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Trypanosoma cruzi-Induced Immunosuppression: Selective Triggering of CD4$^+$ T-Cell Death by the T-Cell Receptor–CD3 Pathway and Not by the CD69 or Ly-6 Activation Pathway

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In a model of experimental Chagas' disease induced with metacyclic forms of *Trypanosoma cruzi*, CD4$^+$ but not CD8$^+$ T cells undergo T-cell receptor (TCR)-CD3-mediated activation-induced cell death (AICD) in vitro. CD4$^+$ T cells from *T. cruzi*-infected mice also develop unresponsiveness in proliferative responses to TCR-CD3-mediated stimulation. A linear correlation was found between extent of proliferative unresponsiveness and loss of CD4$^+$ T-cell viability. CD4$^+$ T-cell activation through the CD69 or Ly-6 A/E pathway, on the other hand, did not result in proliferative unresponsiveness compared with controls. Lack of suppression in proliferation assays correlated with lack of AICD by cells stimulated through the CD69 or Ly-6 A/E pathway. Concomitant stimulation through CD69, however, did not rescue CD4$^+$ T cells from CD3-induced death. Flow cytometry study of cells stimulated in vitro showed no defect in interleukin-2 receptor expression by CD4$^+$ T cells from infected donors, which escaped TCR-mediated AICD. In vivo injection of anti-CD3 into acutely infected mice, but not into control mice, led to splenocyte DNA fragmentation and failed to increase splenic CD4$^+$ T-cell numbers. These results show that TCR-CD3-mediated AICD is involved in CD4$^+$ T-cell unresponsiveness in vitro following infection with *T. cruzi*. In addition, successful activation of these cells through the CD69 and Ly-6 pathways is due to differences in the inability of these stimuli to trigger AICD. Since TCR-CD3-mediated AICD can be induced in vivo in infected mice, these findings may be relevant for the onset of immunological disturbances in the host.

Infection of humans with the intracellular protozoan parasite *Trypanosoma cruzi* causes Chagas' disease, which affects 18 million people in Latin America (5). To understand pathogenic mechanisms linking abnormal functioning of the immune system to infection by *T. cruzi*, a great effort has been devoted to developing experimental models of the disease. Experimental infection of mice with *T. cruzi* leads to disturbances in the host immune system and to cardiac inflammatory damage associated with a reduced but persistent load of parasites within the host tissues (23). Immunosuppression of the T-cell compartment in vivo (27) and in vitro (8, 9, 19, 23, 24, 30, 32) is a hallmark of the experimental disease and has been implicated in both parasite persistence and late consequences of the chronic infection (9, 19). Different T-cell-suppressive mechanisms which could play a role in abnormal immune responses of the host have been identified. These mechanisms include suppressor cell activity by CD8$^+$ T cells (30), macrophages (8, 30), and suppressive factors released by the parasite (2). Apart from suppressive factors released during the inflammatory response, previous studies (8, 30, 31) also observed an intrinsic T-cell defect during acute infection, but its cellular and molecular bases were not identified. We have recently demonstrated that in a model of experimental murine infection with *T. cruzi* metacyclic trypomastigotes, CD4$^+$ T-cell unresponsiveness develops in vitro in the absence of CD8$^+$ T cells or accessory cells (15). In this model, the functional T-cell defect can be induced through the T-cell receptor (TCR)-CD3 pathway but not through the CD69 activation pathway (15). More recently, we found in the same model infection that CD4$^+$ but not CD8$^+$ T cells undergo activation-induced cell death (AICD) by apoptosis when stimulated in vitro with either mitogens or anti-TCR-CD3 monoclonal antibodies (MAbs), in the absence of any exogenously added lymphokines (16). Infection with insect vector-derived metacyclic forms also primes host T cells for AICD in vitro (6). These results prompted us to investigate whether TCR-CD3-mediated AICD could be one intrinsic defect leading to in vitro unresponsiveness in CD4$^+$ T cells from *T. cruzi*-infected hosts. In this investigation, we found that TCR-CD3-mediated AICD is a determinant factor of CD4$^+$ T-cell unresponsiveness in vitro and that CD4$^+$ T-cell stimulation through accessory CD69 and Ly-6 pathways results in activation but not in programmed cell death. We also demonstrate that infection primes the host lymphocytes for TCR-CD3-mediated apoptosis in vivo.

MATERIALS AND METHODS

Mice. Male BALB/c mice (6 to 8 weeks old) were obtained from the Animal Section, Institute of Microbiology, Federal University of Rio de Janeiro.

