

Peritoneum from *Trypanosoma cruzi*-infected mice is a homing site of Syndecan-1^{neg} plasma cells which mainly provide non-parasite-specific antibodies

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Abstract

Humoral immunity during experimental Chagas disease has been considered a double-edge sword, critical to control *Trypanosoma cruzi* spreading but also associated to tissue damage. Peritoneal B-1 cells have been linked to the pathogenesis of Chagas disease; however, they may also help to control the infection by providing a fast wave of antibodies. In the present work, we determined that peritoneal B-cell response to *T. cruzi* is characterized by a marked reduction of CD19⁺ B cells due to plasma cell differentiation rather than to cell death. Both peritoneal B-2 and B-1 cells decrease after parasite infection, but with different kinetics. Thus, the reduction in B-2 cell number can be detected from day 4 postinfection while the number of B-1 cells decreases only after 15 days of infection. Differentiation of peritoneal B-1 and B-2 cells into IgM-secreting cells was triggered by parasites but not by cytokines produced by peritoneal cells. Electron microscopy studies showed that peritoneum of infected mice lodges plasma cells with typical morphology as well as atypical plasma cells named 'Mott-like cells' containing high number of cytoplasmatic Ig⁺ granules. The plasma cells induced during the infection showed a phenotype that may allow their persistence in peritoneum and they may contribute to the high levels of antibodies exhibited at the chronic phase of infection. We also showed that the peritoneal B-cell response is scarcely specific for the invading pathogen and rather constitute an important source of non-parasite-specific IgM and IgG in the infected host.

Keywords: B cells, differentiation, infection, polyclonal response

Introduction

Antibody responses are a key element of the immunity against circulating microorganisms, helping to clear acute infections, to protect against re-infections and to control reactivation of persistent pathogens. These effector functions are mediated by antibodies secreted by antibody-secreting cells known as plasmablasts and plasma cells (1).

Plasma cells secreting IgG isotypes and high-affinity antibodies are the result of the germinal center reactions. Germinal centers are formed in secondary lymphoid organs after activated B cells receive co-stimulatory signals by specialized CD4⁺ T_H cells and are the place where class switching, somatic hypermutation and affinity maturation occur (2, 3). T-cell dependent antibody responses generate a humoral memory provided by circulating memory B cells as well as by long-lived plasma cells that home to the bone marrow (1, 4).

In contrast to T-dependent antibody responses, T-independent responses are fast, do not require T cell help and do not generate detectable germinal centers or immunological memory (5). During T-independent responses, antibody-secreting cells arise few days after microorganism encounter and mostly secrete low-affinity antibodies of IgM isotype (6).

Antibodies of low affinity and broad specificity are produced by the immune system in the absence of apparent infection or immunization. These so-called 'natural' antibodies, characterized by the lack of N region additions and somatic hypermutations, are principally secreted by peritoneal B-1 cells and often recognize highly conserved T-independent type 2 microorganism antigens (7, 8). Hence, B-1 cells can easily sense the presence of bacterial challenge and rapidly differentiate into plasma cells in the absence of T cell help

(9, 10). Lately, it has been reported that B-1 cells can also undergo Ig class switching releasing different IgG isotypes or IgA (11). Due to the distinctive features of B-1 cells and the antibodies produced by them, these cells are very efficient in the control of bacterial and viral infections and in the clearance of apoptotic cells (12). However, given the broad specificity and slight autoreactivity of the B-1 cell-derived antibodies, B-1 cells have been also involved in autoimmunity (13, 14).

In addition to B-1 cells, the peritoneal cavity lodges a population of B-2 cells that remains poorly studied. Recently, peritoneal B-2 cells have been reported to present some B-1b cell-like features (15). However, peritoneal B-2 cells show a substantially reduced ability to respond to T-independent antigens in comparison to B-1 cells and behave like splenic follicular B cells in response to Toll-like receptor agonists (11). Although the contribution of peritoneal B-2 cells to humoral response is not completely elucidated, they would have a role complementary to B-1 cells in the secretion of protective antibodies.

Trypanosoma cruzi, the causative agent of Chagas disease induces the production of parasite-specific and autoreactive antibodies in the infected hosts (16, 17). Humoral immunity was found to be critical for the control of parasite spreading during *T. cruzi* infection and, consequently, for preventing acute myocarditis produced by the parasite (18, 19). In agreement, we reported that signals that enhance plasma cell differentiation and antibody secretion promote parasite clearance (20). However, the parasite-specific immune response mounted during *T. cruzi* infection is inadequate to completely eradicate the pathogen, allowing chronic infection and pathogenesis (21). On the other hand, autoreactive antibodies produced during the infection were proposed to be responsible for much of the Chagas disease pathological damage (17, 22). The production of the autoreactive antibodies has been ascribed to CD5⁺B-1 cells because they are increased in spleen during *T. cruzi* infection (23). Mice lacking CD5⁺B-1 cells (Xid mice) did not show the wasting observed in wild-type BALB/c mice and developed almost no pathology early in the chronic phase. However, the resistance of Xid mice to experimental Chagas disease was associated with the absence of IL-10-secreting B-1 cells and the consequent production of high levels of IFN- γ (24) rather than to the absence of B-1 cell-derived autoreactive antibodies.

Due to the lack of previous data concerning peritoneal B-2 cells fate during Chagas disease and the controversial role of B-1 cells in the pathogenesis or in the control of *T. cruzi* infection, we investigated the development and the role of the peritoneal B-cell response during this parasite infection.

