Experimental Infection with *Trypanosoma cruzi* Increases the Population of CD8⁺, but not CD4⁺, Immunoglobulin G Fc Receptor-Positive T Lymphocytes

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It is well established that activating-type Fc receptors for immunoglobulin G (FcγR), such as FcγRI and FcγRIII, are essential for inducing inflammatory responses. On the other hand, a unique inhibitory FcγR, FcγRIIB, inhibits intracellular signaling upon engagement of immunoglobulin G-immune complexes, suppressing inflammation and autoimmunity. The expression of FcγRIIB on B lymphocytes, natural killer cells, macrophages, mast cells, and a number of other cell types has been demonstrated for many years. However, the expression on T lymphocytes is probably restricted to activated cells in a narrow window of time. The controversy regarding the FcγR expression on T lymphocytes is attributable to considerable heterogeneity of cellular subpopulations and activation stages during immune responses in vivo. We addressed here this question by using mice experimentally infected with *Trypanosoma cruzi*, and we found an increase in the CD8⁺ FcγR⁺ population but not in the CD4⁺ FcγR⁺ population. Moreover, CD8⁺ FcγR⁺ T cells predominantly composed the cardiac inflammatory infiltration induced by the infection. These results indicate a novel pattern of FcγR expression on T cells in a pathological situation, and possible functional roles of this phenomenon are discussed.

Experimental infection with *Trypanosoma cruzi*, the causative agent of Chagas disease, induces dramatic changes in immune cell populations and lymphoid organs. These include splenomegaly, thymus atrophy (16), and polyclonal activation of both T and B cells (20) associated with hypergammaglobulinemia. Cellular inflammatory infiltrations composed mainly of CD8⁺ T cells have been observed in many tissues, including the heart (24) and the esophagus and colon (26) in both humans and murine models. Experiments with nude (*nu/nu*) mice (15, 11), thymectomized mice (25), and mice depleted of CD4⁺ (21) or CD8⁺ T cells (28, 29) have shown that parasite load, mortality, and inflammation depend on T cells. However, T-cell activity during infection can be downregulated by reduced secretion of interleukin-2 and expression of interleukin-2 receptor (12, 27) and activation-induced cell death (AICD) (18) of CD4⁺ T lymphocytes through Fas-based mechanisms (17).

Many stimuli and surface molecules are involved in the regulation of T cells activation and in the full acquisition of effector functions. Receptors for the Fc portion of immunoglobulin G (FcγR) expressed by T lymphocytes were indicated to be among the receptors that control T-cell proliferation and cytokine secretion (9). Three classes of FcγR, namely, FcγRI, FcγRII, and FcγRIII, were identified on many cells of hematopoietic origin (8, 14). FcγRI and FcγRIII are associated with

intracytoplasmic domains that contain immunoreceptor tyrosine-based activation motifs (ITAMs) and activate cells when aggregated at the cell surface. FcγRII are single-chain receptors represented as FcγRIIA/C on human cells and FcγRIIB on both mice and human lymphocytes. FcγRIIA/C also contains an ITAM that can trigger cell activation, whereas FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (31) that can inhibit cell activation when coaggregated with receptors that contain ITAMs (9).

The functional expression of FcγR on T cells in vivo has not been well documented. This is due to considerable variation in T-cell differentiation, maturation, and activation during early development of the murine fetal thymus and adult life. Most Thy⁺ cells at 13 to 16 days of gestation express FcγR (22); however, the receptor was no longer found at 17 days of gestation after the onset of T-cell receptor (TCR) complex expression (19). In adult lymphoid organs and blood, most T lymphocytes are FcγR[−] resting cells, and engagement of the TCR will transiently trigger FcγR expression. There are some exceptions that include some CD4⁺ Thy1⁺ T cells and subpopulations of TCR⁺ intraepithelial lymphocytes of the intestinal tract, where TCR-based cellular activation failed to induce FcγR expression (23).

We have previously described an expansion of total FcγRII/III⁺ lymphocytes in BALB/c mice infected with the Tulahuen strain of *T. cruzi* (1), but the specific cellular populations that were expressing those receptors were not defined. In the present study we examined FcγRII/III expression in splenic lymphocytes during *T. cruzi* infection and observed a selective pattern of FcγR expression on CD8⁺ but not in CD4⁺ lym-

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phocytes. In addition, the majority of CD8⁺ cells in cardiac inflammatory infiltrates were FcγRII/III⁺ cells. Some possible physiopathological implications of this selective expression of FcγR by CD8⁺ T cells during experimental *T. cruzi* infection are discussed.