Infection with *T. cruzi*. Mice were infected subcutaneously (10^5 parasites per 0.1 ml) with chemically induced metacyclic trypomastigotes from the *T. cruzi* Dm28c clone (4). Uninfected littermates were used as controls. Chemically induced metacyclic forms were obtained with triatomine artificial urine-proline medium as described by Contreras et al. (4). Parasitemia peaked between 18 and 26 days of infection.

T cells. Primary T-cell-enriched populations, from control or infected donors, were obtained by nylon wool filtration of unfractionated splenic cell suspensions previously depleted of erythrocytes by treatment with Tris-buffered ammonium chloride. CD4$^+$ T cells were obtained from enriched T cells by antibody and complement lysis of CD8$^+$ T cells as previously described (16). Viable cells were recovered after centrifugation in Ficoll-Hypaque. By flow cytometry, 92 to 97% of the resulting T cells were CD4$^+$ and 1 to 3% were CD8$^+$ in both control and infected groups. In most experiments, endogenous non-T cells were not depleted and were used as accessory cells (AC) for stimulating CD4$^+$ T cells with mitogenic antibodies. In some experiments, highly purified CD4$^+$ and/or CD8$^+$ T cells were obtained by magnetic bead separation as described previously (16). Contaminating CD8$^+$ and major histocompatibility complex class II$^+$ cells were less than 1.0% of T cells in control and infected groups. Proliferation in this case

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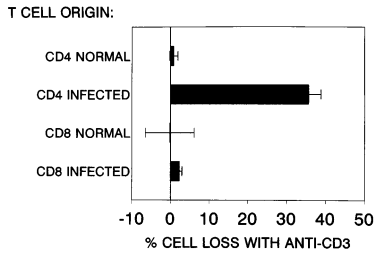

FIG. 1. TCR-CD3 stimulation of cells from *T. cruzi*-infected donors results in $CD4^+$ but not $CD8^+$ T-cell death. Highly purified $CD4^+$ or $CD8^+$ T cells were obtained as described in Materials and Methods. Cells were cultured with PMA alone or in anti-CD3-coated wells plus PMA for 20 h, and viable cells were counted. Mean counts of cultures with PMA alone were taken as the 100% control. Percent cell loss was calculated as described in the text. The means and standard deviations of replicate cultures are shown.

was induced in the presence of normal syngeneic, irradiated (3,000 rads) splenocytes as AC (10^5 $CD4^+$ T cells plus an equal number of AC).

T-cell proliferation and viability assays. $CD4^+$ T cells from either control or infected donors were cultured (1×10^5 to 2×10^5) in Dulbecco modified Eagle medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 10 μg of gentamicin per ml, sodium pyruvate, minimal essential medium nonessential amino acids, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Cultures (0.2 ml) were established in 96-well flat-bottom microtiter plates (Corning) and incubated for either 20 h or 3 days at 37°C and 5% CO_2 in a humid incubator. All cultures were treated with the phorbol ester phorbol myristate acetate (PMA; Sigma) at 5 ng/ml. Some of the wells were previously treated with anti-CD3ε MAb 145-2C11 (0.2% ascites) in medium without serum for 4 h at 37°C, washed with Hanks' solution–10% FCS, and incubated with Hanks' solution–10% FCS to saturate available sites on the plastic surface. Other cultures received soluble MAbs directed to CD3 (145-2C11; 1/20,000 dilution of ascites fluid), TCR αβ (H57.597; 5% culture supernatant), CD69 (H1.2F3; 10 μg of ascites salt cut per ml), and Ly-6 A/E (D7; 0.2% ascites fluid). In additional experiments (not shown), cultures were stimulated with anti-CD69 and anti-Ly-6 MAbs at doses of up to 100 μg of ascites salt cut protein per ml. For proliferation assays, cultures were pulsed with 0.5 μCi of tritiated thymidine (Sigma) 8 h before harvest. Cultures were harvested with a cell-harvesting device, and the amount of thymidine incorporated into DNA was assessed by liquid scintillation spectroscopy. All cultures were done in triplicate. The standard error of the mean was less than 10% of the mean values and is omitted for simplicity. Results are presented as the ratio between proliferation values (counts per minute) and the number of viable cells (either added at the start of culture or counted in replicate cultures after 20 h). In one study (Fig. 3), proliferation is shown as the ratio of counts per minute between infected and control donors for each stimulus used. Viability in this case was the viable cell count ratio between infected and control donors. For all viability assays, replicate cultures identical to those used for proliferative responses were gently dispersed with a Pasteur pipette after 20 h, and the mean counts of two aliquots of each individual well were determined by trypan blue exclusion. Three or two independent replicate cultures were counted for each treatment. Results were normalized as percent viable cell recovery (means and standard deviations), taking the mean viable cell count in unstimulated (PMA alone) cultures as the 100% control. In some experiments, results were expressed as percent cell loss with immobile anti-CD3 plus PMA, taking viable cell recovery in the presence of PMA alone as 100% recovery, according to the following formula: % cell loss = 100 − (cell number with anti-CD3) × 100/(cell number with PMA).