Methods

Antibodies and flow cytometry reagents

PE-labeled anti-mouse CD19 mAb, PE-labeled anti-mouse CD5 mAb, PE-labeled anti-mouse Syndecan-1 mAb, PE-labeled anti-mouse IA^d mAb, FITC-labeled anti-mouse IgM mAb, FITC-labeled anti-mouse CD3 mAb, PE-labeled anti-mouse CD4 mAb, PE-labeled anti-mouse CD8 mAb, biotin-labeled anti-mouse CXCR4 mAb, peridinin chlorophyll-

a protein-labeled-streptavidin (PerCP-St), and Purified anti-mouse F4/80 mAb were purchased from BD PharMingen (Palo Alto, CA, USA). Allophycocyanin (APC)-labeled anti-mouse B220 mAb, FITC-labeled anti-mouse Mac-1 mAb, PE-labeled anti-mouse CD21 mAb, FITC-labeled anti-mouse CD23 mAb, biotin-labeled anti-mouse F4/80 mAb, APC-labeled streptavidin, biotin-labeled anti-mouse Fc gamma receptor II B (FcgammaRIIb) mAb and purified anti-mouse CD23 mAb were purchased from e-Biosciences (San Diego, CA, USA). Purified anti-mouse IgM and FITC-labeled anti-mouse IgM for intracellular staining were purchased from Southern Biotech (Birmingham, AL, USA). PE-labeled anti-mouse CXCR3 mAb was purchased in R&D systems (Minneapolis, MN, USA).

Infection with *T. cruzi*

BALB/c mice, 6–8 weeks old (Comisión Nacional de Energía Atómica, Buenos Aires, Argentina) were intraperitoneally infected with 500 trypomastigotes of *T. cruzi* (Tulahuén strain) diluted in PBS as previously described (25). After different days postinfection (p.i.), mice were sacrificed by CO₂ asphyxiation and peritoneal cells were obtained. In some experiments, spleen, lymph nodes and bone marrow were also obtained as previously reported (26). Non-infected (NI) control normal littermates were processed in parallel and mentioned as non-infected control mice in the text. The studies are approved by the Institutional Review Board and Ethical Committee of the School of Chemical Sciences, National University of Córdoba, Argentina.

Trypomastigotes obtention

Trypomastigotes from the Tulahuén strain of *T. cruzi* were obtained from blood of acutely infected BALB/c mice. Briefly, the blood was centrifuged (3000 r.p.m. for 10 min) and then incubated at 37°C and 5% CO₂ for 2 h to allow parasites concentrate in the plasma. The plasma was then centrifuged at 13 000 r.p.m. during 3 min. The pellet containing parasites was washed three times, counted and resuspended in complete medium adjusting the concentration to 0.5×10^6 trypomastigotes ml⁻¹ (27).

Cell preparation and culture

Peritoneal cells from *T. cruzi*-infected (I) or non-infected (NI) control normal mice were obtained by peritoneal washouts. For peritoneal B-lineage cells purification, monocytes and T cells were depleted by magnetic cell sorting using rat IgG anti-mouse F4/80 mAb, followed by anti-rat IgG-coated magnetic beads and anti-Thy 1.2-coated magnetic beads (DynaL Biotech, Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. This procedure yielded an enriched B-cell population with <2% CD3⁺ cells and <3% F4/80⁺ cells, as determined by flow cytometry analysis (data not shown), 95% intracellular IgM⁺ and >95% of viable cells as determined by Trypan blue exclusion. These cells were analyzed for their surface marker expression and Ig production.

In some experiments, peritoneal cells from NI control or I mice were co-cultured with purified B cells from normal mice separated by transwells (0.4- μ m tissue culture-treated polycarbonate membrane; Costar, Corning, NY, USA).

Briefly, peritoneal B cells from normal mice (0.5×10^6 in a volume of 0.5 ml in the lower chamber) were co-cultured with peritoneal cells from infected mice (0.5×10^6 in a volume of 0.5 ml in the upper chamber) for 72 or 120 h.

Purified peritoneal B-1 and B-2 cells were obtained by cell sorting using APC-labeled anti-mouse B220, FITC-labeled anti-mouse CD11b. B-1 and B-2 cells were obtained by gating on the B220⁺CD11b⁺ and B220⁺CD11b⁻ population, respectively. Splenic B-2 cells were obtained by cell sorting using APC-labeled anti-mouse B220, PE-labeled anti-mouse CD21 and FITC-labeled anti-mouse CD23. Splenic B-2 cells were obtained by gating on the B220⁺CD23^{high}CD21^{low} population using a FACS Aria (BD Biosciences).

In some experiments, B-cell subpopulations were adjusted to a final concentration of 1×10^6 cells ml⁻¹ and cultured in the presence of medium alone, CpG ($1 \mu\text{g ml}^{-1}$) or 0.5×10^6 *T. cruzi* trypomastigotes ml⁻¹ during 5 days.

Flow cytometry analysis

Total peritoneal cells or peritoneal B-lineage cells or purified B-cell subpopulations freshly explanted or harvested after culture were washed in ice-cold flow cytometry (FCM) buffer (HBSS, 1% FBS, 0.1% NaN₃). Then, for surface staining, cells were incubated with PE, FITC or biotin-conjugated antibodies at 4°C for 30 min and washed with FCM buffer. When biotin-labeled antibodies were used, an extra 30-min incubation with PerCP-St was performed. Data were acquired on a Cyturon Absolute® cytometer (Ortho Diagnostic System, Raritan, NJ, USA) and analyzed using FlowJo software version 5.7.2. The analysis was performed gating lymphocytes from forward scatter versus side scatter dot plots.

For intracellular IgM detection, surface IgM was blocked by incubating the cells with purified anti-mouse IgM mAb (Southern Biotech) ($1 \mu\text{g per } 1 \times 10^6$ cells) during 30 min on ice. After that, the cells were resuspended in CytoFix/CytoPerm™ Solution (BD Pharmingen) 30 min at room temperature and permeabilized by two washes with Perm/Wash™ solution (BD Pharmingen). Then, the cells were incubated with FITC-conjugate anti-mouse IgM diluted in Perm/Wash™ solution during 30 min at room temperature and washed twice with Perm/Wash™ solution. Data were acquired as indicated before.

