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*J Immunol* 2002; 168:3965-3973; doi: 10.4049/jimmunol.168.8.3965
http://www.jimmunol.org/content/168/8/3965

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Elina Zuniga,* Claudia C. Motran,* Carolina L. Montes,* Hideo Yagita,† and Adriana Gruppi2*

The control of B cell expansion has been thought to be solely regulated by T lymphocytes. We show in this study that Trypanosoma cruzi infection induces up-regulation of both Fas and Fas ligand (FasL) molecules on B cells and renders them susceptible to B cell-B cell killing (referred to as fratricide throughout this paper) mediated via Fas/FasL. Moreover, by in vivo administration of anti-FasL blocking mAb we demonstrate that Fas-mediated B cell apoptosis is an ongoing process during this parasitic infection. We also provide evidence that B cells that have switched to IgG isotype are the preferential targets of B cell fratricide. More strikingly, this death pathway selectively affects IgG+ B cells reactive to parasite but not self Ags. Parasite-specific but not self-reactive B cells triggered during this response are rescued after either in vitro or in vivo FasL blockade. Fratricide among parasite-specific IgG+ B lymphocytes could impair the immune control of T. cruzi and possibly other chronic protozoan parasites. Our results raise the possibility that the blockade of Fas/FasL interaction in the B cell compartment of T. cruzi-infected mice may provide a means for enhancing antiparasitic humoral immune response without affecting host tolerance. The Journal of Immunology, 2002, 168: 3965–3973.

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Received for publication June 19, 2001. Accepted for publication January 18, 2002.

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1 This work was supported by grants from Consejo de Investigaciones Científicas y Técnicas, Fundación Antorchas, Agencia Córdoba Ciencia, and Agencia Nacional de Promoción Científica y Técnica (to A.G.). A.G. is a member of the Scientific Career of Consejo de Investigaciones Científicas y Técnicas. E.Z., C.C.M., and C.L.M. are the recipients of Consejo de Investigaciones Científicas y Técnicas fellowships.

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3 Abbreviations used in this paper: FasL, Fas ligand; SMC, spleen mononuclear cell; CM, culture medium; FCM, flow cytometry; TcAg, Trypanosoma cruzi Ag; SM, skeletal muscle; AFC, Ab-forming cell; SAv, streptavidin; AICD, activation-induced cell death; PI, propidium iodide.

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interaction and preferentially affects IgG\(^+\) highly activated B cells specific for parasite Ags.

**Materials and Methods**

**Infection with *T. cruzi* and anti-FasL treatment**

BALB/c mice (6–8 wk old, obtained from Comisión Nacional de Energía Atómica, Buenos Aires, Argentina) were infected i.p. with 500 trypomastigotes from *T. cruzi* (Tulahue strain) as described (24). Age-matched uninfected normal littermates were used as controls. Spleen cells were harvested 15 days after infection. For in vivo treatment with anti-FasL, neutralizing anti-FasL mAb MFL-4 (0.5 mg per mouse) or normal hamster IgG (0.5 mg per mouse) was injected i.p. into infected mice twice a week starting on day 4 postinfection.

**Abs and reagents**

PE-labeled anti-mouse MHC class II (2G9), PE-labeled and biotinylated anti-mouse CD19 (1D3), FITC-labeled anti-mouse CD3 (145-2C11), FITC-labeled anti-mouse Mac-1 (M1/70), PE-labeled anti-mouse Fasl (MFL-3), FITC-labeled anti-mouse Fas (J02), FITC-labeled anti-mouse IgM mAbs, anti-mouse FasL mAb (NA/LE, MFL-3), PE-labeled anti-mouse Syndecan-1 (281-2), as well as streptavidin (SAv)-CyChrome and SAv-FITC, were purchased from BD PharMingen (San Diego, CA). FITC-labeled anti-mouse IgG, peroxidase-conjugated or nonconjugated anti-mouse IgG, biotinylated anti-goat IgG, SAv-alkaline phosphatase, FITC-, or biotin-conjugated Ab for 30 min at 4°C and washed with FCM buffer. Cells were then incubated with each PE-, FITC-, or biotin-conjugated Ab for 30 min at 4°C and washed with FCM buffer. For apoptotic cell detection, PI staining was performed as described (30). Briefly, cells were washed twice with HBSS and fixed in 1 ml 70% ethanol at 4°C. Cell pellets were gently resuspended in 1 ml hypotonic fluorochrome solution (50 µg/ml PI diluted in 4 mM sodium citrate plus 0.3% Nonidet P-40) and kept at 4°C for 18 h in the dark. Data were acquired on a Cytometer Sharing System (Ortho Diagnostics, Raritan, NJ) and analyzed using WinMDI 2.7 software (J. Trotter, Scripps Research Institute, La Jolla, CA).