In vivo injection of anti-CD3. Both infected and control mice were injected into the hind footpads with a total of 4 μg of anti-CD3 MAb 145-2C11 or left uninjected. Twenty hours later, animals were sacrificed and spleen cell counts were performed. Whole splenocytes were analyzed for CD4 staining by flow cytometry, and total $CD4^+$ T-cell numbers were determined. In addition, DNA was extracted from freshly explanted splenocytes (2×10^6) and run on agarose gels as described previously (16).

Flow cytometry analysis. Nylon-passaged spleen cell suspensions were either directly stained or cultured (4×10^6/ml) for 2 days in the presence of PMA (5 ng/ml) with either anti-TCR MAb H57 (5% supernatant) or anti-CD69 MAb H1.2F3 (10 μg/ml). Viable cells were separated from dead cells by centrifugation over a Ficoll gradient, washed, and preincubated with a saturating dosage of anti-Fc receptor MAb 2.4G2 (10 μg/ml). Cells were then directly stained (10^6/0.1 ml) with fluorescein isothiocyanate (FITC)-labelled anti-CD25 and phycoerythrin (PE)-labelled anti-CD4 or PE-labelled anti-CD8 MAbs (all at 10 μg/ml) in sorter buffer consisting of phosphate-buffered saline, 0.002% azide, and 3% FCS for 30 min on ice. Cells were washed and fixed (1% paraformaldehyde) until analysis. Cells were acquired (7,000 events per group) and analyzed for two-parameter immunofluorescence on a Becton Dickinson FACScan flow cytometer.

MAbs. Anti-CD8 MAb 53.6.7, anti-B220 MAb 6B2, anti-rat immunoglobulin MAb MAR 18.5, anti-major histocompatibility complex class II MAb AMS 32.1 (Pharmingen, San Diego, Calif.), and anti-MAC 1 MAb M1/70 (Pharmingen) were used to purify T-cell subsets. FITC-labelled anti-CD25 MAb 7D4, PE-labelled anti-CD4 MAb GK 1.5, PE-labelled anti-CD8 MAb 53.6.7, and anti-FcγII/III receptor MAb 2.4G2, used in flow cytometry studies, were purchased from Pharmingen. Hamster MAbs 145-2C11, directed to murine CD3ε (13), H1.2F3, directed to the murine homolog of the human CD69 molecule (34), and H57.597, directed to murine TCR αβ (10), and rat MAb D7, directed to the murine Ly-6 A.2/E.1 molecule (17), were used to activate T cells. These activating MAbs were kindly donated by Ethan Shevach, National Institutes of Health, Bethesda, Md., except for MAb H57, which was obtained from Maria Bellio, Institut Pasteur, Paris, France.

RESULTS

TCR-CD3-mediated AICD leads to in vitro $CD4^+$ T-cell unresponsiveness. In this metacyclic model of *T. cruzi* infection, TCR-CD3-mediated AICD is a prominent feature seen in vitro at peak parasitemia. This phenomenon can be observed when highly purified T-cell subsets are stimulated by immobilized anti-CD3 antibody in the absence of AC (Fig. 1). Only $CD4^+$ T cells from infected donors are killed to a significant extent (Fig. 1). This result was reproduced in two additional experiments. Cell killing is variable, depending on the form and dose of the anti-TCR-CD3 MAb used. Immobile anti-CD3 induced comparable $CD4^+$ T-cell AICD in the infected group in the absence of PMA (not shown). To investigate the contribution of AICD to $CD4^+$ T-cell unresponsiveness (15), $CD4^+$ T cells, containing endogenous AC, were cultured in PMA with anti-TCR-CD3 MAbs or with MAbs directed to either CD69 or Ly-6 A/E accessory molecules, and viability was measured after 20 h in culture (Fig. 2A). The use of endogenous AC, PMA, and soluble MAbs is necessary for triggering