For apoptosis detection, the cells were first stained with either PE-labeled anti-mouse CD5 or PE-labeled anti-mouse CD23 and later with Annexin V-Biot and 7-AAD following BD Pharmingen protocol (BD Pharmingen). Then, the cells were washed with binding buffer and incubated with $0.5 \mu\text{g}$ FITC-St diluted in $100 \mu\text{l}$ of binding buffer and $5 \mu\text{l}$ of 7-AAD (BD Pharmingen) for 15 min at RT. Finally, the cells were resuspended in $400 \mu\text{l}$ of binding buffer. Data were acquired as indicated before. UV-induced apoptotic cells were used as positive control for Annexin V staining (28). Induced necrotic cells by either heating or freezing/thawing were used as positive control for 7-AAD staining (29).

RNA isolation and reverse transcription-PCR

Total RNA was extracted, using TRIzol (Life Technologies, Brooklyn, NY, USA), from peritoneal plasmoblast/plasma (intracellular IgM⁺CD19⁺F4/80⁺CD3⁻) cells or splenic

Syndecan-1⁺ cells. Peritoneal plasmoblast/plasma cells were obtained by negative cell sorting (CD19⁺F4/80⁺CD3⁻) using FITC-labeled anti-mouse CD3, PE-labeled anti-mouse CD19, biotin-labeled anti-mouse F4/80 and APC-St. The purity of this population was controlled by intracellular IgM staining. Syndecan-1⁺ cells were positively selected from spleen by cell sorting using PE-labeled anti-mouse Syndecan-1.

B-lymphocyte-induced maturation protein 1 (Blimp-1) and interferon regulatory factor-4 (IRF-4) mRNA expression was determined as previously described (30). The levels of mRNAs IRF-4 and Blimp-1 are presented as the relative expression of mRNA from peritoneal plasmoblast/plasma cells (CD19⁺) referred to splenic Syndecan-1⁺ plasma cells. Relative expression levels of the transcripts were estimated by standardization with internal control of GAPDH gene and evaluated by densitometry using GelPro analyzer 3.2.

Measurements of antibody production

For Ig determination in culture supernatant, peritoneal cells, peritoneal purified B-1 or B-2 cells, splenic B2 cells, splenocytes, lymph nodes cells or bone marrow were incubated in the conditions indicated in the legends for figures, at a density of 2×10^6 cell ml⁻¹ in a volume of 1 ml. Supernatants were collected and assayed in an IgM or IgG isotype-specific ELISA as described before (30). The reaction was developed with 3, 3', 5, 5'-tetramethylbenzidine substrate-chromogen (Dako, CA, USA). Each sample was assayed in triplicate and the values were expressed as concentration (ng ml⁻¹).

For *T. cruzi*-specific antibodies detection, half-area 96-well plates (Costar; Sigma Aldrich) were coated with $40 \mu\text{l}$ of *T. cruzi* F105 antigen diluted in carbonate-bicarbonate buffer ($15 \mu\text{g ml}^{-1}$) for 18 h at 4°C and then washed with $80 \mu\text{l}$ of PBS containing Tween 0.05% (PBS-Tween). They were blocked by adding $80 \mu\text{l}$ of PBS-Tween-BSA 1% for 1 h at 37°C. After washing, $40 \mu\text{l}$ of each sample (culture supernatant or sera) were added to the wells and the plates were incubated for 30 min at 37°C. Then, the plates were washed and $50 \mu\text{l}$ of peroxidase-conjugated goat anti-mouse IgM or IgG diluted 1:1500 and 1:2000 (Sigma-Aldrich, St Louis, MO, USA), respectively, was added and incubated for 30 min at 37°C. The plates were then washed and $50 \mu\text{l}$ of 3, 3', 5, 5'-tetramethylbenzidine substrate-chromogen (Dako) was added to the wells. The reaction was stopped by adding $50 \mu\text{l}$ 1N H₂SO₄. Each sample was assayed in triplicate and the values were expressed as mean of OD read at 450 nm in an ELISA reader (Bio-Rad, Hercules, CA, USA). Sera from normal and infected mice were assayed in parallel as negative and positive controls, respectively.

ELISpot assay

The frequency of IgM- or IgG-secreting cells was determined as previously described (31). Briefly, peritoneal, spleen or lymph node single-cell suspensions were distributed at various dilutions onto MultiScreen®-HA Plate (Millipore, Bedford, MA, USA) precoated with $7.5 \mu\text{g ml}^{-1}$ goat anti-mouse IgM or IgG (Southern Biotech) and then incubated for 3 h at 37°C and 5% CO₂. Plates were then treated with biotin-labeled goat anti-mouse IgM or IgG (Southern Biotech), followed by incubation with alkaline phosphatase-labeled streptavidin

(Southern Biotech). The plates were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP®)/nitro blue tetrazolium liquid substrate system (Sigma-Aldrich). Colored spots were counted in a stereomicroscope.

Transmission electron microscopy

Peritoneal B-lineage cells were fixed in 2% glutaraldehyde diluted in 0.1 M cacodylate buffer (pH 7.3). Samples were post-fixed in 1% OsO₄, dehydrated in acetone and embedded in araldite (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections were cut in a Porter-Blum MT2 ultramicrotome (Munich, Germany), examined and photographed in a Zeiss LEO 906 electron microscope (Zeiss, Overkochen, Germany).

Immunoelectron microscopy

Peritoneal B-lineage cells were fixed with glutaraldehyde (1% for 15 min) in 0.1 M cacodylate buffer (pH 7.3). Osmium postfixation was omitted. Cell pellets were dehydrated in ethanol and embedded in an acrylic resin (LR-White-London Resin Corp.). Thin sections were mounted in nickel grids and incubated in PBS-BSA 1% to block inespecific bindings and then on a drop of 16 nm colloidal gold/anti-mouse IgG complex (Pelco International) for 30 min at 37°C.