Transmission electron microscopy

Purified splenic B cells, from control or infected mice, were fixed in 1% glutaraldehyde diluted in 0.1 M cacodylate buffer (pH 7.3). Samples were postfixed in 1% OsO\(_4\), dehydrated, and embedded in araldite (Ciba-Geigy, Summit, NJ). Thin sections were cut in a Porter-Blum MT2 ultramicrotome (Manch, Germany) and examined in a Zeiss 109 electron microscope (Zeiss, Overkochen, Germany). Photographs were taken on Kodak electron imaging film (Eastman Kodak, Rochester, NY).

**B cell culture and spontaneous proliferation in vitro**

B cells (1 × 10\(^6\) cells/well) were cultured (1 ml) in flat-bottom 48-well tissue culture plates (Techno Plastic Product, Trasadingen, Switzerland) in culture medium (CM), which consisted of RPMI 1640 medium supplemented with 10% FBS and 40 µg/ml gentamicin, for the indicated periods in the presence of either anti-mouse FasL mAb (10 µg/ml) or a control hamster IgG mAb (10 µg/ml). To assess spontaneous proliferation, B cells (2 × 10\(^5\) cells/well) were cultured in CM for 6 h in the absence of any exogenous stimuli in the presence of [\(^3\)H]TdR (1 µCi/well; New England Nuclear, Boston, MA) following the protocol of Minoprio et al. (31). Cells were harvested and [\(^3\)H]TdR incorporation into DNA was measured by liquid scintillation spectroscopy. Results are mean and SD of triplicate cultures.

**Ag preparation**

*T. cruzi* Ags (TcAg) were prepared from epimastigote (Tulahue strain) harvested from cultures in monophasic medium (32). The epimastigote homogenate was centrifugated at 105,000 × g, and the supernatant was used for ELISA and ELISPOT assays. Mouse SM extract was from muscle tissue homogenized in 10 vol of ice-cold KCl buffer (0.3 M KCl, 15 mM K\(_2\)HPO\(_4\) (pH 6.5), 5 mM MgCl\(_2\), 20 mM EDTA, 1 mM PMSF). After centrifugation at 10,000 × g for 20 min at 4°C, the supernatant was collected and stored at −70°C (33).

**ELISPOT assay**

The presence of anti-TcAg or anti-mouse SM Ab-forming cells (AFCs) in large and small B cells was determined by ELISPOT as described (34). In brief, serial dilutions of B cells or SMC (starting from 200,000 cells/well) were cultured in CM in wells coated with 10 µg/ml TcAg or mouse SM.
Following overnight incubation at 37°C, the wells were washed and incubated overnight at 4°C with anti-IgG or anti-IgM Abs, and then with alkaline phosphatase-conjugated anti-goat IgG, and washed after each incubation. AFCs were visualized by adding a 1/4 mix of distilled water containing 1.25 mg/ml 4-bromo-4-chloro-indolyl phosphate in 2-aminomethyl-1-propanol buffer (1 M 2-amino-2-methyl-1-propanol, MgCl2, Triton X-450, NaNO3 (pH 10.2)).

ELISA

After a 96-h cell culture either in the absence or in the presence of anti-FasL blocking mAb (MFL-3), supernatants from B cells (from either normal or infected mice) were collected for TcAg or mouse SM IgM and IgG determination. Levels of specific Abs were detected by ELISA following the procedure described previously (35). Each sample was assayed in triplicate and the values were expressed as mean of OD read at 490 nm in an ELISA reader (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical comparisons were performed by using unpaired Student’s t test or ANOVA test as indicated in the figures. All data were considered statistically significant if p values were <0.05.