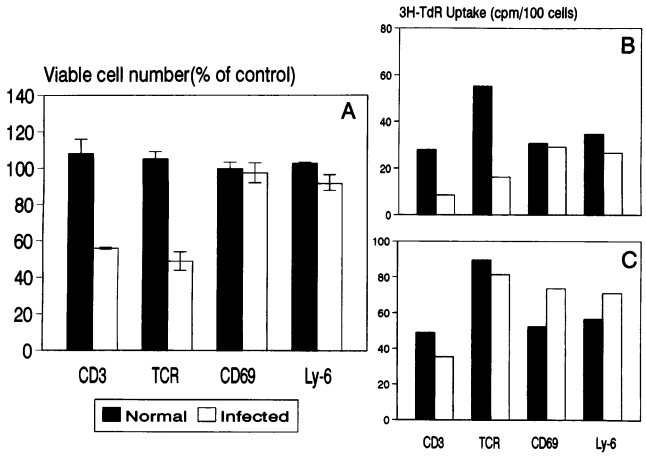

FIG. 2. Differential effects of TCR-CD3, CD69, and Ly-6 A/E pathways on $CD4^+$ T-cell AICD. Viable $CD4^+$ T cells containing endogenous AC were isolated from infected (day 22) and control littermates. Cells were cultured with PMA alone or with PMA plus the following stimuli (at the doses specified in Materials and Methods): CD3, anti-CD3-coated wells; TCR, soluble anti-αβ TCR MAb; CD69, soluble anti-CD69 MAb; Ly-6, soluble anti-Ly-6 A/E MAb. Viability was assessed after 20 h in culture. Proliferation was assessed in sister cultures by tritiated thymidine uptake after 3 days in culture. (A) TCR-CD3, but not CD69 or Ly-6, stimulation causes AICD. Viable cell counts (shown as percentage of viability in unstimulated cultures) in control and infected groups were compared for each stimulus. (B) Proliferative responses of cultures identical to those in panel A, as assessed after 3 days in culture. Results are the ratio of proliferation (counts per minute) values per 100 viable cells. Unstimulated (PMA alone) cultures gave 3.3 (control) and 2.2 (infected) cpm/100 cells. (C) Proliferative responses measured after 3 days in culture, but expressed as a function of the actual number of viable cells, remaining after 20 h in culture. Unstimulated cultures gave 5.6 (control) and 5.5 (infected) cpm/100 viable cells.

mitogenicity with both anti-CD69 and anti-Ly-6 MAbs (17, 34). Stimulation of $CD4^+$ T cells from infected donors with either anti-CD3 or anti-TCR MAbs resulted in a substantial degree of AICD (Fig. 2A; 48.9% viable cell recovery for soluble anti-TCR and 59.0% for immobile anti-CD3). Stimulation of $CD4^+$ T cells from control littermates did not result in AICD (Fig. 2A; 105.1% viable cell recovery for anti-TCR and 97.2% for immobile anti-CD3). On the other hand, stimulation with either anti-CD69 or anti-Ly-6 A/E MAbs (Fig. 2A), although inducing T-cell proliferation, did not result in AICD by $CD4^+$ T cells from either infected (97.5 and 92.2% viable cell recovery for anti-CD69 and anti-Ly-6, respectively) or control (99.7 and 102.7%) mice. The results shown in Fig. 2A were reproduced in additional experiments; both anti-CD69 and anti-Ly-6 failed to induce AICD even at doses of 20 and 100 μg/ml, nor did they do so in an immobilized form (not shown). These doses are much in excess of the amounts needed to induce T-cell activation. Maximal proliferative responses were obtained at 1 μg/ml for anti-CD69 and 10 μg/ml for anti-Ly-6 MAbs. A reduced proliferation in response to TCR-CD3-dependent stimuli in the infected group resulted largely from AICD. The results of proliferative responses were compared either as a function of the original number of viable cells added at the start of culture (Fig. 2B) or as a function of the actual number of viable cells measured after 20 h of incubation in replicate cultures (Fig. 2C). Suppression of the proliferative response of $CD4^+$ T cells to either a CD3 (30.3% of control) or a TCR (29.2% of control) stimulus, but not to CD69 (94.8% of control) or Ly-6 A/E (77.6% of control), was observed (Fig. 2B). However, if proliferation after 3 days was correlated with the actual number of viable cells found after 20 h in culture (Fig. 2C), $CD4^+$ T cells from infected donors gave responses comparable to those of the control group for both CD3 (84.9% of control) and TCR (90.9% of control) stimuli. In this case, the responses to both CD69 (133.1% of control) and Ly-6 A/E (125.6% of control) were slightly greater than in the controls. These results indicate that cell death accounts for $CD4^+$ T-cell unresponsiveness. In some but not all experiments, there is also spontaneous cell death in unstimulated cultures from infected mice. Spontaneous cell death appears to be responsible for the fact that suppression of proliferation (Fig. 2B) was higher than the level of AICD seen in Fig. 2A. To confirm that $CD4^+$ T cells and not non-T cells were proliferating in response to anti-Ly-6 and anti-CD69 stimuli, we used AC-depleted, highly purified $CD4^+$ T cells stimulated with anti-CD3, anti-CD69, and anti-Ly-6 in the presence of PMA along with irradiated splenocytes as AC. The response to anti-CD3 was suppressed by 75% in the infected group (45,323 cpm) compared with the control group (178,466 cpm). The response to anti-CD69 (51,550 cpm), however, was 70% of the control response (74,693 cpm). The response to anti-Ly-6 was higher in the infected (142,861 cpm) than in the control group (104,863 cpm). These results confirmed that differences in proliferation were due to T cells. Overall, the data indicate that those $CD4^+$ T cells from infected mice escaping cell death responded in a manner comparable to or even better than that of control T cells. In addition, data from two separate experiments using different stimuli were analyzed to compare the extent of reduction in $CD4^+$ T-cell proliferation with reduction in cell viability (Fig. 3). A strong linear correlation was found, with a nominal slope approaching 1.0 (estimated slope, 0.97; Fig. 3). This result confirmed that cell death is a cause of in vitro $CD4^+$ T-cell unresponsiveness in this metacyclic model of *T. cruzi* infection.