Parasitemia count

Peripheral blood was collected by eye bleeding at different times p.i. Erythrocytes were lysed in a 0.87% ammonium chloride buffer, and viable trypomastigotes were counted in a Neubauer counting chamber.

Statistics

The statistical significance was analyzed using unpaired Student's *t*-test. Values were considered statistically significant when *P* < 0.05.

Results

Trypanosoma cruzi infection induces a marked decrease in the number of CD19⁺ B cells from peritoneal cavity

To evaluate the development of the peritoneal B-cell response during the experimental *T. cruzi* infection, we first analyzed the number of CD19⁺ B cells within the peritoneal cavity at different times p.i. We observed that the percentage of peritoneal CD19⁺ B cells significantly decreased after 15 days of infection (Fig. 1A) when the parasite load is high [data not shown; (25, 26)]. Because the number of peritoneal cells was also reduced at day 15 p.i. (Fig. 1B), *T. cruzi*-infected mice exhibited a strong and significant reduction in the absolute number of peritoneal CD19⁺ B cells (Fig. 1C).

Considering that the peritoneal cavity lodges both B-1 (CD5⁺IgM⁺) and B-2 cells (CD5⁺IgM⁺), we next determined if the infection-driven decrease in CD19⁺ B-cell number influenced a particular B-cell subset. By flow cytometry, we observed that *T. cruzi* infection affected both peritoneal B-1 and B-2 cells, but with different kinetic and to a different extent (Fig. 1D and E). The percentage as well as the number of the B-2 cell population started to decrease by day 4 p.i. and reached normal levels at the chronic phase (day 65 p.i.) (Fig. 1E and F) while B-1 cell numbers decreased at

later times p.i. (day 15 p.i.) and remained very low throughout the period studied (Fig. 1E and F).

The reduction in B-cell numbers was accompanied by a remarkable increase in the percentage of a CD5⁺IgM⁺ population (Fig. 1D, days 15, 23 and 65 p.i.), likely T cells. Further phenotypic analysis of this population confirmed the augment of CD4⁺ and CD8⁺ T cells in the peritoneum of *T. cruzi*-infected mice (Supplementary Figure 1, available at *International Immunology* Online).

The decrease in peritoneal CD19⁺ B-cell numbers in T. cruzi-infected mice is associated with B-cell differentiation but not with cell death

To test whether the reduction in the number of peritoneal CD19⁺ B cells during *T. cruzi* infection resulted from enhanced cell death, we analyzed the levels of apoptosis in a B-cell-enriched peritoneal population (depleted from macrophages and T cells) referred to hereafter as peritoneal B-lineage cells. As depicted in Figure 2, minimal changes in the percentages of Annexin V⁺/7-ADD⁺ apoptotic cells within B-1 and B-2 cell populations were observed during the course of the infection. These results indicated that *T. cruzi* infection does not induce a strong apoptosis of peritoneal B-1 and B-2 cells, arguing against increased cell death as the cause of peritoneal B cell number reduction.

A second hypothesis to explain the disappearance of peritoneal CD19⁺ cells is the occurrence of terminal differentiation of activated B cells into CD19^{low/-} antibody-secreting cells. To test this, we determined the capability of peritoneal cells obtained from mice at different times p.i. to secrete Igs to the culture supernatants. We observed that after 15 days of infection, peritoneal B cells from infected mice secreted significantly higher amounts of IgM, IgG1, IgG2a, IgG2b and IgG3 than peritoneal cells from non-infected (day 0) or early infected (day 4) mice (Fig. 3A). Thus, the reduction in the numbers of B-1 and B2 peritoneal cells was kinetically correlated with the acquisition of the ability to secrete antibodies. Furthermore and as determined by the amount of secreted antibodies, peritoneal antibody-secreting plasma cells induced during *T. cruzi* infection seemed to be able to remain in the peritoneal cavity for periods longer than day 65 p.i. (Fig. 3A), although by this time parasitemia is already undetectable [data not shown, (25)].

To confirm the presence of plasma cells, we evaluated, by electron microscopy, the ultrastructural characteristics of the peritoneal B-lineage cells from non-infected and infected mice. Peritoneal B-lineage cells from non-infected mice showed a large cytoplasm and enlarged nucleus with diffuse chromatin similar to activated cells (Fig. 3B, left picture). In contrast, peritoneal B-lineage cells from infected mice exhibited the typical morphology of plasma cells (Fig. 3B, middle picture), including increased cytoplasmic volume with eccentric nuclei and abundant mitochondria and rough endoplasmic reticulum. Interestingly, within peritoneal B-lineage cells, we detected a particular population of plasma cells characterized by the presence in the cytoplasm of high number of granules (Fig. 3B, right picture). Cells with these characteristics have been previously named 'Mott-like cells', a pathologic state of plasma cells characterized by the presence of intracellular inclusions of Igs called Russell bodies

