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Trypanosoma cruzi extracts elicit protective immune response against chemically induced colon and mammary cancers

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***Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease, has anticancer effects mediated, at least in part, by parasite-derived products which inhibit growth of tumor cells. We investigated whether immunity to *T. cruzi* antigens could induce antitumor activity, using two rat models which reproduce human carcinogenesis: colon cancer induced by 1,2-dimethylhydrazine (DMH), and mammary cancer induced by *N*-nitroso-*N*-methylurea (NMU). We found that vaccination with *T. cruzi* epimastigote lysates strongly inhibits tumor development in both animal models. Rats immunized with *T. cruzi* antigens induce activation of both CD4$^+$ and CD8$^+$ T cells and splenocytes from these animals showed higher cytotoxic responses against tumors as compared to rats receiving adjuvant alone. Tumor-associated immune responses included increasing number of CD11b/c$^+$ His48$^-$ MHC II$^+$ cells corresponding to macrophages and/or dendritic cells, which exhibited augmented NADPH-oxidase activity. We also found that *T. cruzi* lysate vaccination developed antibodies specific for colon and mammary rat cancer cells, which were capable of mediating antibody-dependent cellular cytotoxicity (ADCC) *in vitro*. Anti-*T. cruzi* antibodies cross-reacted with human colon and breast cancer cell lines and recognized 41/60 (68%) colon cancer and 38/63 (60%) breast cancer samples in a series of 123 human tumors. Our results suggest that *T. cruzi* antigens can evoke an integrated antitumor response involving both the cellular and humoral components of the immune response and provide novel insights into the understanding of the intricate relationship between parasite infection and tumor growth.**

Key words: cancer, parasite, *Trypanosoma cruzi*, vaccination

Abbreviations: ADCC: antibody-dependent cellular cytotoxicity; DMH: 1,2-dimethylhydrazine; NK cells: Natural Killer cells; NMU: N-nitroso-N-methylurea

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The correlation between parasitic infection and lower cancer incidence in humans has been reported in some epidemiological studies. A significantly lower prevalence of cancer was observed in patients with hydatid cyst disease.[1] In addition, colorectal cancer was not observed in patients with chagasic megacolon,[2] suggesting the existence of protection against colon cancer in patients with Chagas' disease. Development of megacolon during the chronic phase of *Trypanosoma cruzi* infection is associated with increased influx of cytotoxic T cells into enteric lymph nodes and loss of muscle innervations.[3] Furthermore, the survival of adult T-cell leukemia patients infected with *Strongyloide stercoralis* was significantly longer than noninfected patients.[4] In line with these observations, infections by parasites, including *T. cruzi*,[5] *Toxoplasma gondii*,[6] and *Plasmodium yoelii*,[7] has conferred resistance to tumor growth in several animal models.

It has been demonstrated that the anticancer activity of parasites is mediated, at least in part, by the induction of antitumor immunity. An intratumoral injection of a live attenuated strain of *T. gondii* evoked robust anti-tumor immune responses

What's new?
The *Trypanosoma cruzi* parasite, the cause of Chagas' disease, naturally antagonizes cancer, a phenomenon discovered in the 1930s. Yet, despite decades of research, precisely how the parasite prevents tumor growth is not fully understood. Here, vaccination with *T. cruzi* epimastigote lysates was found to trigger strong cytotoxic responses against tumors, with activation of CD4+ and CD8+ T cells and augmented NADPH-oxidase activity, in rats developed to recapitulate colonic and mammary carcinogenesis. In rat tumor cells, the antigens stimulated the production of antibodies that cross-reacted with human colon and breast cancer tissue samples.

in vivo that promoted regression of established primary melanoma B16-F10 tumors in mice.[8] Treatment with this parasite in an immunosuppressive ovarian tumor environment restores the ability of $CD11c^+$ antigen-presenting cells to trigger potent antitumor $CD8^+$ T-cell responses.[9] Moreover, it was also found that malaria infection suppresses Lewis lung cancer growth via induction of innate and specific adaptive antitumor responses characterized by heightened production of Th1-type cytokines.[7] We have recently found that immunization with mucin peptides derived from the helminth parasite *Echinococcus granulosus* can potently induce antitumor activity by increasing the frequency of activated NK cells and endowing splenocytes with the capacity to mediate killing of tumor cells.[10] In addition, we observed that vaccination with *E. granulosus* antigens (human hydatic cyst fluid) significantly inhibits colon cancer growth (both in prophylatictic and therapeutic settings) via induction of antitumor immunity.[11] Interestingly, mice immunized with hydatic cyst fluid developed specific antibodies capable of recognizing tumor cells,[11] consistent with previous reports indicating that parasites and cancer cells share common antigens.[12]

Roskin and Exempliarskaia were the first to report that *T. cruzi* inhibits tumor cell growth.[13] However, in spite of considerable advances on the antineoplastic effects of *T. cruzi*,[14–17] the mechanisms underlying tumor growth inhibition are poorly understood. The possibility that the antitumor effects of *T. cruzi* could be due to a toxic substance is supported by certain evidence.[18] The antitumor effects may be associated to the proapoptotic activity of *T. cruzi* components.[19] Indeed, a recombinant protein equivalent to the *T. cruzi* surface molecule gp82, specifically induced apoptotic death of melanoma cells without altering the fate of normal melanocytes.[20] On the other hand, *T. cruzi* calreticulin (TcCRT) has antiangiogenic properties,[21] which could explain the *in vivo* antitumor effects of TcCRT in the murine mammary TA3 MTXR tumor model.[22] It has been proposed that the immune response elicited by *T. cruzi* could be effective toward tumor cells.[16] This hypothesis is supported by the observation that Ehrlich's adenocarcinoma growth is significantly impaired in mice injected with splenocytes from animals immunized with *T. cruzi* lysate.[23] Inhibition of tumor growth by the passively transferred immunocompetent cells suggests that *T. cruzi* and Ehrlich's adenocarcinoma cells display common epitopes. The authors confirmed the presence of some common surface antigens between *T. cruzi* and Ehrlich's adenocarcinoma cells. A candidate common structure could be the mucin-type cancer-associated sialyl-Tn antigen (sialic acid-GalNAc-*O*-Ser/Thr), previously characterized in *T. cruzi*.[24]

To better understand the role of *T. cruzi* antigens in the induction of antitumor effects, in this study, we investigated whether immunizations with *T. cruzi* extracts protect against cancer development in two rat models which reproduce human carcinogenesis: the colon tumorigenesis model induced by administration of 1,2-dimethylhydrazine (DMH), and the mammary carcinogenesis model induced by N-nitroso-N-methylurea (NMU). DMH-induced carcinogenesis is one of the most widely used colorectal cancer models as it resembles sporadic human colon cancer in terms of morphology, pattern of cell growth and clinical manifestations.[25] NMU-induced mammary cancer is one of the most frequently used models of human breast cancer.[26] The rat tumor's histopathology indicates their origin from mammary ductal epithelial cells and their dependency on ovarian hormones for tumor development (estrogen receptor positive), all features that correlate with human breast cancer.[27] In this work, we present data showing that vaccination with *T. cruzi* antigens significantly reduces colon and mammary cancer development in rats. We also demonstrate that immunization with *T. cruzi* induces cellular cytotoxicity and the presence of cross-reactive antibodies against cancer cells, providing antitumor effects through mechanisms involving a*ntibody*-dependent cell-mediated cytotoxicity (ADCC).