MATERIALS AND METHODS

Mice and parasites. Male C57BL/6 perforin knockout mice (C57BL/6 background), BALB/c mice, and BALB/c *gld/gld* mice were bred at the animal facilities of BioManguinhos, FIOCRUZ, Rio de Janeiro, Brazil, and were used at 8 to 10 weeks of age (13, 17). The BALB/c and BALB/c *gld/gld* mice were infected intraperitoneally (i.p.) with 5×10^3 metacyclic trypomastigotes of *T. cruzi* clone Dm28c (7). C57BL/6 and perforin knockout mice were infected i.p. with 10^4 blood trypomastigote forms of *T. cruzi* Y strain, and control mice were always uninfected littermates. The FIOCRUZ Committee of Ethics in Research approved this project in accordance with resolution 196/96 of the National Health Council of Brazilian Ministry of Health.

Flow cytometry. (i) Spleen cells. Splenocytes were obtained by mechanical dissociation, and erythrocytes were lysed by hypotonic shock in culture medium RPMI (Gibco, Paisley, Great Britain) diluted 1:10 in water for 10 s on different days postinfection (dpi). The cells were washed in cold phosphate-buffered saline and cells were >95% viable when counted in hemocytometer using trypan blue exclusion. For phenotypic analysis, 10^6 viable cells per sample were incubated with supernatant of rat 2.4G2 hybridoma cells (rat anti-mouse FcγRII/III) in ice and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat F(ab')₂ fragments. For double labeling, the samples were then extensively washed in RPMI and incubated with phycoerythrin (PE)-conjugated CD4- or CD8-specific MAb developed in rat (Caltag Laboratories, Burlingame, Calif.). The cells were washed and fixed in 2% paraformaldehyde (Sigma, St. Louis, Mo.) for subsequent analysis in a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif.). A total of 10,000 events were acquired for each sample, and the lymphocytes were gated for analysis by a combination of forward and side light scatter (FSC and SSC, respectively) and phenotypic T-cell markers (CD3 or CD8).

(ii) Heart inflammatory cells. Cardiac inflammatory cells were collected from adult *T. cruzi*-infected C57BL/6 mice (24). Briefly, at 15 dpi the mice were sacrificed, and the hearts were cut into small pieces for successive enzymatic dissociations with collagenase (0.023%; Sigma). The cells were then subjected to Ficoll-Histopaque (Sigma) centrifugation and fixed in 1% paraformaldehyde for 20 min in ice. The samples were incubated with FITC-conjugated 2.4G2 monoclonal antibody (MAb; Southern Biotech Associates, Birmingham, Ala.) and with PE-conjugated anti-CD4 or with cytochrome-conjugated anti-CD8 MAb (Pharmingen, San Diego, Calif.). A total of 15,000 cells were acquired for each sample, and the lymphocytes were gated for analysis of FcγR expression in T cells by a combination of FSC and SSC and phenotypic double labeling of CD3⁺ and CD8⁺ T-cell markers. Samples incubated with anti-CD3 and anti-macrophage MAb (anti-Mac1; Southern Biotech) or the appropriate isotype controls were regularly run in parallel. Flow cytometry acquisition was carried out in a FACScalibur flow cytometer, and the analysis was performed in the WinMDI software (Multiple Document Interface Flow Cytometry Application, v2.8).

Statistical analysis. The Student *t* test was used to compare two sets of data, and a *P* value of <0.01 was considered statistically significant.