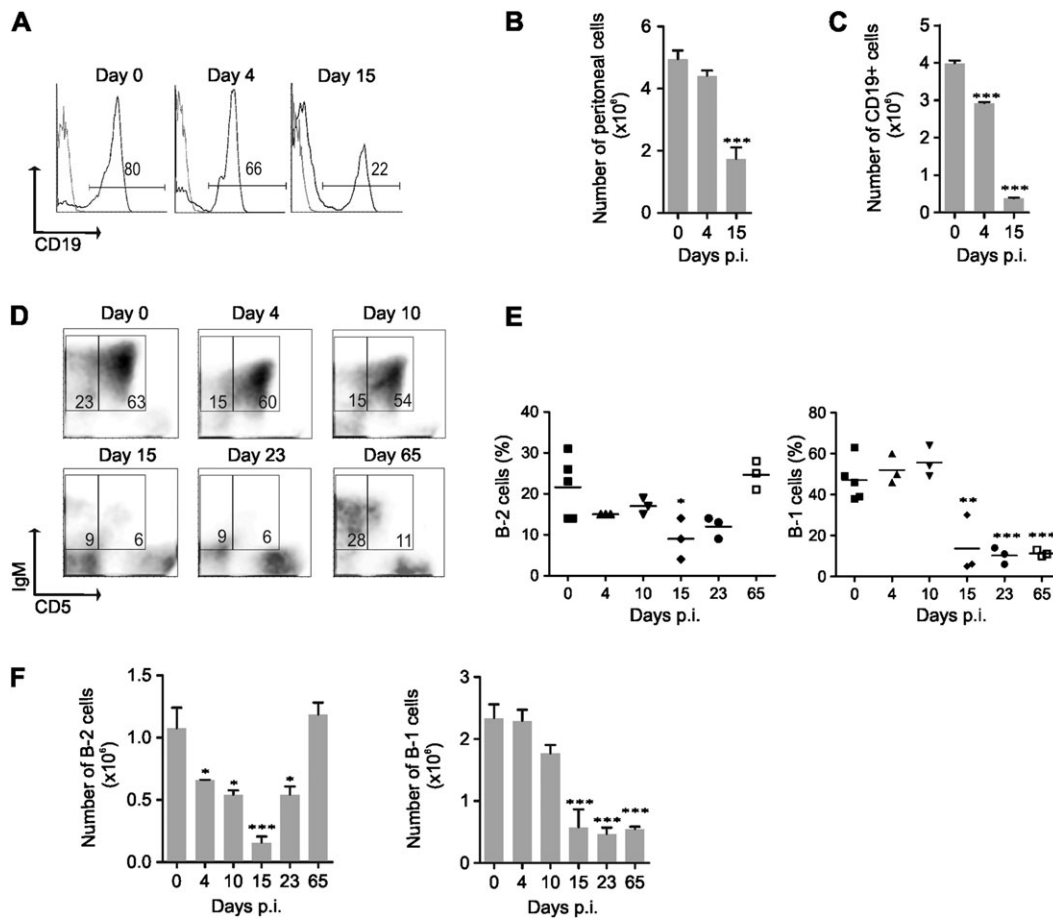


Fig. 1. *Trypanosoma cruzi*-infected mice exhibit a decrease of peritoneal CD19⁺ cells after 12–15 days p.i. Total peritoneal cells were obtained from non-infected control normal mice (day 0; $n = 6$) or *T. cruzi*-infected mice (different days p.i.; $n = 4$ per day). (A) Histograms show the percentage of CD19⁺ cells in peritoneal cells. (B) Number of total peritoneal cells. (C) Number of CD19⁺ cells calculated by referring the percentage obtained in (A) to the number of total peritoneal cells obtained in (B). (D) Representative plots of IgM versus CD5 expression in peritoneal cells. Numbers indicate percentages of cells within each region. (E) Percentage of peritoneal B-2 cells (identified as IgM⁺CD5[−]) and B-1 cells (identified as IgM⁺CD5⁺) as determined in (D). (F) Number of B-2 and B-1 cells calculated by referring the percentage obtained in (E) to the number of total peritoneal cells obtained in (B). The analysis was performed gating on lymphocytes from forward scatter versus side scatter dot plots. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are representative of three to five independent experiments.

(32). By immunoelectronmicroscopy, we determined that the granules observed in the peritoneal plasma cells from *T. cruzi*-infected mice contained Igs, confirming the phenotype of Mott-like cells (Fig. 3C).

The peritoneal cavity of T. cruzi-infected mice lodges plasmablasts/plasma cells expressing typical plasma cell transcription factors

The development of peritoneal plasma cells during the course of *T. cruzi* infection was also determined by flow cytometry. Thus, a marked percentage of cells within the intracellular IgM⁺ peritoneal B-lineage population obtained from infected, but not from non-infected mice, exhibited a CD19^{low/−} phenotype (Fig. 4A). Furthermore, almost all the intracellular IgG⁺ peritoneal B-lineage cells from infected mice showed reduced expression of CD19, compatible with a plasma cell phenotype (Fig. 4A). Further phenotypic analysis revealed that these intracellular IgM⁺ and IgG⁺ populations were also CD5^{low/−} and CD23^{low/−} (data not shown).

Analyzing the expression of MHC-II on total peritoneal B-lineage cells from infected mice, two distinct populations were identified within the CD19^{low/−} peritoneal cells (Fig. 4B). According to the literature (33), these subsets, which were not found in non-infected mice, would correspond to CD19^{low/−} MHC-II⁺ plasmablast and to CD19^{low/−} MHC-II[−] terminally differentiated plasma cells. Unexpectedly, CD19^{low/−} peritoneal B-lineage cells from infected mice did not express the prototypical plasma cell marker Syndecan-1 (Fig. 4C). The lack of Syndecan-1 expression is not a common feature for peritoneal B cell-derived plasma cells because peritoneal B cells up-regulated this marker in response to CpG-ODN (Fig. 4C). Another atypical phenotypic characteristic of peritoneal plasma cells from *T. cruzi*-infected mice is related to the expression of the plasma cell-associated activation marker FcγRIIb involved in the control of plasma cell survival and up-regulated during terminal differentiation (34). As shown in Figure 4D, a proportion of peritoneal plasma cells from infected mice showed a decreased expression of FcγRIIb in comparison to peritoneal cells

from non-infected mice. Although peritoneal plasma cells induced during *T. cruzi* infection lack of the expression of surface markers usually ascribed to antibody-secreting plasma cells, they showed detectable levels of transcripts encoding IRF-4 and Blimp-1, transcription factors involved in plasma cell differentiation (35) and highly expressed in conventional Syndecan-1⁺ plasma cells (Fig. 4E).