Material and Methods

Preparation of *T. cruzi* protein extracts

Dm28c *T. cruzi* strain was used throughout this work. Epimastigotes were grown at 28°C in liver infusion tryptose medium (LIT) supplemented with 10% heat inactivated fetal bovine serum (FBS). For protein extracts, parasites at late exponential growth phase were washed twice in PBS and resuspended in DOC buffer (0.15 M glycine, 1% sodium deoxycholate, 0.5 M NaCl, pH 9). The lysate was incubated at room temperature for 1 hr in agitation, then 30 min at 37°C and finally 30 min at 4°C. The lysate was centrifuged at 20,000*g* for 1 hr and the supernatant was separated and stored at −80°C with 1 mM PMSF and 10 μg/mL aprotinin. Protein concentration was determined by Bradford Assay (Sigma, USA). Low endotoxin levels of *T. cruzi* lysate were detected (<0.4 EU/mg) using the Limulus Amebocyte Lysate kit Pyrochrome (Associates of Cape Cod, MA USA).

Animals and cell lines

Six-week-old female Wistar rats were obtained from URBE (Unidad de Reactivos Biológicos de Experimentación, School of Medicine, Universidad de la República, Uruguay) kept in the animal house with water and food supplied *ad libitum*, and handled in accordance with National guidelines for animal welfare (Committee on Animal Research, CHEA, Uruguay). The NMU breast cancer cell line, derived from a tumor induced in a rat treated with NMU,[28] was obtained from the American Type Culture Collection (ATCC) and grown in complete RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate and 5 mM glucose, at 37°C and 5% CO_2. T47D and MCF-7 human breast cancer cell lines as well as HT29 and LS-174T human colon cancer cell lines were obtained from ATCC and cultured in DMEM medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, in humidified atmosphere at 37°C and 5% CO_2.

Induction of anti-*T. cruzi* antibodies

Rats were immunized i.p. with 100 μg of *T. cruzi* lysate in complete (CFA) or incomplete (IFA) Freund's adjuvant at days 0, 14 and 28. Bleedings were carried out at day 35. Control sera derived from animals inoculated with PBS in Freund's adjuvant. Sera-reactivity was then analyzed by ELISA or flow cytometry. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.1 μg/well of *T. cruzi* lysate in 50 mM carbonate buffer (pH 9.6). After blocking with 1% gelatin in PBS, three washes with PBS containing 0.1% Tween-20 (PBS-T) were performed. Then, serially diluted sera in PBS-T containing 0.5% gelatin were added to the wells for 1 hr at 37°C. Following three washes, samples were added for 1 hr at 37°C with goat anti-rat IgG horseradish peroxidase (HRP) conjugate (Sigma Chemical Co., St. Louis, MO) and *o*-phenylenediamine-H_2O_2 was then added as substrate. Plates were read photometrically at 492 nm in an ELISA microplate-reader (Labsystems Multiskan MS). Rat sera were also tested at a 1:100 dilution by flow cytometry on the NMU, T47D, MCF-7 and HT29 tumor cell lines. Cells were fixed with 4% PFA and eventually for intracellular staining permeabilized with 0.1% Tween-20. Then, cells were incubated for 30 min with sera at 4°C in PBS containing 5% FBS and 0.1% sodium azide and finally with an anti-rat IgG goat antibody conjugated to FITC (Sigma). After washing steps, cells were analyzed on a CyAn™ ADP Flow Cytometer (Beckman Coulter). Data were obtained and analyzed using Summit v4.3 software.

Animal carcinogenesis and experimental designs

Colonic carcinogenesis: DMH (Sigma, St. Louis, MO), freshly dissolved in PBS, was administered to rats at the beginning of the experiment, by s.c. injection (15 mg of DMH per kg body weight) as described previously.[29] Injections were repeated every week for 8 weeks after the initial DMH administration.

Mammary carcinogenesis: NMU (Sigma, St. Louis, MO), dissolved in distilled water (10 mg/0.5 mL) acidified with 3% acetic acid, was administered to rats by i.v. caudal-vein injection (5 mg/100 g body weight) according to Babino *et al.*[30] Injections were repeated 4 and 8 weeks after the initial NMU administration.

The rats were fed on a laboratory chow diet and water *ad libitum* in a room with temperature maintained at 25°C and a 12-h-light/12-h-dark cycle.

The antitumor potential of *T. cruzi*-vaccination was evaluated in rats divided into the following 4 groups: (*i*) DMH-induced colon carcinogenesis in rats receiving only PBS + adjuvant (control colon cancer group); (*ii*) DMH-induced colon carcinogenesis in rats immunized with 100 μg/dose of *T. cruzi* lysate in adjuvant; (*iii*) NMU-induced mammary carcinogenesis in rats receiving only PBS + adjuvant (control mammary cancer group); and (*iv*) NMU-induced mammary carcinogenesis in rats immunized with 100 μg/dose of *T. cruzi* extract in adjuvant. In all cases, the first immunization was done at the beginning of carcinogenesis (*T. cruzi* + CFA), followed by immunizations (*T. cruzi* + IFA) 4 times every 2 weeks. Rats with DMH- or NMU-treatments were sacrificed at 24 or 14 weeks, respectively, after the beginning of carcinogenesis by cervical dislocation. Colon or mammary tissues were then removed, fixed in neutral-buffered formalin and embedded in paraffin to perform histopathological analysis.

Assessment of T-cell responses

T-cell responses were evaluated on spleen cells of rats immunized i.p. three times with *T. cruzi* lysate (100 μg/rat) in CFA or IFA at days 0, 14 and 28. At day 35, rats were sacrificed and spleen cells were cultured in complete culture medium. Cells (1×10^6/well) were labeled with CFSE (Invitrogen, CA USA) and cultured for 72 hr at 37°C and 5% CO_2 in 96-well plates with *T. cruzi* lysate (5 and 25 μg/mL). Proliferation was determined by the number of $CFSE^{low}$ $CD4^+$ or $CD8^+$ T cells. Controls were incubated either with culture medium alone or with concanavalin-A (Con A, 10 μg/mL). The negative control group consisted of spleen cells from rats inoculated with PBS in adjuvant.

Analysis of splenocytes by flow cytometry

Splenocytes from *T. cruzi* and control-immunized rats obtained as described above were washed twice with PBS containing 2% FBS and 0.1% sodium azide. Splenocytes were then stained with the following antibodies: CD4 (OX-35), FoxP3 (FJK-16s), CD45RA (OX-33), CD3 (G4.18), CD8α (OX-8), CD161a (10/78), MHC II (OX-6), CD11b/c (OX-42) and His48 obtained from BD biosciences (NJ, USA). After staining, cells were fixed with 1% formaldehyde and analyzed using a CyAn™ ADP Flow Cytometer (Beckman Coulter).

Cell cytotoxicity assays

Rats were immunized with *T. cruzi* antigens or PBS in CFA or IFA, at days 0, 14 and 28. Splenocytes were removed on day 42, mechanically dispersed and centrifuged at 1500 rpm for 5 min. Effector splenocytes (E) and target NMU cells (T) were cultured in different ratios for 18 hr at 37°C and 5% CO_2 in 96-well plates in complete culture medium. Then, 10

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μL/well of WST-8 (2-(2-methoxy-4-nitrophenyl)−3-(4-nitrophenyl)−5-(2,4-disulfophenyl)−2H-tetrazolium) (Sigma-Aldrich, St. Louis, MO) were added to the media and incubated for 3 hr at 37°C and 5% CO_2. Absorbance at 450 nm was measured using an ELISA microplate reader (Labsystems Multiskan MS). Each condition was analyzed in triplicate. Controls were carried out by culturing tumor cells alone, splenocytes alone and tumor cells in presence of 1% Triton-X100 containing media (100% of cytotoxicity expected).

Percentage of cytotoxicity was calculated as:

$$\%\,\text{cytotoxicity} = [E_{\text{alone}} + T_{\text{alone}} - (\text{E} + \text{T})]/[T_{\text{alone}} - T_{\text{Triton-X100}}],$$

where E corresponds to the absorbance for splenocytes and T for tumor cells respectively, and $(E+T)$ corresponds to the absorbance for splenocytes cocultured with tumor cells.

