitometric analysis were analyzed by Mann-Whitney test, using Graph Pad InStat 3.01 for Windows. All differences with a p value<0.05 or lower were considered significant.

Acknowledgements: This investigation received financial support from Brazilian National Research Council (CNPq), Rio de Janeiro State Science Foundation (FAPERJ), Programa de Núcleos de Excelência (PRONEX), and Howard Hughes Medical Institute (grant # 55003669). M.P.N., V.M.B., M.F.L. and G.A.D.R. are investigators from CNPq.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviation: **FcγR:** Fc gamma-receptor

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Received: 12/5/2009
Revised: 30/10/2009
Accepted: 16/11/2009

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