RESULTS

FcγRII/III expression in *T. cruzi*-infected mice. Parasitemia increased during the second week of infection in acutely infected BALB/c mice peaking at 21 dpi and decreasing thereafter. A subpatent level of infection was reached at 35 dpi (Fig. 1, parasitemia). Experimental *T. cruzi* infection also induced splenomegaly and a marked blast cell transformation with proliferation of B and T lymphocytes in the spleen. The number of total spleen cells progressively increased during the acute phase of infection peaked at 1 month and returned to normal levels after 6 weeks (Fig. 1, total cellularity). The number of FcγRII/III⁺ lymphocytes also increased progressively until 28 dpi (~8-fold) and decreased thereafter (Fig. 1, FcR⁺ lymphocytes). However, the percentages of FcγRII/III⁺ lymphocytes

FIG. 1. Parasitemia and FcγR expression in spleen lymphocytes. BALB/c mice were infected with Dm28c clone of *T. cruzi*, and parasitemia (dashed line) was evaluated. The number of total spleen cells (open symbols) and lymphocytes expressing FcγR (closed symbols) were analyzed by flow cytometry in gated lymphocytes, determined according to the expression of CD3 in FSC×SSC dot plots, in uninfected mice (dpi 0) and other days postinfection as indicated.

were not significantly different in uninfected and infected mice at 21 dpi (ca. 41 and 43%, respectively [data not shown]). Similar results were obtained in our previous study with BALB/c mice infected with the Tulahuen strain of *T. cruzi* (1).

To study FcγRII/III expression on subpopulations of T cells, we double labeled spleen cells with 2.4G2 and either anti-CD4 or anti-CD8 MAb. A reproducible increase in CD8⁺ T cells expressing FcγRII/III was observed only after infection (Fig. 2A and B), in contrast to CD4⁺ FcγRII/III⁺ T cells (Fig. 2C and D). The absolute number of CD8⁺ FcγRII/III⁺ cells increased almost 20-fold on dpi 21 (Table 1), ranging from 3×10^5 to nearly 6×10^6 cells after infection. The number of CD8⁺ FcγRII/III⁻ T cells also increased ~5-fold on the same dpi (Table 1). In contrast, a very modest increase in CD4⁺ FcγR II/III⁺ cells was observed in infected mice (Table 1), although we found a 4.8-fold increase in the population of CD4⁺ FcγRII/III⁻ cells, probably induced by splenomegaly. The kinetic analysis of cell number ratio (infected/uninfected) showed an expansion of both CD8⁺ FcγRII/III⁺ and CD8⁺ FcγRII/III⁻ populations (Table 1), but the expansion of the CD8⁺ FcγRII/III⁺ population was more pronounced. We observed no relative expansion of CD4⁺ FcγRII/III⁺ cells and a moderate expansion of CD4⁺ FcγRII/III⁻ from 17 to 35 dpi (Table 1). These results are representative of five independent experiments with at least five mice per group. Although the CD8 molecule is not an exclusive marker of T lymphocytes, we are confident that the CD8⁺ cells examined were primarily, if not exclusively, T cells, since we confirmed our flow cytometry analysis using CD3 and CD8 double labeling and MAC-1 labeling (data not shown).

Using another model of infection with C57BL/6 mice and the Y strain of *T. cruzi*, we also observed a splenomegaly that was completely recovered by the day of spleen cells harvesting (dpi 21) (data not shown). The CD8⁺ FcγRII/III⁺ population showed also a greater expansion over the CD8⁺ FcγRII/III⁻ population. We observed no increase in FcγRII/III⁺ or FcγRII/III⁻ CD4⁺ cells after infection. Taken together, these data confirm the differential FcγRII/III expression in T-cell

FIG. 2. Expression of Fc γ R in subpopulations of T lymphocytes after *T. cruzi* infection. Spleen cells were collected from uninfected (A and C) or BALB/c mice infected with the clone Dm28c of *T. cruzi* (B and D) at 21 dpi. The cells were labeled with 2.4G2 (anti-Fc γ R/III) and anti-CD4 or -CD8 MAb as depicted. The profiles were obtained from gated lymphocytes as described in the text. Numbers inside the dot plots correspond to the percentage of cells in each quadrant.

subpopulations during *T. cruzi* infection, with no interference of splenomegaly.

Since experimental infection with *T. cruzi* elicits AICD of CD4 $^{+}$ spleen T cells triggered by Fas-based mechanisms (17), we examined the possibility that the CD4 $^{+}$ Fc γ R/III $^{+}$ population could have died in vivo before they were collected. We thus infected *gld/gld* mice, which are deficient in FasL-mediated

cytotoxicity (17), and examined Fc γ R/III expression by T-cell subpopulations (data not shown). Again, we observed an increase in CD8 $^{+}$ Fc γ R/III $^{+}$ cells after infection but not in CD4 $^{+}$ Fc γ R/III $^{+}$ cells. Moreover, activated CD8 $^{+}$ T cells play important roles in cytotoxic events mediated by perforin in association with granzymes (30). We therefore infected perforin-deficient mice to evaluate whether the lack of this cytotoxic molecule would alter the expression of Fc γ R/III in CD8 $^{+}$ cells and/or bring forth compensatory mechanisms that could induce Fc γ R expression in CD4 $^{+}$ T cells. However, we observed once more the expansion of only CD8 $^{+}$ cells expressing Fc γ R/III (data not shown).