Flow cytometry analysis of $CD4^+$ T-cell activation in vitro. We assessed expression of interleukin-2 (IL-2) receptors following in vitro stimulation with either anti-TCR or anti-CD69 MAbs (Fig. 4). Flow cytometric analysis of freshly explanted nylon-passaged splenic cells from control (Fig. 4A) and *T. cruzi*-infected (Fig. 4B) mice shows only 21.2% $CD4^+$ T cells in the infected sample, compared with 48.7% $CD4^+$ T cells in the control donor, in this and two additional experiments. Depletion of $CD4^+$ T cells could also be seen in whole splenocytes from infected donors. After in vitro stimulation with anti-TCR for 2 days, the resulting $CD4^+$ viable T cells displayed markedly increased levels of surface IL-2 receptors in both control and infected groups (Fig. 4C and D). The proportion of IL-2 receptor-positive $CD4^+$ T cells was higher in infected (95.1%) than control (71.8%) samples. Similar observations were made in two additional experiments. When the same unfractionated, nylon-passaged cells (containing endogenous AC) were stimulated with anti-CD69, a somewhat lower proportion of $CD4^+$ T cells became activated than after anti-TCR stimulation (Fig. 4E and F). Viable $CD4^+$ T cells from infected donors also responded better (72.5% IL-2 receptor positive) than those from the control group (47.6% IL-2 receptor positive). The finding that the mean fluorescence intensities of $CD4^+$ T cells responding to anti-CD69 and to anti-TCR were similar (Fig. 4) further indicates that the dose of anti-CD69 used (10 μg/ml) was optimal for T-cell activation. Together with results of proliferation assays, these results indicate the absence of any functional defect in the remaining viable $CD4^+$ T cells from infected mice. In the infected group, stimulation with anti-CD69 resulted in 30% enrichment of $CD4^+$ cells after 2 days compared with anti-TCR stimulation. This relative enrichment is, however, underestimated, since anti-CD69 stimulates $CD8^+$ cells better than $CD4^+$ cells, as observed in cultures of control mice (not shown).

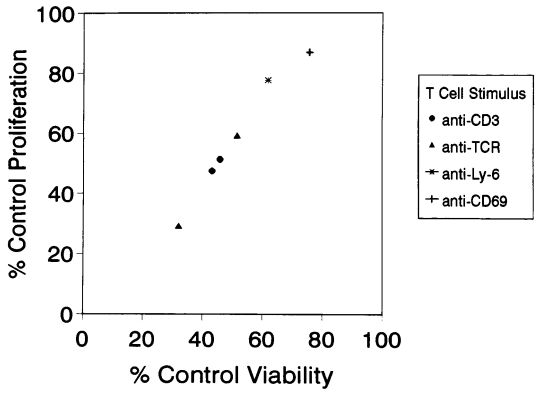

FIG. 3. Linear correlation between $CD4^+$ T-cell death and suppression of $CD4^+$ T-cell proliferation in *T. cruzi*-infected donors. $CD4^+$ T cells from infected and control donors were assayed for proliferative responses to the indicated stimuli after 3 days, and viable cell counts were determined after 20 h. Mean proliferation and viability data from control donors were taken as 100%. Data represent percentages of control values determined for infected donors in response to different stimuli in two independent experiments. Different levels of suppression in response to anti-TCR were obtained by using soluble anti-TCR (which is more potent for the induction of suppression) in one experiment and immobilized anti-TCR in the other. All experiments were done in the presence of PMA. Data fitted a straight line, with a correlation coefficient of 0.98 and an estimated slope of 0.97.