Alternatively, ADCC was performed using ConA-stimulated splenocytes from naïve rats (E), starved for 10 min in culture medium without FBS and then cocultured with NMU cells (T) and with heat-inactivated anti-*T. cruzi* or control antibodies at 1/50 dilution. In this case, the following formula was used:

$$\%\,\text{cytotoxicity} = \left[(E+T) - (E+T)_{\text{w/antibody}}\right]/[T_{\text{alone}} - T_{\text{Triton-X100}}],$$

where $E+T$ corresponds to the absorbance for splenocytes cocultured with tumor cells, and $(E+T)_{\text{w/antibody}}$ corresponds to the absorbance for splenocytes cocultured with tumor cells in presence of antibody.

Determination of NADPH-oxidase activity

Splenocytes from *T. cruzi* and control-immunized rats were dispersed in flasks and cultured for 72 hr. Nonadherent cells were removed at 2, 6 and 24 hr from the cell culture so as to enrich in adherent cells, shown to be CD11b/c^+ His48^- by flow cytometry. These cells were seeded in 24-well plates at 5×10^5 cells/well. The next day, cells were incubated in PBS containing p*horbol* 12-myristate 13-acetate (PMA; 10 μg/mL), Amplex Red (50 μM) and HRP (10 μg/mL). The H_2O_2-dependent oxidation of Amplex Red was measured in a microplate fluorometer (Fluostar Galaxy) over 30 min. NADPH-oxidase activity is shown as fluorescence relative units (FRU) and corresponded to the slope of the curve measured for 30 min (excitation 540 nm, emission 580 nm).

Clinical samples

Paraffin-embedded tumor tissues were obtained from 63 patients who had undergone surgical treatment for a breast lump at Pereira Rossell Hospital (Montevideo, Uruguay) with histopathological diagnosis of breast cancer, and 60 patients who had undergone surgical treatment for colon cancer at Maciel Hospital (Montevideo, Uruguay). The study was examined and approved by the Ethical Review Board of the School of Medicine, Montevideo, Uruguay. Normal breast tissues were obtained from reduction mammoplasties (n = 5) and normal colon tissues were obtained from the proximal resection border of the surgical sample (n = 15). All specimens were formalin-fixed (4%) paraffin-embedded tissues, and histological sections were subjected to hematoxylin and eosin staining (standard procedure) as well as immunohistochemical evaluation using anti-*T. cruzi* serum. All breast tumors were conventionally classified by histological type and grading (Scarff–Bloom–Richardson system with Nottingham modification), and all colon tumors were classified according to the Duke's method

Immunohistochemical staining

Sections were deparaffinized, rehydrated and then blocked with 10% goat serum, 1% BSA in PBS for 1 hr at room temperature. The quenching of endogenous peroxidase activity was performed with 3% H_2O_2 in PBS for 20 min, and blocked with normal goat serum for 20 min. Anti-*T. cruzi* rat serum (diluted 1:300 in 0.1% Tween-20, 1% BSA in PBS) was incubated overnight at 4°C. For every assay, negative controls using preimmune rat serum or PBS in the absence of primary antibody were included. Sections were treated with goat polyclonal anti-rat IgG biotin conjugate (Sigma), for 1 hr at room temperature. Finally, peroxidase-labeled avidin (Sigma) was added for 45 min. Reactions were revealed with 3,3′-diaminobenzidine (Sigma), washed in water, counterstained in Mayer's hematoxylin, dehydrated in ethanol and xylene and mounted.

Statistical Analysis

Correlation between anti-*T. cruzi* reaction and clinicopathological factors were analyzed using Fisher's exact test. Univariate analysis of disease-free survival was performed with the use of log-rank test: P values <0.05 were considered statistically significant.

Results

T. cruzi immunization protects against colon and mammary cancer development in models of chemical carcinogenesis

To investigate whether vaccination with *T. cruzi* antigens protects rats against colon or mammary cancer development, we induced carcinogenesis with DMH and NMU mutagens, respectively. The first immunization was performed at the beginning of carcinogenesis (*T. cruzi* + CFA), followed by 4 immunizations (*T. cruzi* + IFA) every 2 weeks. For the study in the colon cancer model, 30 rats were treated with DMH and divided in two groups: 15 animals were injected with PBS plus adjuvant (control group) while 15 rats were immunized with a *T. cruzi* lysate (immunized group) as described in *Material and Methods*. Twenty-four weeks after the initiation of carcinogenesis, 12 out of 15 rats (80%) developed malignant tumors in the control group while only 5 animals (33%) presented colon cancer in the immunized group ($p = 0.009$) (Table 1). In addition, most of the animals with tumors in the control group presented an advanced grade of tumor dissemination to muscle (n = 9) and peritoneum

Table 1. Colon tumors induced by DMH

Group	Rat number	Tumors (*n*)	Grade of invasion	Histological type
	1	0		
	2	2	Muscle	MDA
	3	1	Muscle	MDA
	4	1	Muscle	MDA
	5	1	Muscle	MDA
	6	1	Muscle	PDA
	7	1	Muscle	PDA signet ring
Control	8	1	Peritoneum	PDA ascitis
group	9	1	Muscle	MDA
	10	1	Muscle	MDA
	11	2	Muscle	PDA + PDA
	12	0		
	13	1	Peritoneum	MDA
	14	0		
	15	1	Submucose	WDA
	16	0		
	17	0		
	18	0		
	19	0		
	20	0		
	21	1	Submucose	WDA
	22	0		
Immunized	23	1	Muscle	MDA
group	24	0		
	25	1	Submucose	MDA
	26	0		
	27	0		
	28	1	Muscle	MDA
	29	0		
	30	1	Submucose	WDA

WDA, well-differentiated adenocarcinoma; MDA, moderately differentiated adenocarcinoma; PDA, poorly differentiated adenocarcinoma.

($n = 2$), while only 2 out of 5 tumor-bearing animals which were previously immunized with *T. cruzi* lysate showed muscle tumor invasion (Table 1).

Next, we evaluated the antitumor protective effect of *T. cruzi* vaccination in a mammary cancer model induced with NMU. Fourteen weeks after the first injection of NMU, animals were sacrificed and analyzed. Six out of 9 animals (66%) in the control group (PBS plus adjuvant) developed multiple (~2–6) large tumors (15–25 mm of diameter), which showed skin infiltration and ulceration. In contrast, only 1 out of 6 rats immunized with *T. cruzi* antigens (16%) exhibited a small tumor (4 mm) (Table 2). Assessment of mean differences in tumor size was performed using nonparametric Wilcoxon Rank Sum Test that evaluated tumor diameters. We have assigned null

Table 2. Mammary tumors induced by NMU

Group	Rat number	Tumors (*n*)	Size (mm)	Histological type
	31	4	26	Cribiform
	32	4	23	Papilary
	33	0	-	-
	34	3	23	Cribiform
Control	35	2	15	Papilary
group	36	6	25	Mixed
	37	0	-	-
	38	0	-	-
	39	2	25	Cribiform
	40	0	-	-
	41	0	-	-
Immunized	42	0	-	-
group	43	1	4	Papilary
	44	0	-	-
	45	0	-	-

diameter for animals which did not develop tumors. Results showed differences that reached statistical significance between control and treated animals ($p = 0.0345$). Altogether these results indicate that *T. cruzi* vaccination protects from tumor growth, as nonimmunized animals developed a more aggressive disease in number, size and degree of dissemination as compared to the single tumor developed in the *T. cruzi*-immunized group.