Expression of Fc γ R/III in cardiac inflammatory cells. Since the cardiac inflammatory infiltrates are enriched in CD8 $^{+}$ T cells after *T. cruzi* infection (26), we evaluated the expression of Fc γ R/III on these cells. Figure 3A shows that heart inflammatory cells recovered from the dissociated tissue are identified by flow cytometry using a combination of morphological data (FSC $\times$ SSC) and the labeling with T-cell markers (CD3 and CD8), which allowed the setting of a lymphocyte window (polygon R1 in Fig. 3A). Phenotypic characterization of macrophages (anti-Mac1) in parallel samples confirmed that they do not fit in the same region (data not shown). In accordance with the negative control (Fig. 3B), the data indicated that CD8 $^{+}$ T cells are a most common T-cell subset in cardiac inflammatory infiltrates (Fig. 3C and D). In addition, we observed that virtually all CD8 $^{+}$ cells expressed Fc γ R/III (Fig. 3C), whereas <10% of the cells were CD4 $^{+}$ (Fig. 3D). The high frequency of Fc γ R/III expression by cardiac inflammatory cells could also be observed in 2.4G2-stained heart sections from infected mice (Fig. 3E). Results obtained with a negative isotype control are shown in Fig. 3F.

DISCUSSION

In the present study we investigated the expression of Fc γ R/III on subpopulations of T cells during the acute phase of experimental *T. cruzi* infection. We found increased numbers of spleen CD8 $^{+}$ Fc γ R/III $^{+}$ T cells, but not of CD4 $^{+}$ Fc γ R/III $^{+}$ T cells, and that CD8 $^{+}$ Fc γ R/III $^{+}$ T cells mostly compose cardiac inflammatory infiltrates. The increased numbers of CD8 $^{+}$ Fc γ R/III $^{+}$ T cells observed during *T. cruzi* infection could have several explanations. It could be the mere consequence of the increased cellularity (splenomegaly) resulting from polyclonal activation that is induced by the parasite. However, we observed a comparable increase in CD8 $^{+}$ Fc γ R/III $^{+}$ T cells in an infection model with no interference of splenomegaly (data not shown). This indicates that the numbers of CD8 $^{+}$ Fc γ R/III $^{+}$ T cells selectively increased during infection and to our knowledge this is the first time that the receptor is observed only in a subpopulation of T cells. This increase could be due to (i) the expansion of a preexisting pool of CD8 $^{+}$ Fc γ R/III $^{+}$ T cells, (ii) a differentiation of CD8 $^{+}$ Fc γ R/III $^{-}$ T cells into Fc γ R/III $^{+}$ T cells, or (iii) a targeted death of CD4 $^{+}$ Fc γ R/III $^{+}$ T cells. The third possibility was really conceivable, since during experimental infection with *T. cruzi*, CD4 $^{+}$ splenocytes die in vitro by AICD and in vivo through apoptotic mechanisms triggered by Fas/Fas-L interaction (17, 18). To investigate whether the CD4 $^{+}$ Fc γ R/III $^{+}$ population died in vivo before being harvested for phenotypic

TABLE 1. Spleen T-cell subsets in relation to FcR expression during the course of *T. cruzi* infection in BALB/c mice

Subset	Status ^a	Absolute number of cells (10 ⁶) or ratio of infected/uninfected cells at:			
		17 dpi	21 dpi	28 dpi	35 dpi
CD8 $^{+}$ Fc $^{+}$	No infected	4.3	5.6	6	4.4
	No uninfected	0.3	0.3	0.2	0.2
	Ratio INF/UN	14.3	18.7	30.0	22.0
CD8 $^{+}$ Fc $^{-}$	No infected	10.7	21.2	40.1	27.6
	No uninfected	1.8	3.9	2.5	4.1
	Ratio INF/UN	5.9	5.4	16.0	6.7
CD4 $^{+}$ Fc $^{+}$	No infected	ND ^b	1.4	1.8	0.5
	No uninfected	ND	0.4	0.4	0.1
	Ratio INF/UN		3.5	4.5	5.0
CD4 $^{+}$ Fc $^{-}$	No infected	24.7	31.4	43.1	36.3
	No uninfected	3.7	6.6	7	10.7
	Ratio INF/UN	6.7	4.8	6.2	3.4

^a Values are absolute numbers of cells (10⁶). Ratio INF/UN, ratio of infected to uninfected mice.