Activation mediated by CD69 fails to rescue cells from CD3-mediated AICD. We also investigated whether concomitant activation mediated by both CD3 and CD69 could rescue $CD4^+$ T cells from death (Fig. 5). To avoid competition of different soluble antibodies for Fc receptor-bearing AC, cells were concomitantly stimulated with immobilized anti-CD3 and soluble anti-CD69. $CD4^+$ T cells from *T. cruzi* infection are

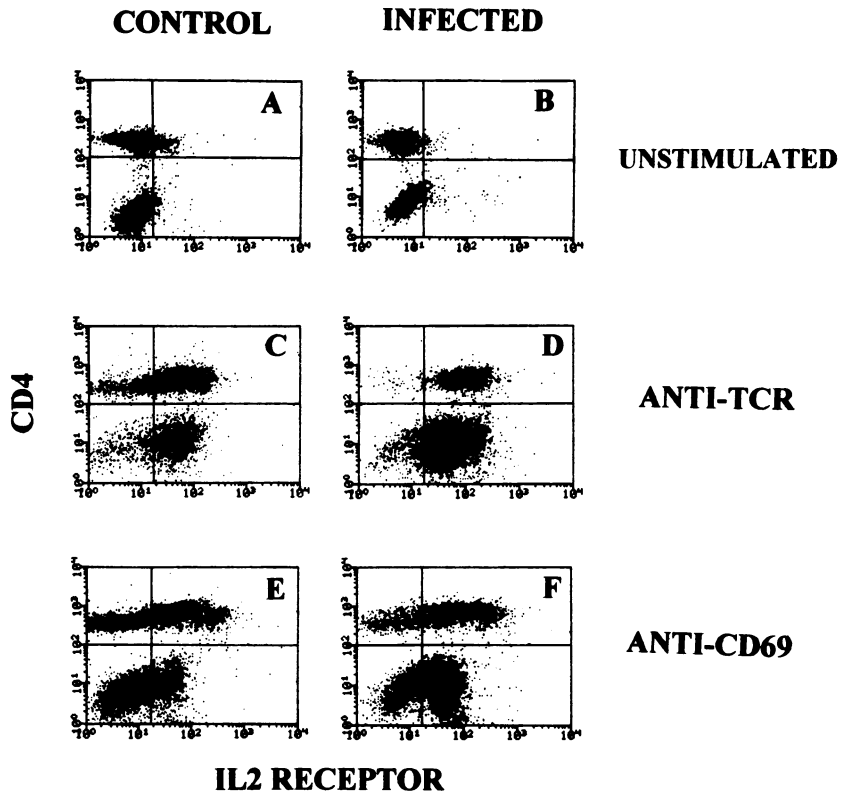

FIG. 4. Flow cytometry analysis of IL-2 receptor expression by $CD4^+$ T cells. Nylon-passaged splenic cells from control or *T. cruzi*-infected donors were analyzed for simultaneous CD4 (*y* axis) and IL-2 receptor (*x* axis) expression, either freshly explanted from donors (A and B) or after a 3-day stimulation in vitro (C to F). (A and B) Control littermate (A) and infected donor (B) at day 19 of infection; cells passaged through nylon columns and directly stained. (C to F) Control littermate (C and E) and infected donor (D and F) at day 25 of infection; nylon-passaged cells cultured for 3 days with 5% supernatant of anti-TCR MAb (C and D) or with 10 μg of anti-CD69 MAb per ml (E and F) in the presence of PMA (5 ng/ml). Viable cells were isolated from the Ficoll interface, washed, and stained. All groups received anti-Fc receptor MAb 2.4G2 and a mixture of PE-labelled anti-CD4 MAb GK 1.5 plus FITC-labelled anti-IL-2 receptor MAb 7D4. Seven thousand events were acquired. Total $CD4^+$ cell counts (upper left plus upper right quadrants) were 48.7% (A), 21.1% (B), 63.8% (C), 30.7% (D), 57.6% (E), and 39.7% (F). Levels of IL-2 receptor-positive cells within $CD4^+$ cells were 7.6% (A), 15.2% (B), 71.8% (C), 95.1% (D), 47.6% (E), and 72.5% (F). Note that analyses in panels A and B are independent of those in panels C to F.

killed after 20 h in culture with immobile anti-CD3 plus PMA but not with soluble anti-CD69 plus PMA. Control $CD4^+$ T cells were not killed by any treatment. Concomitant stimulation with both anti-CD3 and anti-CD69 plus PMA, however, did not rescue $CD4^+$ T cells from CD3-mediated death (Fig. 5). This result was reproduced in a repeat experiment using even higher doses of anti-CD69.