CD11b/c$^+$ His48$^-$ splenocytes from *T. cruzi*-immunized rats mediate tumor cell cytotoxicity *in vitro*

To examine the cellular mechanisms underlying tumor protection induced by *T. cruzi* antigens in rats with chemically induced cancer, we analyzed specific cellular responses triggered by *T. cruzi* antigens. First, we evaluated specific T-cell proliferation in splenic cells from *T. cruzi*-immunized rats exposed *ex vivo* to different concentrations of *T. cruzi* antigens. We found robust proliferation of both CD4$^+$ and CD8$^+$ T cells in the presence of 25 μg/mL of *T. cruzi* lysate (Figs. 1*a* and 1*b*), suggesting that specific T cells are generated upon immunization. Furthermore, both CD4$^+$ and CD8$^+$ T cells from *T. cruzi*-immunized animals proliferated when stimulated with a tumor protein lysate from NMU tumors (Figs. 1*a* and 1*b*), indicating that T cells were capable of proliferating following recognition of tumor-derived components. Taking these results into account, we next sought to determine whether splenocytes from immunized mice were capable of killing tumor cells. Although tumor cell killing is often mediated by NK cells or cytotoxic CD8$^+$ T lymphocytes, it may also involve macrophages[31] or CD4$^+$ T cells[32] as effectors. Total spleen cells from *T. cruzi*- or control-rats were coincubated with NMU tumor cells at different ratios. Determination of cell viability showed

that splenocytes from *T. cruzi*-immunized rats induced significantly higher levels of tumor cytotoxicity than splenocytes from control rats, at all evaluated cell ratios (Fig. 1*c*). Strikingly, the cytotoxicity of NMU cells did not increase when the *E*:*T* cell ratio increased beyond 66:1, indicating that under these conditions, the increased number of effector cells did not lead to increased cytotoxicity. On the other hand, the fact that splenocytes from control animals showed lower levels of cytotoxicity might imply that the components present in the complete Freund's adjuvant activated effectors cells from innate immunity, in particular considering that splenocytes from naïve animals did not induce detectable levels of cytotoxicity against NMU tumor cells (Fig. 1*c*).

To study whether *T. cruzi* antigens could modify the frequency of innate or adaptive immune cells, we phenotyped splenocytes from immunized rats. The number of B cells (CD45RA$^+$ CD3$^-$), CD4$^+$ T cells (CD45RA$^-$ CD3$^+$ CD4$^+$), CD8$^+$ T cells (CD45RA$^-$ CD3$^+$ CD8$^+$), Treg cells (CD25$^+$ CD4$^+$ FoxP3$^+$), NK cells (CD161$^+$ CD3$^-$ MHCII$^-$) and granulocytes (CD11b/c$^-$ His48$^+$) was not modified in *T. cruzi*-primed animals as compared to spleens of control or naïve rats (data not shown). However, CD11b/c$^+$ His48$^-$ MHC II$^+$ cells were considerably augmented in spleens from rats immunized with *T. cruzi* antigens (Fig. 1*d*).

As CD11b/c$^+$ His48$^-$ MHCII$^+$ cells may correspond to macrophages and/or dendritic cells, we analyzed the NADPH-oxidase activity on macrophage/dendritic-enriched cells. NADPH-oxidase is involved in the production of reactive oxygen species (ROS) during the respiratory burst of phagocytes and is crucial for pathogen killing.[33] Additionally, ROS production by dendritic cells can play a critical role in the regulation of antigen processing and presentation.[34] We found that macrophage/dendritic-enriched cells from *T. cruzi*-immunized animals displayed higher levels of NADPH-oxidase activity than control or naïve rats (Fig. 1*e*), which could directly influence the capacity of *T.cruzi*-primed splenocytes to mediate cytotoxicity of tumor cells.

T. cruzi-specific antibodies recognize colon and mammary rat tumors and mediate tumor cell cytotoxicity *in vitro*

We next evaluated the capacity of anti-*T. cruzi* polyclonal antibodies to recognize tumor tissues from tumor-bearing rats. Antibodies induced by *T. cruzi* specifically reacted with colon and mammary tumors, showing a cytoplasmatic and membrane staining (Figs. 2*a*,*b*,*g*,*h*, and *i*). Of note, the normal colon epithelium of naïve rats was negative in all cases (Fig. 2*d*). Furthermore, morphologically normal colon tissues of immunized animals was strongly positive at 16 weeks of colon carcinogenesis (Figs. 2*e* and 2*f*), suggesting an early expression of the antigen/s involved in the immune reaction. However, no staining was observed in normal mammary tissues of naïve rats (data not shown).

Because antibodies can mediate tumor cell killing through a process known as antibody-dependent cell cytotoxicity (ADCC), we analyzed the capacity of *T. cruzi*-specific antibodies to mediate killing of the NMU tumor cell line. Thus, sera from *T. cruzi*-immunized animals were first evaluated for their recognition of tumor-derived components or tumor cells. These sera exhibited a high titer of specific *T. cruzi* antibodies that did not recognize protein lysates derived from rat NMU tumors or normal breast by ELISA (Fig. 3*a*). On the contrary, *T. cruzi*-specific antibodies recognized native NMU tumor cells both on the surface and intracellularly by flow cytometry (Fig. 3*b*). The fact that anti-*T. cruzi* antibodies recognized tumor tissues or cells by both immunohistochemistry and flow cytometry, but did not react with tumor lysates by ELISA, might suggest that the physicochemical properties of these antigens in tumor cell lysates did not favor their coating on the hydrophobic surface of ELISA plates. Finally, we incubated sera from *T. cruzi*-immunized rats or preimmune sera with NMU tumor cells together with ConA-stimulated splenocytes and evaluated cell viability after 24 hr. Splenocytes incubated with anti-*T. cruzi* antibodies induced tumor cytotoxicity, while splenocytes incubated with control sera did not (Fig. 3*c*).

Anti-*T. cruzi* antibodies specifically recognize human colon and breast cancer tissues

To further characterize the relevance of these antibodies, we evaluated the reactivity of anti-*T. cruzi* antibodies against a variety of colon and breast human carcinoma cells. All tumor cell lines were recognized by these antibodies, with considerable differences in the intensity and distribution of the staining. In fact, HT29, MCF7 and T47D cells showed an intense and widespread staining (Fig. 4*a*), whereas the staining of LS174T cells was focal and weaker (data not shown). These observations were confirmed by flow cytometry, as a strong and specific recognition of MCF-7, T47D and HT29 was observed (Fig. 4*b*). Two populations were clearly recognized in the case of LS174T (data not shown) and a homogenous population for the other 3 tumor cell lines when they were not permeabilized. When permeabilized, a specific and homogeneous signal was observed in the four cell lines analyzed (Fig. 4*b*). Importantly, preimmune sera did not react with any of the tumor cells (Fig. 4*b*, thin line).

Finally, we evaluated the potential of *T. cruzi*-specific antibodies to recognize human tumor tissues. To this end, we performed immunohistochemical analysis on 60 colon cancer samples and 63 breast cancer samples, comparing the reactivity with nonmalignant tissues. Anti-*T. cruzi* antibodies recognized 41 out of 60 colon cancer samples (68%) (Table 3), displaying a staining pattern similar to that described for rat colon tumors with a diffuse cytoplasmic staining and strong membrane labeling (Figs. 4*c*, A–C). The association between immunostaining and clinicopathologic factors is shown in Table 3. Positive staining showed no significant association within different stages: stage I (1/4; 25%), stage II (13/18; 72%), stage III (15/21; 71%) and stage IV (8/11; 72%). No correlation was found between staining and tumor differentiation. Normal colon epithelia adjacent to cancer was found in 21 out of 60 cases analyzed revealing no staining or a slight focal staining in 9 out of 21 cases (36%), (Fig. 4*c*).