^b ND, not determined.

FIG. 3. Phenotypic profile of cardiac inflammatory cells. (A) For flow cytometry analysis, the hearts were collected from C57BL/6 mice on the dpi 15, and the inflammatory cells were obtained by collagenase digestion as described in Materials and Methods. (C to D) The samples were incubated with isotype control (B) or FITC-conjugated 2.4G2 (rat anti-murine FcγRII/III) (C and D) and PE-conjugated goat anti-mouse CD8 (C) or anti-mouse CD4 (D). For immunohistopathology, the slices were obtained from BALB/c mice infected with Dm28c clone of *T. cruzi* on dpi 21. (E) The samples were incubated with 2.4G2 and then with anti-rat peroxidase-conjugated immunoglobulin. (F) The negative control represents the incubation with IgG2a isotype with further incubation with anti-rat peroxidase-conjugated immunoglobulin. Arrows indicate nuclei, and arrowheads indicate specific labeling.

analysis, we infected Fas-L-deficient *gld/gld* mice. However, we found essentially the same polarized expression of FcγR only on CD8⁺ T cells. The undetectable expression of FcγR on CD8⁺ T cells in uninfected mice favors the second possibility and that may be the most likely interpretation.

Instead, with regard to CD4⁺ T cells, it is possible that FcγR expressed by these cells had been released from the cell membrane as a soluble molecule. We found previously that large amounts of soluble FcγR are released in vitro by splenocytes from *T. cruzi*-infected mice (1). If so, it will be interesting to explore the selective release of FcγR from CD4⁺ T cells. Finally, the most attractive possibility is that, indeed, CD4⁺ T cells do not express FcγR in *T. cruzi* infection, which selectively upregulates FcγR on CD8⁺ T cells. The receptors were detected by using the MAb 2.4G2 that recognizes both mouse FcγRII and FcγRIII. If FcγRIII was reported on T-cell precursors (22), it were not found, to our knowledge, on mature T

lymphocytes. In contrast, only FcγRIIB was positively identified on murine hybridoma, thymomas, and lymphoma T cells at both the level of transcripts and the level of membrane proteins (3, 9, 2).

FcγR and immunoglobulin G are pivotal elements in the regulation of both physiological and pathological immune responses. Negative regulation exerted by FcγRIIB was first demonstrated in vitro on B-cell activation (5) and later confirmed in vivo with genetically modified mice. FcγRIIB-deficient mice were indeed shown to mount enhanced antibody responses (32). FcγRIIB were also shown to critically determine the outcome of antibody-based immunotherapy of experimental tumors (6). FcγRIIB-dependent negative regulation of FcγRI-mediated mast cell activation was also demonstrated first in vitro (10) and later confirmed in vivo. Finally, in vitro experiments also demonstrated that T-cell activation could be negatively regulated upon coaggregation of TCR with

Fc γ RIIB (9). Besides, we observed the in vivo binding of immunoglobulin G to cardiac epitopes after infection (data not shown) in accordance with previous data published (4). In the present study we have shown that the receptor is expressed on T cells and we can hypothesize that Fc γ R also plays a negative role in the regulation of T-cell activity during *T. cruzi* infection, particularly in the development of myocarditis. When bound to cardiac cells by their Fab portions, immunoglobulin G antibodies would expose their Fc portion that could interact with Fc γ R-bearing CD8⁺ T lymphocytes during TCR/major histocompatibility complex peptide engagement. The concomitant gathering of TCR and Fc γ R with their respective ligands could coaggregate both receptors on T cells and, as a consequence, modulate cytotoxic and/or secretory functions of inflammatory T cells. This possibility is currently under investigation in our laboratory.

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