Considering that antibody-secreting cells remain in the peritoneal cavity of infected mice during extended periods

(longer than 65 days after infection), we analyzed in these cells the expression of some chemokine receptors involved in plasma cell migration. Peritoneal B-lineage cells from non-infected mice expressed CXCR4, the peritoneal cavity homing receptor (36), but not CXCR3, the IFN- γ -induced receptor that mediates migration to inflamed tissues. Similar levels of CXCR4 and CXCR3 expression were observed on the peritoneal B-lineage cells obtained from 15-day infected and control mice (Fig. 4F). This result indicated that, in contrast to what is observed during the terminal differentiation of memory B cells (37), *T. cruzi* infection did not affect the profile of chemokine receptor expression on peritoneal B-lineage cells.

*Peritoneal B cell differentiation is triggered by the *T. cruzi* itself but not by cytokines produced by peritoneal cells*

To determine the possible factors involved in the terminal differentiation of peritoneal B cells induced by *T. cruzi* infection, we first evaluated whether peritoneal cells from infected mice are able to secrete cytokines implicated in B cell differentiation (38). We observed that after 15 days of infection, peritoneal cells from infected mice cultured during 48 h spontaneously released higher amounts of IL-10, tumor necrosis factor and IFN- γ than peritoneal cells from non-infected mice. Moreover, while the amount of IL-5 released was similar for both groups studied, the levels of IL-6 secreted by peritoneal cells from infected mice were significantly decreased (Fig. 5A). Next, we studied whether these cytokines or other soluble factors could mediate peritoneal B cell differentiation. Co-culture experiments using CD19 down-regulation as read-out of differentiation showed that soluble mediators derived from infected-mice peritoneal cells were not able to induce the differentiation of peritoneal B cells purified from non-infected mice after 72 or 120 h of culture (Fig. 5B).

Because the presence of antibody-secreting cells was strongly correlated with the parasite burden (data not

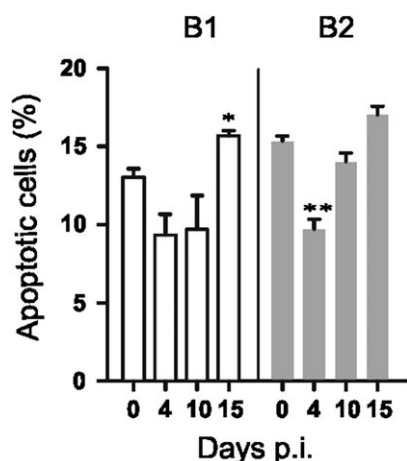


Fig. 2. The decrease in peritoneal CD19⁺ B cell number in *T. cruzi*-infected mice is not associated with cell death by apoptosis. Peritoneal B-lineage cells were obtained from non-infected control normal mice (day 0, $n = 6$) or *T. cruzi*-infected mice (different days p.i., $n = 4$ per day). After 10 h of culture, the cells were harvested and stained with Annexin V and anti-mouse CD23 or CD5 mAbs. The percentage of 7 ADD⁻ Annexin V⁺ B-1 cells (white bars) or 7 ADD⁻ Annexin V⁺ B-2 cells (gray bars) determined by flow cytometry is depicted. * $P < 0.05$, ** $P < 0.01$. Data are representative of three independent experiments.

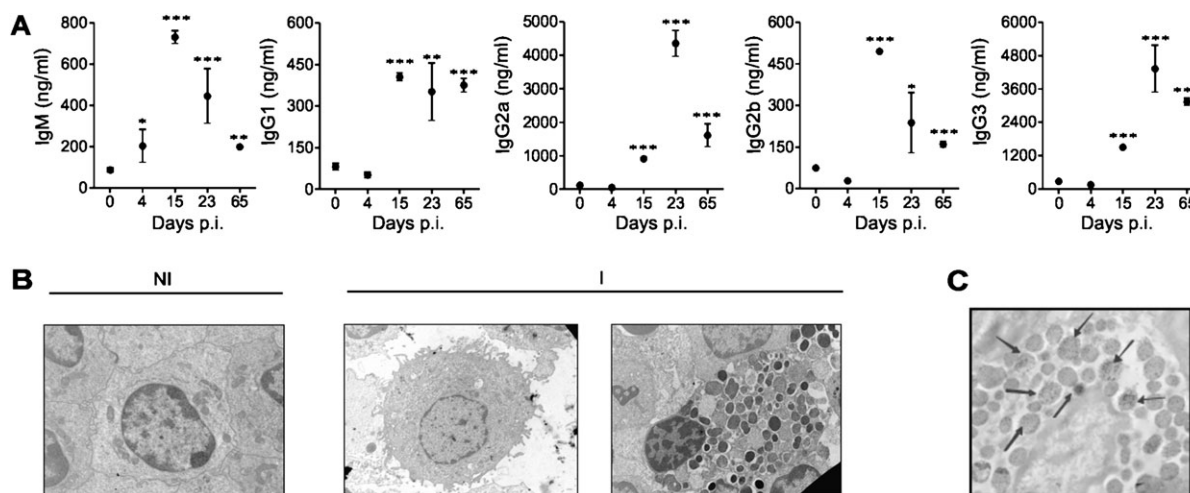


Fig. 3. Peritoneal B cells decrease in *T. cruzi* infection is associated with B cell differentiation. (A) Concentration of IgM, IgG1, IgG2a, IgG2b and IgG3 was determined by ELISA in the supernatant of peritoneal cells from non-infected control normal (day 0; $n = 5$) or infected mice (different days p.i.; $n = 4$ per day) cultured during 48 h with medium. Results are expressed as mean concentration of duplicates \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) Electron microscopy image of purified peritoneal B-lineage cells from non-infected control normal mice (NI, left panel) and 15-day infected (I, middle and right panels) mice. (C) Immunoelectronmicroscopy of peritoneal B-lineage cells from 15-day-infected mice showing intracellular granules containing Igs (arrows). Data are representative of three independent experiments.