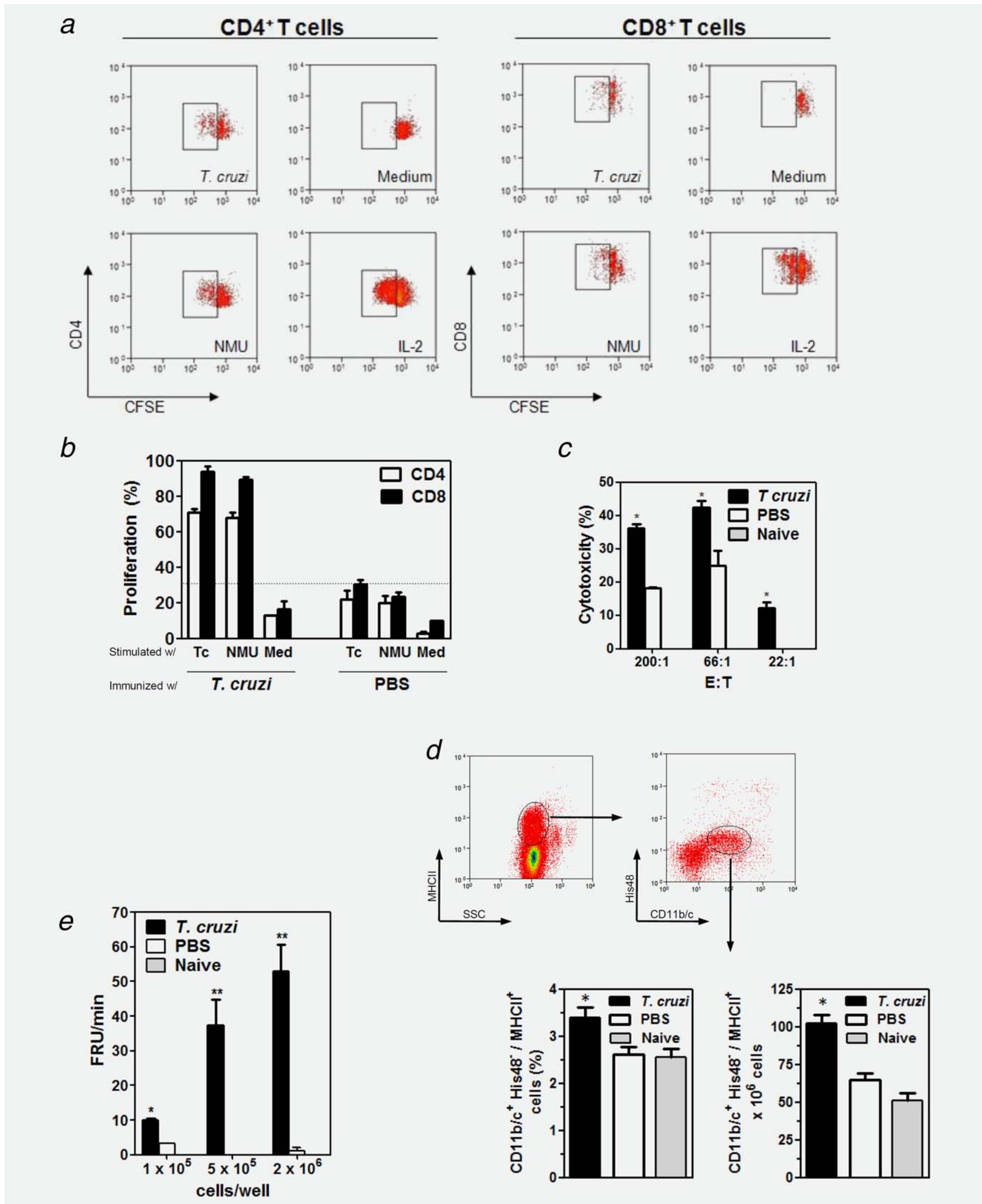

Figure 1.

Remarkably, all 15 normal colon specimens analyzed showed negative immunoreactivity (Fig. 4*c*).

In breast cancer samples, anti-*T.cruzi* antibodies stained 38 out of 63 (60%) specimens (Table 3). The staining pattern was predominantly cytoplasmic (Figs. 4*d*–4*d*-c, A–C). The relationship between staining and clinicopathologic parameters is shown in Table 3. T1 (24/40; 60%) and T2 (9/17; 53%) tumors were more frequently positive than T3 tumors (2/6; 33%) without reaching statistical significance. No difference was observed in the nodal status or following the UICC staging procedure. Ductal carcinoma in situ (DCIS) was observed adjacent to malignant tissues in 13 samples and found to be strongly positive in all the cases (Fig. 4*d*, D). On the other hand, fibroadenoma, a nonmalignant breast pathology, also showed negative immunoreaction (Fig. 4*d*, E). The five normal breast samples analyzed were found to be negative (Fig. 4*d*, F). Altogether these results indicate that *T. cruzi*-specific antibodies specifically recognize both rat and human colon and breast tumors and set the bases for exploring the relevance of anti-*T. cruzi* antibodies in human cancer.

Discussion

It has been previously demonstrated that chronic infection with *T. cruzi* attenuates DMH-induced colon carcinogenesis compared to noninfected animals.[5] This observation raised several questions regarding the mechanisms of antitumor activity mediated by this parasite *in vivo*. Here, in the same carcinogenesis model but vaccinating with a *T. cruzi* epimastigote lysate, we demonstrate that an immune response elicited by *T. cruzi* is responsible, at least in part, for the strong protective effect evoked by immunized rats against tumor development. Moreover, we confirmed this immune-mediated protection in NMU-treated animals, an experimental model that closely reproduces human breast carcinogenesis.

The fact that anti-*T. cruzi* antibodies, cross-reacting with cancer cells, can perform ADCC against the NMU rat mammary cancer cell line acquires importance in the design and implementation of diagnostic and/or therapeutic strategies. In this line, we found that *T. cruzi*-specific antibodies cross-react with human colon and breast cancer cell lines. More interestingly, in a series of 123 human tumors, these antibodies recognized 68% of colon cancer samples and 60% of breast cancer tumors, while no staining was found in normal tissues. These data strongly suggest the presence of common antigens between *T. cruzi* and malignant colon and breast tissues. The occurrence of cross-reactivity between *T. cruzi* and human tissues is not new; this is a widely reported subject and discussed in the physiopathology of the Chagas' disease, which is considered as a paradigm of infection-induced autoimmune disease.[35] Molecular mimicry has been proposed as an important mechanism leading to autoimmunity of this disease, because various *T. cruzi* antigens, such as B13, cruzipain and Cha, cross-react with host antigens through mechanisms involving either B or T cells.[36] The antigens involved in the cross-reaction between this parasite and cancer cells have not yet been identified. In this regard, the cancer-associated mucin-type *O*-glycan antigen sialyl-Tn, previously identified in *T. cruzi*[24] as well as in rat DMH-induced colon cancer[37] could be a good candidate. In spite of the remarkable specificity of *T. cruzi*-specific antibodies for tumor tissues, in the initial clinical assessment, we could find no relationship between antibody staining and clinicopathologic parameters at diagnosis of the disease. Nevertheless, the potential clinical utility of this diagnostic approach should be validated in a larger number of patients with known clinical follow-up.