Results

B cells from T. cruzi-infected mice undergo apoptosis in vivo and in vitro

B cells from T. cruzi-infected mice show increased spontaneous apoptosis in vitro (24). To investigate whether B cell apoptosis occurs in vivo, purified B cell populations were obtained from normal or T. cruzi-infected mice and immediately processed for analysis of DNA content and transmission electronic microscopy. Around 18% freshly of explanted B cells from T. cruzi-infected mice undergo apoptosis, as evidenced by the presence of cells bearing hypodiploid nuclei (Fig. 2A, right panel), which is 3-fold the background levels seen in uninfected mice (Fig. 2A, left panel). A representative electron micrograph of B cells from T. cruzi-infected mice is shown in Fig. 2B, right panel, in comparison with B cells from normal mice (Fig. 2B, left panel). B cells from infected mice showed the typical ultrastructural features of apoptosis, including reduction of the cytoplasmic volume, loss of surface microvilli, chromatin condensation, and margination along the inner surface of the nuclear envelope.

To investigate whether B cell apoptosis correlated with the cell activation stage, B cells from either normal or T. cruzi-infected mice were further separated on Percoll gradient into large and small B cells. As shown in Table I, large B cells from infected mice exhibited the highest degree of activation, as demonstrated by the marked increase in percentages of blast cells and MHC class I<sup>high</sup> cells, and their spontaneous proliferation. B cells from non-infected mice showed background levels of activation. Each of these cell populations was analyzed for apoptosis by PI staining in either freshly explanted or cultured B cells.

As expected, large and small B cells from normal mice show background levels of apoptosis (Fig. 3, A and B), while a significant proportion of large and small B cells from infected mice underwent increased levels of apoptosis in vivo (Fig. 3, C and D, left panels) and in vitro (Fig. 3, C and D, right panels). Moreover, freshly explanted large B cells from T. cruzi-infected mice showed an increase in the percentage of apoptotic cells compared with small B cells (Fig. 3, C vs D, left panels). Consistently, after 18 h of culture in medium alone strongly activated large B cells from infected mice show higher levels of spontaneous apoptosis (40%) compared with small B cells, which exhibit 28% of hypodiploid nuclei (Fig. 3, C vs D, right panels). In summary, B cells strongly activated by T. cruzi infection present the highest levels of apoptosis both in vivo and in vitro, and the death rate is associated with the B cell activation stage.

Influence of Fas/FasL pathway in regulation of T. cruzi-induced B cell apoptosis

In the next series of experiments we attempted to examine the role of Fas/FasL interactions in regulating B cell survival during T.

Table I. Activation grade of B cells obtained from normal and T. cruzi-infected mice

<table>
<thead>
<tr>
<th>B Cells</th>
<th>MHC Class I&lt;sup&gt;high&lt;/sup&gt; B Cells (%)</th>
<th>Spontaneous Proliferation (cpm ± SD)</th>
</tr>
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<tbody>
<tr>
<td>Purified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small</td>
<td>8.8</td>
<td>22</td>
</tr>
<tr>
<td>Large</td>
<td>12.3</td>
<td>27</td>
</tr>
<tr>
<td>Infected</td>
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<tr>
<td>Small</td>
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<td>58</td>
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<tr>
<td>Large</td>
<td>45.8</td>
<td>71</td>
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cruzi infection. First, we analyzed the expression of Fas and FasL on B cells from control or T. cruzi-infected mice. By FCM analysis we detected that T. cruzi infection induced marked up-regulation of both Fas (data not shown) and FasL (Fig. 4A) on CD19<sup>+</sup> B cells. Moreover, CD19<sup>+</sup> B cells with FSC<sub>high</sub> show the highest expression of these molecules (data not shown). Accordingly, Percoll-separated large B cells from infected mice exhibit the highest expression of both Fas (Fig. 4B) and FasL (Fig. 4C). In contrast, small B cells from infected mice had only slightly up-regulated Fas and FasL expression (Fig. 4, B and C) compared with controls.

**Figure 3.** Large B cells from T. cruzi-infected mice exhibit the highest level of apoptosis in vivo and in vitro. Small (A and C) and large (B and D) B cell subpopulations from normal (A and B) or from T. cruzi-infected (C and D) mice were separated by a discontinuous Percoll gradient. Hypodiploid DNA content analysis for each isolated subpopulation was performed in freshly explanted (in vivo, left panels) cells or after an 18-h culture with medium alone (in vitro, right panels). M1 indicates the percentage of cells with hypodiploid DNA content. Data are representative of two independent experiments.
Hence, during *T. cruzi* infection Fas and FasL expression is strongly associated with B cell activation status.