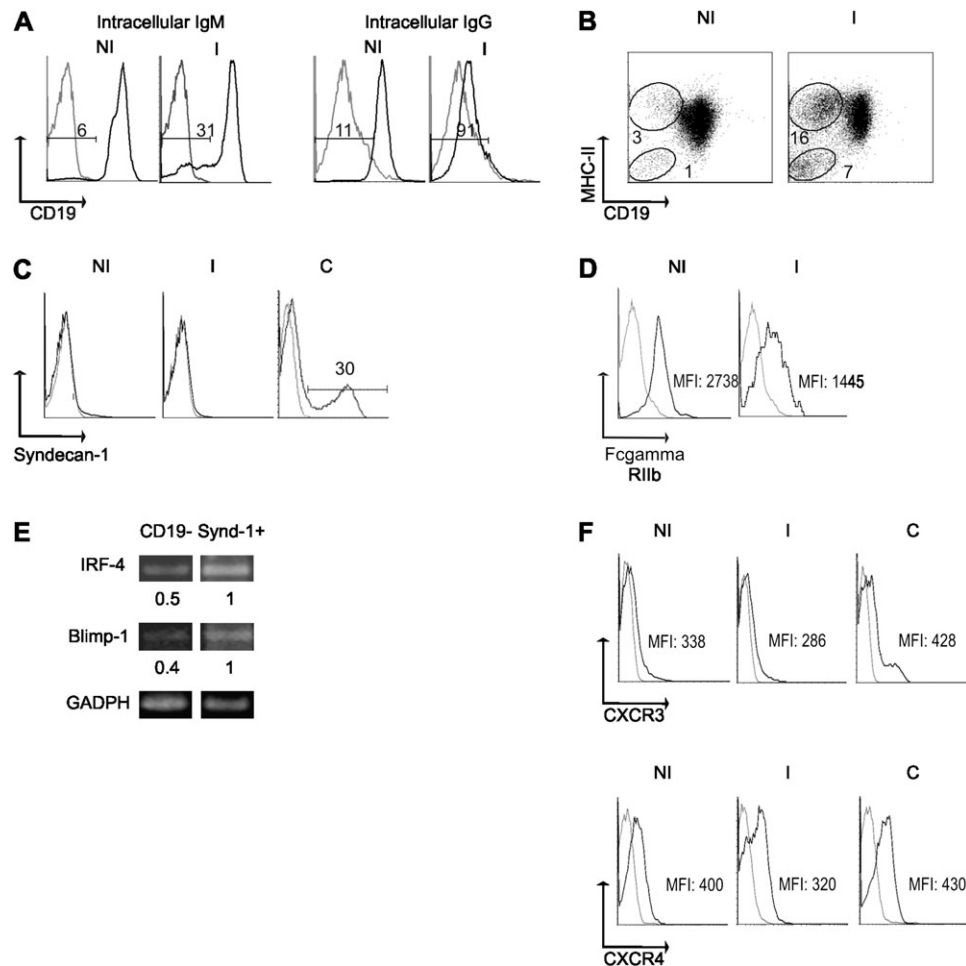


Fig. 4. Antibody-secreting cells from peritoneal cavity of *T. cruzi*-infected mice show a 'non-conventional' phenotype. Peritoneal B-lineage cells were obtained from non-infected control normal mice (NI) ($n = 4$) or *T. cruzi* 15-day-infected (I) mice ($n = 8$). (A) Representative histograms of CD19 expression gated on intracellular IgM⁺ or intracellular IgG⁺ cells. Numbers indicate the percentage of intracellular Ig⁺CD19⁺ cells. (B) Representative plots of MHC-II versus CD19 expression on peritoneal B-lineage cells. Numbers represent the percentage of cells within each region. (C) Representative histograms of Syndecan-1 expression on peritoneal B-lineage cells. Peritoneal B-lineage cells cultured with CpG were used as positive control (indicated with C) of Syndecan-1 expression. (D) Representative histograms of FcγRIIb expression on peritoneal B-lineage cells. Numbers indicate the mean fluorescence intensity (MFI). (E) Peritoneal plasmablast/plasma cells (CD19⁺) and splenic Syndecan-1⁺ (Synd-1⁺) plasma cells from infected mice were purified by sorting from peritoneum and spleen, respectively, as described in Materials and Methods ($n = 15$). Reverse transcription-PCR of IRF-4, Blimp-1 or GADPH (control for RNA integrity and loading). Numbers indicate the relative expression of mRNA from peritoneal plasmablast/plasma cells (CD19⁺) referred to splenic Syndecan-1⁺ plasma cells. (F) Representative histograms of CXCR3 and CXCR4 expression. Numbers indicate the MFI. Splenic T lymphocytes or total peritoneal cells from normal mice were used as positive controls (indicated with C) for CXCR3 and CXCR4 expression, respectively. Data are representative of three independent experiments.

shown) and live trypomastigote of *T. cruzi* are found in the peritoneal washout of infected mice after 15 days p.i. (Fig. 5C and Supplementary Figure 2, available at *International Immunology* Online), we evaluated if peritoneal B-1 and B-2 cell differentiation may be mediated by direct interaction with the parasite. Sorted peritoneal B-1 and B-2 cells and splenic B-2 cells from non-infected mice were incubated with live *T. cruzi* trypomastigotes during 5 days and the concentration of IgM and IgG was determined by ELISA. After stimulation, increased concentrations of IgM were detected in the supernatants of peritoneal B-1 and B-2 cells, but not splenic B-2 cells (Fig. 5D) while IgG secretion was undetectable in all cases (data not shown). These results indicate that *T. cruzi* parasites were able to trigger terminal

differentiation but not Ig-class switching of purified peritoneal B-1 and B-2 cells in the absence of any other stimuli.