We also found that the immune response elicited by *T. cruzi* lysates induces the activation of specific $CD4^+$ and $CD8^+$ T cells. In addition, splenocytes from *T. cruzi*-immunized rats induced

Figure 1. *T. cruzi* antigens induce specific $CD4^+$ and $CD8^+$ T-cell responses and tumor cytotoxicity associated with an increase in $CD11b/c^+$ $His48^-$ cells. (*a*) T-cell responses induced by *T. cruzi* lysate were evaluated by incubating splenocytes from *T. cruzi*-immunized or control rats for 72 hr in triplicates in presence of *T. cruzi* antigens, NMU tumor lysate (25 μg/mL), IL-2 (as positive control of proliferation) or medium alone. Splenocytes were previously labeled with CFSE. Cell proliferation was assessed by flow cytometry by gating on CD4+ or CD8+ CFSElow cells. (*b*) Proliferation index (%) of splenoctyes from *T. cruzi*-immunized or control rats was calculated as the % of $CD4^+$ or $CD8^+$ $CFSE^{low}$ T cells in relation to the control condition (the % of T cells when stimulating with IL-2 was considered as 100%). A pool of splenocytes from *T. cruzi*- or PBS-immunized animals as wells as naïve rats (n = 5) was used. Control rats consisted in animals immunized with PBS in adjuvant. Results are expressed as mean value of biological triplicates (±SD, indicated by error bars) and are representative of two independent experiments. Asterisks (*) represent statistically significant differences ($p < 0.01$) with respect to medium alone. (*c*) Splenocytes from rats immunized with *T. cruzi* antigens (E) were cultured with NMU cells (T) for 18 hr at 37°C. Then, 10 μl/well of WST-8 were added to the media and absorbance was measured at 450 nm. The percentage of cell cytotoxicity was calculated as indicated in *Materials and Methods*. A pool of splenocytes from *T. cruzi*- or PBS-immunized animals as well as naïve rats ($n = 5$) was used. Results are expressed as mean value of technical triplicates (±SD, indicated by error bars) and are representative of three independent experiments. Asterisks (*) represent statistically significant differences ($p < 0.01$) with respect to medium alone. (d) Splenocytes from rats immunized with *T. cruzi* antigens were analyzed by flow cytometry to determine the frequency (%) and total numbers of $CD11b/c^+$ $His48^{-}/MHCII^+$ cells on individual spleens. Results expressed as mean value of 5 biological replicates (±SD, indicated by error bars) and are representative of two independent experiments. Asterisks (*) represent statistically significant differences ($p < 0.01$) with respect to the PBS group. (e) Adherent cells from splenocytes derived from rats immunized with *T. cruzi* antigens were incubated at different concentrations with Amplex Red and HRP. Hydrogen peroxide-dependent oxidation of Amplex Red was measured in a microplate fluorometer. NADPH-oxidase activity is shown as fluorescence relative units (FRU). Basal levels obtained with naïve animals were subtracted. Results are expressed as mean value of technical triplicates (±SD, indicated by error bars) and are representative of three independent experiments. Asterisks represent statistically significant differences (*$p < 0.01$, **$p < 0.05$) with respect to medium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

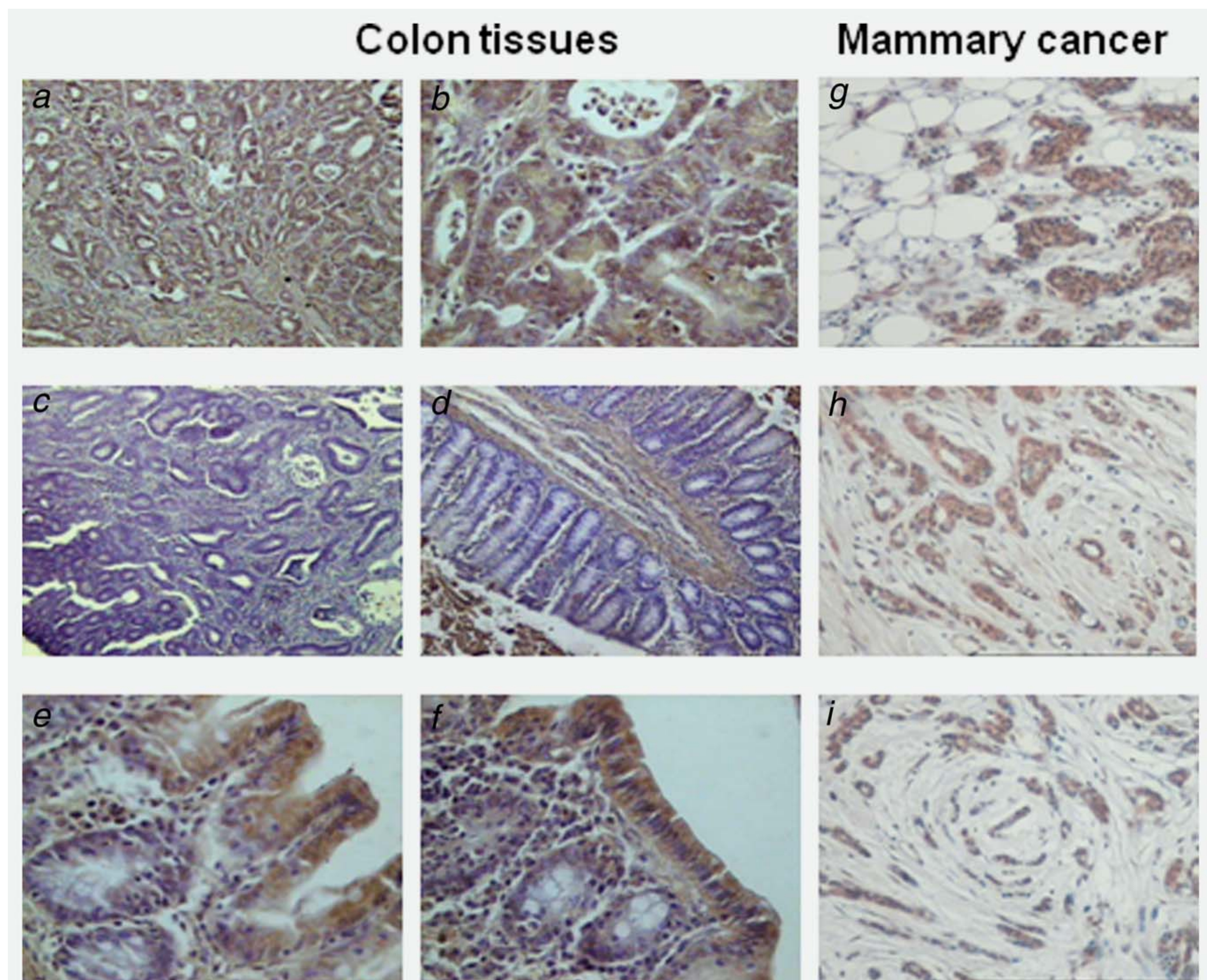

Figure 2. Immunohistochemical evaluation of anti-*T. cruzi* antibodies in rat colon and mammary tissues. (*a*) Immunoreactivity of anti-*T. cruzi* antibodies in rat colon adenocarcinoma (magnification 100×). (*b*) Colon adenocarcinoma (magnification 400×); (*c*) Colon adenocarcinoma incubated with preimmune serum (magnification 100×). (*d*) Normal rat colonic mucosa (magnification 100×). (e,f) Normal colon tissues at 16 weeks of DMH-treatment (magnification 400×). (g) Mammary carcinoma (magnification 200×). (h) Mammary carcinoma (magnification 200×). (i) Mammary carcinoma (magnification 100×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly higher levels of cytotoxicity than rats receiving adjuvant alone. It is well known that host resistance to *T. cruzi* infection depends on both a CD8 and a Th1-mediated response characterized by a strong production of IL-12 and IFN-γ.[38] This can be explained by the activation of dendritic cells by TLR agonists expressed by *T. cruzi*,[39] which produce large amounts of IL-12 and can induce the differentiation of IFN-γ-producing Th1 cells. Th1 cells will in turn enhance the antitumor immunity through the activation of cytotoxic $CD8^+$ T lymphocytes, natural killer (NK) cells or macrophages. We found that tumor cell cytotoxicity was associated with an increased number of $CD11b/c^+$ $His48^-$ MHC II^+ cells, which represent antigen-presenting cells like macrophages and/or dendritic cells. An increase in dendritic cells in the spleen of *T. cruzi*-immunized animals may contribute to the activation and differentiation of $CD4^+$ or $CD8^+$ T cells, as well as the killing of tumor cells. Indeed, IFN-γ-secreting killer dendritic cells have been described and are capable of drive cytolysis of tumor cells.[40] Also, macrophages are able to kill tumor cells by apoptosis, in a process that requires production of ROS and secretion of serine proteases.[41] We demonstrate here that an enriched population of macrophage/dendritic cells produces higher levels of NADPH-oxidase activity, an effect that is associated with ROS production.[33] Thus, it is likely that these cells mediate tumor cell killing as a result of an augmented NADPH-oxidase activity. These results also suggest that these macrophages could differentiate into an M1-type phenotype, able to display proinflammatory and antitumor activities.[42] Finally, an increased number of macrophages would also enhance ADCC of tumor cells *in vivo*,[43] as *T. cruzi*-specific antibodies could mediate tumor cell killing *in vitro*. Furthermore, tumor cytotoxicity mediated by