To investigate whether Fas/FasL interactions limit B cell expansion during infection in vivo, a neutralizing anti-FasL mAb was administered into infected mice. Anti-FasL mAb was injected twice a week starting on day 4 postinfection and the total number of CD19<sup>+</sup> B cells was evaluated by FCM on day 15. As clearly shown in Fig. 5A, *T. cruzi*-infected mice treated with anti-FasL mAb presented a significant increase in the total number of B cells compared with mice treated with control hamster IgG or PBS. According to this, we observed that in vivo anti-FasL treatment markedly reduces the amount of B cell apoptosis compared with
mice treated with control hamster IgG (data not shown). These results support the idea that Fas/FasL interaction triggers B cell death during in vivo acute T. cruzi infection.

Considering that during T. cruzi infection B cells undergo spontaneous apoptosis in the complete absence of T cells (24), we next investigated whether FasL-mediated B cell-B cell fratricide accounted for the increased rate of B cell apoptosis. Results are shown as the mean of B cell apoptosis ± SD. The results were statistically evaluated by Student’s t test. C, Small and large B cells from normal or T. cruzi-infected mice were cultured during 18 h in presence of 10 μg/ml anti-FasL mAb or isotype-matched control mAb and processed for apoptotic detection by staining with PI. Results are shown as the mean of B cell apoptosis ± SD. The results were statistically evaluated by Student’s t test. D, B cells from T. cruzi-infected mice were cultured during 18 h in presence of 10 μg/ml anti-FasL, (filled histogram) or isotype-matched control (open histogram) mAb. The cells were then analyzed for IgG expression by FCM using FITC-labeled anti-mouse IgG.

**Fas/FasL-mediated fratricide preferentially targets IgG⁺ highly activated B lymphocytes**

In an attempt to evaluate the relative importance of FasL-mediated fratricide in T. cruzi infection, we determined by FACS the percentage of IgG⁺ and IgM⁺ B cells after culture in the presence of anti-FasL. FasL blockade barely decreased the percentage of IgM⁺ B cells from infected mice compared with control isotype (data not shown) but rescued 10% of IgG⁺ B cells (Fig. 5D). These data correspond to the overall antiapoptotic effect of FasL blockade seen in our cultures and indicate that B cells strongly activated by T. cruzi infection develop a killer capacity that preferentially targets cells that have switched to IgG.

**Influence of Fas/FasL B cell fratricide on the humoral immune response profile during T. cruzi infection**

Apoptosis of B cell population may be beneficial or detrimental for the host, depending on the specificity of the clones affected. We evaluated the effect of FasL blockade on the frequency of AFCs.
reactive with either TcAg or mouse SM self Ag. We selected as self Ag mouse SM antigenic fraction because several studies in humans and animals have detected humoral and cellular autoreactivity against this tissue during *T. cruzi* infection and demonstrated that SM is one of the target structures involved in the pathogenesis of this disease (36). The AFC frequency before and after FasL blockade was compared with an isotype control and evaluated by ELISPOT. Cells were processed freshly explanted (open bars) or after culturing during 36 h in the presence of anti-FasL mAb (III) or isotype-matched control mAb (III). The results were statistically evaluated by repeated measures ANOVA and Tukey-Kramer multiple comparisons test.

![FIGURE 6. In vitro FasL blockade selectively rescues *T. cruzi*-specific IgG-secreting cells. The presence of AFCs secreting IgM (upper panels) or IgG (lower panels) reactive to parasite Ags (TcAg, left panels) or self Ags (SM, right panels) were determined by ELISPOT. Cells were processed freshly explanted (open bars) or after culturing during 36 h in the presence of anti-FasL mAb (III) or isotype-matched control mAb (III). The results were statistically evaluated by repeated measures ANOVA and Tukey-Kramer multiple comparisons test.](http://www.jimmunol.org/)

These results indicate that FasL-mediated fratricide selectively targets parasite-specific IgG-producing cells.

**Discussion**

In this study, we demonstrate that B cell apoptosis is an ongoing process in vivo during *T. cruzi* infection that is B cell sufficient and is mediated by the Fas/FasL pathway. Moreover, our data indicate that FasL-mediated B cell fratricide selectively eliminates IgG + activated B cell reactive against parasite but not self Ags. We have previously reported that B cells from *T. cruzi*-infected mice undergo an increased rate of apoptosis in culture with medium alone (24). Herein, we extend these findings by showing that B cells also undergo apoptosis in vivo during infection. These results are consistent with the notion that B lymphocyte death is a hallmark event during the acute phase of many infectious processes (17, 38, 39). We found that susceptibility to apoptosis is influenced by the activation state of the B cell. Our data agree with the proposal that activated B lymphocytes are more prone to die than resting ones (40). Cell death of proliferating lymphocytes is necessary to preserve a healthy and balanced immune system (6). Nevertheless, during an infectious process pathogen-specific lymphocyte apoptosis, at a stage when the pathogen has not been cleared, would be deleterious, as it would not only restrict the magnitude of the effector response but it would also facilitate establishment of the microorganism and thus chronicity of the infection (41). In this regard, the delaying of pathogen-specific lymphocyte apoptosis would be an attractive strategy to enhance protective immunity and eradicate the infectious agent. Therefore,
identification of lymphocyte apoptotic mechanisms triggered during chronic infections emerges as an important issue from a clinical point of view.