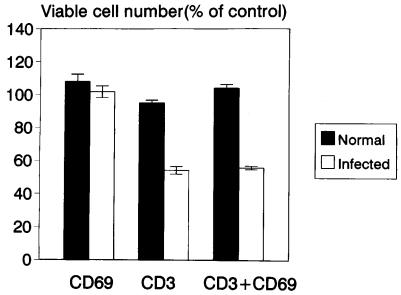

FIG. 5. Stimulation through CD69 does not rescue $CD4^+$ T cells from CD3-induced death. $CD4^+$ T cells (containing endogenous AC) were obtained from control and infected (day 26) littermates as for Fig. 2. Cells were cultured for 20 h in PMA alone, with PMA plus anti-CD3-coated wells, with PMA plus soluble anti-CD69, or with PMA plus both immobile anti-CD3 and soluble anti-CD69 in the same wells. Viable cell counts were determined for control and infected groups as described in Materials and Methods. Each antibody alone induced a vigorous proliferative response in the control group.

Apoptosis in vivo induced by anti-CD3 injection. Since TCR-CD3-mediated AICD was characterized only in vitro, we also investigated whether *T. cruzi* infection primes for anti-CD3-mediated apoptosis in vivo. Infected and control mice were injected (or not) with anti-CD3 at a time point (day 25) when infection-related splenocyte apoptosis was already diminished (16). Anti-CD3 injection into control mice resulted in a 2.3-fold increase in splenic $CD4^+$ T-cell numbers after 20 h, but no significant DNA fragmentation could be detected in spleens (Fig. 6). The splenic $CD4^+$ T-cell number of infected mice was already increased, and anti-CD3 injection had no significant effect on this parameter (Fig. 6). On the other hand, anti-CD3 induced DNA fragmentation in the spleen of the infected recipient (Fig. 6). These results show that infection with *T. cruzi* also primes for TCR-CD3-mediated AICD in vivo.

DISCUSSION

In this study, we demonstrate that TCR-CD3-mediated AICD causes in vitro $CD4^+$ T-cell unresponsiveness in mice infected with metacyclic forms of *T. cruzi*. On the other hand, both CD69 and Ly-6 A/E T-cell activation pathways were unable to induce cell death over a wide range of concentrations of

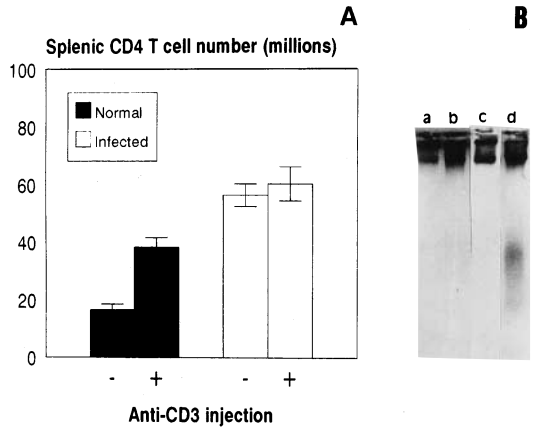

FIG. 6. Infection primes for anti-CD3-mediated apoptosis in vivo. Infected and control mice (two per group) were either uninjected or injected in the hind footpads with a total of 4 μg of anti-CD3 MAb. Twenty hours later, animals were sacrificed. Spleen cell number was determined, and percentage of $CD4^+$ T cells was evaluated by flow cytometry (A). The standard error of the mean is also shown. Splenocyte DNA was isolated and run on an agarose gel (B). Lanes: a, uninjected control DNA; b, anti-CD3-injected control DNA; c, uninjected infected DNA; d, anti-CD3-injected infected DNA.