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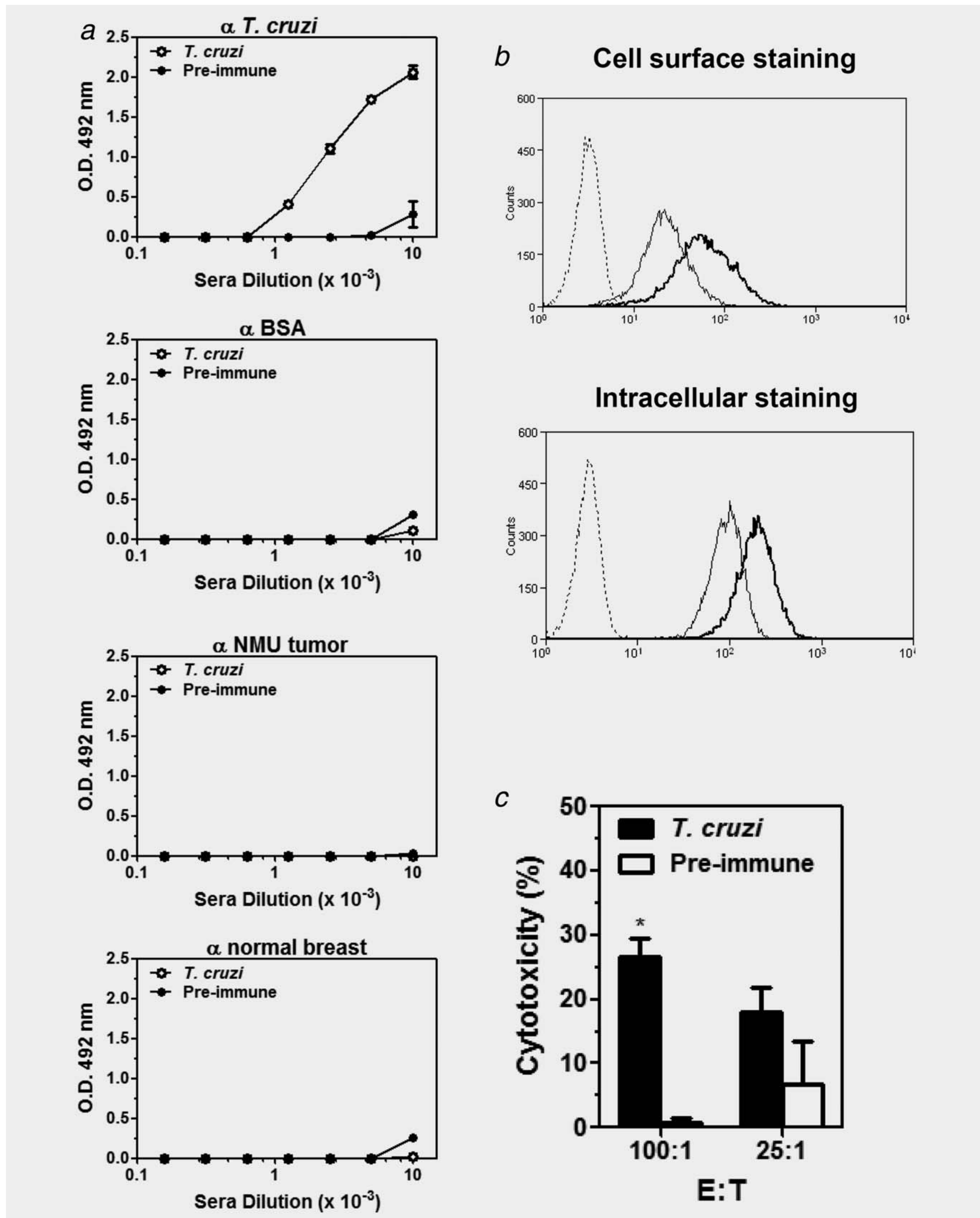

Figure 3. *T. cruzi*-specific antibodies mediate tumor cytotoxicity *in vitro*. (*a*) Rats (5 per group) were immunized i.p. at days 0, 14 and 28 with *T. cruzi* lysate (100 μg/rat). Bleedings were carried out at day 35. Specific IgG antibodies against *T. cruzi* antigens, BSA (negative control), NMU tumor lysate or normal rat breast lysate were detected by ELISA using an anti-rat IgG antibody conjugated to peroxidase. Results are representative of four independent experiments. (*b*) Antibody binding to NMU-cancer cells was analyzed by flow cytometry by using an anti-rat IgG antibody conjugated to FITC (solid line: immune sera; thin line: preimmune sera; dashed line: unstained). Results are representative of two independent experiments. (*c*) Decomplemented sera from *T. cruzi*-immunized or control rats were incubated with naïve splenocytes (E, effector cells) overnight in triplicates in the presence of different ratios of NMU tumor cells (T, target cells). Then, 10 μl/well of WST-8 (2-(2-*methoxy-4*-nitrophenyl)−3-(4-nitrophenyl)−5-(2,4-disulfophenyl)−2H-tetrazolium) were added and incubated for 1 hr. WST-8 reduction by dehydrogenases in living cells was followed by absorbance measurement at 450 nm. Results are expressed as percentage of cytotoxicity, as described in the *Materials and methods* section. They correspond to the mean value of triplicates (±SD, indicated by error bars) and are representative of two different experiments.