Fas-induced apoptosis contributes to the maintenance of homeostasis in both B and T lymphocyte-mediated immunity (42). Fas-mediated apoptosis of T lymphocytes has been reported in several infections (39, 43, 44), including T. cruzi infection, where CD4 T cells up-regulate both Fas and FasL and undergo AICD upon stimulation (23). B cells increase Fas expression and become susceptible to Fas-mediated apoptosis in response to activation signals (45, 46). Accordingly, we found increased Fas expression on B lymphocytes activated in vivo by T. cruzi infection. Our study provides the first experimental evidence of the influence of Fas/FasL pathway on B cell killing in the context of an infectious process. However, the partial reduction of B cell death after FasL blockade suggests that other apoptotic mechanisms also contribute to B cell apoptosis.

B cell killing through Fas was initially thought to be executed only by T cells expressing FasL (1, 9). However, this idea is now controversial, because several recent reports indicate that activated B cells can express FasL (3, 13–17). Furthermore, FasL expressed on B cells activated by S. mansoni soluble egg Ags is functional, because it is able to kill a Fas-bearing target cell line (4). We show that FasL is expressed on highly activated large B cells during acute T. cruzi infection and provide evidence that T. cruzi infection programs B cells to induce Fas/FasL-mediated B cell fratricide. Furthermore, we have found that this death pathway preferentially targets highly activated B cells that have switched to IgG, the main isotype involved in defense and pathogenesis of this parasitic disease (36). Further work is required to elucidate why isotype switching influences susceptibility to FasL-induced death.

The main targets of Fas-mediated B cell apoptosis have so far been shown to be autoreactive lymphocytes (9, 11). Fas-mediated AICD has been shown to be important for eliminating T cells reactive against self Ags but not foreign Ags (47). Nevertheless, these experiments have used simple foreign Ag, and this may not be representative of infectious processes where pathogens replicate intensively in host cells and display a complex antigenicity (including molecular mimetism and mitogens). In keeping with this, it has been proposed that the type of homeostatic process terminating an immune response may vary according to the nature of the Ag (40). Our data indicate that only IgG+ AFCs specific for parasite Ags are rescued by FasL blockade, indicating that during T. cruzi infection this apoptotic mechanism preferentially targets B cells reactive against parasite but not self Ags. The question as to why Ag specificity determines B cell susceptibility to FasL-mediated fratricide remains unanswered. Considering that B cell apoptosis is an immune-regulatory mechanism triggered by the host to control the excessive expansion of these cells, it is likely that T. cruzi-induced massive B lymphocyte activation would be an evasive mechanism developed by this parasite to trigger host homeostatic control and interfere with protective Ab response. Furthermore, a comparable mechanism might also reduce the efficiency of the immune control of other protozoan parasites. Because parasite-specific Ab production is required for full resistance during T. cruzi infection, the increment of T. cruzi-specific IgG production raised by FasL blockade would favor the control of this infection. Our results suggest that the blockade of Fas/FasL pathway specifically in the B cell compartment of T. cruzi-infected mice could provide a means for enhancing antiparasitic humoral responses without affecting host tolerance. However, our findings should be considered with caution. First, this treatment should be conducted during a limited period of acute infection to enhance the protective immunity without promoting immunopathological damage. Second, we do not know whether B cells reactive to tissue-restricted and/or widely disseminated self Ags other than SM could be affected by FasL blockade. Fas-mediated elimination of parasite-specific B cells during acute infection adds to other reports showing that death of effector T lymphocytes limits the ability of the host to achieve complete elimination of the pathogen (43, 48–51).
Acknowledgments
We thank Dr. Carol G. Vinuesa and Dr. George A. Doherty for critically reading the manuscript. We are grateful to Eva Acosta for idiomatic corrections.

References