anti-CD69 and anti-Ly-6 MAbs. In addition, these alternative activation pathways could mediate activation in $CD4^+$ T cells from infected mice in a manner comparable to that in controls. These conclusions derived from the following observations: (i) both CD69 and Ly-6 A/E pathways failed to mediate AICD and also failed to induce a significant suppression of proliferative $CD4^+$ T-cell responses in infected mice; (ii) there was a linear correlation (slope of 1.0) between percent reduction of proliferative response and percent reduction in viability of $CD4^+$ T cells from infected mice, using different stimuli; (iii) TCR-CD3-dependent proliferative responses were suppressed when the initial number of added viable cells was considered but not if proliferation was correlated with the actual number of viable cells remaining after 20 h in culture. However, viable cell count after overnight culture was not sufficient to restore homogeneity in T-cell responses between control and infected groups (Fig. 2C). The most likely explanation for the differences still observed is that remaining viable $CD4^+$ T cells from infected mice are more responsive to stimulation than controls, but the opposing process of induced cell death has not been completed. This assumption is supported by flow cytometric analysis of IL-2 receptor expression, showing a higher proportion of activated cells in the infected group. It is also in agreement with the notion that $CD4^+$ T cells from infected donors have been primed in vivo as a result of polyclonal activation caused by infection (14, 18, 19), as in vivo-primed T cells respond better and with less stringent costimulatory requirements than naive T cells (22, 28). In the present study, CD69 and Ly-6 pathways stimulated $CD4^+$ T-cell proliferation and IL-2 receptor expression (in the case of CD69), but did not induce cell death, even if anti-CD69 or anti-Ly-6 was added in an immobilized form or at an additional 5- to 10-fold excess (not shown). However, concomitant CD69 stimulation did not rescue $CD4^+$ T cells from death induced through TCR-CD3. Absence of anti-CD69-mediated AICD could explain sparing of T-cell activation through CD69 in cells with an already suppressed response to TCR-CD3-mediated stimuli, as previously found (15). We are currently investigating lack of AICD through CD69 and Ly-6 pathways. It is possible that there are differences in intracellular signaling compared with TCR-CD3. Interestingly, a previous study (20) showed that CD69 is unable to trigger cytolysis when there is concomitant expression of the α/β TCR. Also, another report showed that growth inhibition, now attributed to AICD, was less efficient through the Ly-6 than through the CD3 pathway in T-cell hybridomas (29). Our findings with the CD69 and Ly-6 pathways open the possibility of immunomodulation attempts through accessory molecule activation in situations in which the T-cell defect results from AICD.

The relevance of AICD for immunosuppression during in vivo infection by *T. cruzi* is unknown. We have previously demonstrated (16) that splenic T-cell apoptosis occurs in vivo in the course of acute infection with Dm28c metacyclic forms. We have now shown that *T. cruzi* infection primes host splenic cells for apoptosis following anti-CD3 injection in vivo. Anti-CD3 injection did not induce apoptosis in control mice but led to a rapid increase in splenic $CD4^+$ T-cell numbers. In contrast, anti-CD3 injection did not affect splenic $CD4^+$ T-cell numbers in infected mice. Splenocyte apoptosis following anti-CD3 injection in vivo has also been found in murine AIDS (3). Under conditions of high antigenic load and repeated proliferative stimulation, TCR-mediated T-cell death has been termed propriocidal regulation (12), to indicate a feedback mechanism for regulating T-cell activation in the course of vigorous immune responses. It is interesting that sustained polyclonal lymphocyte activation is a feature of both *T. cruzi* and viral infections. Thus, it is possible that apoptosis is a common lymphocyte response following vigorous antigen stimulation in these infectious states. The results showing that in vitro $CD4^+$ T-cell unresponsiveness can result from AICD could help to explain paradoxical findings of in vitro suppression, in spite of massive polyclonal T- and B-cell activation in vivo, following *T. cruzi* infection (18, 19). It will be important to investigate whether *T. cruzi* antigens can also induce AICD. However, direct demonstration of antigen-induced AICD may be difficult because of dilution of *T. cruzi*-specific clones within polyclonally activated populations. On the other hand, in a transgenic TCR murine model (11, 21), a clear relationship between the size of the viral inoculum or antigen dose and the degree of clonal deletion of the responding cytolytic T cells has been demonstrated.

It has been proposed that in acute viral infections, programmed cell death of polyclonally activated T lymphocytes could be involved in a deficiency of the immune response to unrelated antigens (25). A similar condition has also been described for *T. cruzi* infection (24), raising the same possibility. However, it is important to note that apoptosis is a normal event following immune responses, including those to noninfectious antigens. Therefore, it is important to compare the levels of apoptosis induced by the parasite infection with the levels induced by immunization with noninfectious *T. cruzi* antigens. This comparison is necessary to determine any possible relevance of AICD for the pathogenesis of Chagas' disease. Finally, the occurrence of AICD in human Chagas' disease remains to be investigated. Cell-mediated immunodepression has been observed in acute-phase human infection (33), and increased numbers of $CD45RA^-$ T cells were found at the chronic stage of the disease (7). Since this subset is prone to undergo apoptosis in viral infections (1), a similar investigation in Chagas' disease seems justified. In conclusion, our results suggest that AICD could be one possible determinant of immunosuppression in experimental Chagas' disease. Other factors described include deficient IL-2 production (8, 26, 30, 32), suppressor macrophages (8, 30) or $CD8^+$ T cells (30), and suppressive factors released by the parasite (2). All of these

mechanisms are probably relevant for the establishment of T-cell defects in the infected host.

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