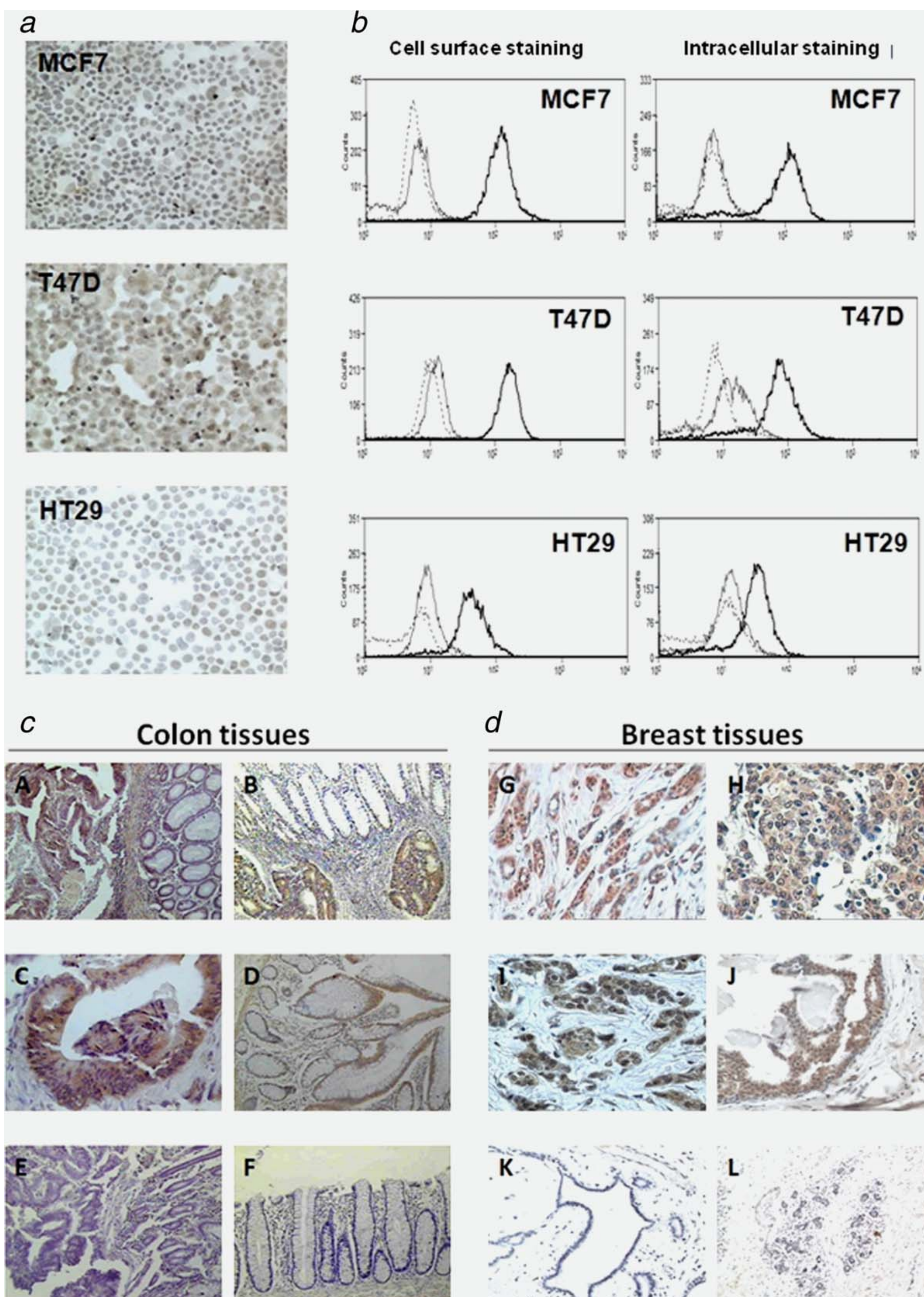

Figure 4. Recognition of human colon and breast cancer cells by anti-*T. cruzi* antibodies. (*a*) Immunocytochemistry of HT29 human colon cancer cells, and MCF7 and T47D human breast cancer cells using anti-*T. cruzi* antibodies, revealed with goat polyclonal anti-rat IgG biotin conjugate and peroxidase-labeled avidin. (*b*) Flow cytometry recognition with anti-*T. cruzi* polyclonal antibodies in nonpermeabilized (left column) and permeabilized (right column) human colon and breast cancer cell lines (solid line: immune sera; thin line: preimmune sera; dashed line: unstained). Results are representative of two independent experiments. (*c*) Immunohistochemical evaluation in human colon tissues. (A and B) Colon adenocarcinoma and adjacent normal colonic mucosa (magnification 100×); (C) colon adenocarcinoma (magnification 400×); (D) normal colon adjacent to cancer (magnification 400×); (E): colon adenocarcinoma incubated with preimmune serum (magnification 100×); (F) normal colonic mucosa (magnification 100×); (*d*) Immunohistochemical evaluation in human breast tissues. (G, H and I) Infiltrating ductal carcinoma (magnification 100×); (J) ductal carcinoma *in situ* (magnification 100×); (K) breast fibroadenoma (magnification 100×); (L) normal breast (magnification 100×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 3. Immunohistochemical staining of human colon and breast tissues using anti-*T. cruzi* antibodies

	n (%)
Colon adenocarcinoma (*n* = 60)	
Age in years	66.6 (23–90)
Negative	19 (31.6)
Positive	41 (68.4)
Normal colon epithelia adjacent to cancer	
Negative	12 (64)
Focally positive	9 (36)
Positive	0
Normal colon epithelium	
Negative	15 (100)
Positive	0
UICC stage	
I	4 (6,6)
II	18 (30)
III	27 (45)
IV	11 (18.4)
Histological grade	
WDA	3/5 (60)
MDA	28/43 (65)
PDA	5/9 (55)
Breast cancer (*n* = 63)	
Age in years	59.1 (26–82)
Tumor size (mm)	31.8 (9–105)
Negative	25 (40)
Positive	38 (60)
Tumor stage	
pT1	24/40 (60)
pT2	9/17 (53)
pT3	2/6 (33)
Nodal status	
Negative	17/27 (63)
Positive	16/36 (44)
UICC stage	
I	16/24 (66)
II	15/28 (54)
III	4/11 (36)
Histological grade	
I	4/7 (57)
II	18/26 (69)
III	12/28 (43)
DCIS (*n* = 13)	
Negative	0 (0)
Positive	13 (100)
Normal breast epithelium (*n* = 5)	
Negative	5 (100)
Positive	0

NK cells should not be excluded, as they are strongly activated during *T. cruzi* infection,[44] and often displays potent antitumor activity.

In recent years, the use of parasites has attracted the attention of immunologists as a novel cancer therapy strategy. Nonpathogenic live parasites, such as *Leishmania tarentolae*, *T. gondii* and *T. cruzi* are emerging as novel candidates for gene delivery in anticancer therapeutic approaches.[45] In fact, it was found that a recombinant nonpathogenic clone of *T. cruzi* expressing a cancer testis antigen (NY-ESO-1) induces antigen-specific T-cell responses and effective protection against tumors in a mouse model.[46] The use of *T. cruzi*-derived TLR agonists as immunological adjuvants also resulted in a significant delay in the growth of the B16-F10 melanoma cell line expressing NY-ESO-1 antigen.[47] Interestingly, protective immunity correlated with the magnitude of $CD8^+$ T-cell responses induced by a specific TLR agonist. One of the major challenges in the development of an efficient anticancer vaccine is overcoming immune tolerance to tumor-associated antigens (TAA), as well as the active immune evasion strategies developed by tumors.[48] Vaccination with TAA coming from evolutionary distant organisms (such as parasites) should be useful to override tolerance problems encountered with human TAA-based cancer therapeutic approaches.[49] We found the presence of *O*-glycosylated TAA in larval and adult tissues of the helminth parasite *E. granulosus*.[50]. Recently we observed that anti-*E. granulosus* antibodies cross-react with molecules expressed on CT26 colon cancer cells, and found that immunization with *E. granulosus* antigens significantly inhibits colon cancer growth via induction of antitumor immunity.[12]. In the same line, *T. cruzi* components should be further explored in the development of antitumor vaccine formulations. Our results support the notion that *T. cruzi* lysate elicits multimodal antitumor mechanisms, activating several components of the immune system.

In conclusion, we report that *T. cruzi* immunization significantly suppresses cancer development via induction of both innate and adaptive immune responses. Although previous works demonstrated similar effects using transplanted tumor models, our study is the first report demonstrating that vaccination using parasite antigens induces an effective cross-reacting antitumor response in an animal model that reproduces human carcinogenesis, There is still much to learn about the immune-mediated mechanisms induced by *T. cruzi* against cancer, as well as other forms of antitumor immunotherapy using parasite components. Further studies are warranted to characterize the nature of the antigens involved in the cross-reaction between *T. cruzi* and colon and breast cancer cells. Nevertheless, our results suggest that vaccination using *T. cruzi* antigens increases tumor immunity in two different carcinogenesis models and opens new horizons for the development of adjuvant anticancer strategies.

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