Antibody-secreting plasma cells are found at low frequency in peritoneal cavity of T. cruzi-infected mice but secrete high amounts of unspecific Igs

As an indirect way to evaluate the contribution of peritoneal B cells to the humoral response in *T. cruzi* infection, we compared the frequency of antibody-secreting cells as well as the production of IgM and IgG in different secondary lymphoid tissues. As depicted in Fig. 6A, IgM- and IgG-secreting plasma cells were found in peritoneal cavity, spleen and lymph nodes from *T. cruzi*-infected mice. The frequency of antibody-secreting cells varied among the tissues, being

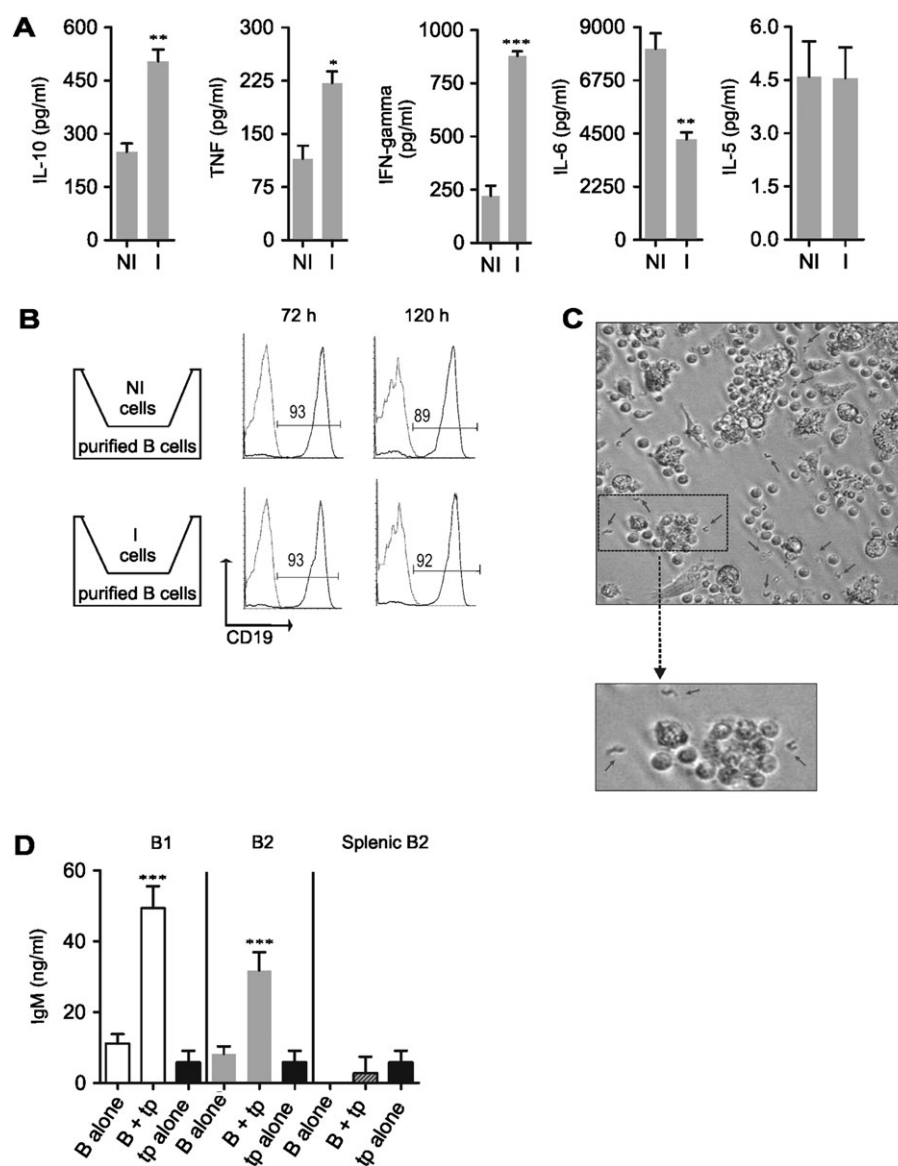


Fig. 5. Peritoneal B-1 cell differentiation is triggered by the *T. cruzi* itself but not by cytokines produced by peritoneal cells. (A) Concentration of IL-10, tumor necrosis factor, IFN- γ , IL-6 and IL-5 determined by ELISA in the supernatant of peritoneal cells from non-infected control normal mice (NI; $n = 3$) or 15-day-infected mice (I; $n = 5$) cultured during 48 h with medium. Results are expressed as mean concentration of duplicates \pm SD. (B) Expression of CD19 in purified B cells co-cultured during 72 or 120 h with peritoneal cells from non-infected control normal mice (NI, upper panels; $n = 6$) or 15-day-infected (I, lower panels; $n = 8$) mice. Numbers indicate the percentage of CD19⁺ cells. (C) Phase-contrast microscopy image of 15-day-infected mice peritoneal washout showing living trypomastigotes (arrows). Shown in inset are magnified details with cells surrounded by trypomastigotes. (D) Concentration of IgM determined by ELISA in the supernatant of purified peritoneal B-1 (white bars), purified peritoneal B-2 (gray bars) or purified splenic B2 cells (gray striped bars) ($n = 6$) after 5 days of culture with trypomastigotes. Trypomastigotes alone were cultured as control (black bars). Results are expressed as mean concentration of duplicates \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are representative of three independent experiments.

higher in the spleen and lymph nodes than in peritoneum. Despite the marked reduction in the total number of peritoneal cells from *T. cruzi*-infected mice, we determined that the absolute number of antibody-secreting cells in the peritoneum from infected mice (calculated as spots in ELISpot assay per total cell number) was increased in comparison to normal mice (data not shown).

Regardless of their low frequency, peritoneal cells secreted higher amounts of all Ig isotypes than cells from

spleen, lymph nodes and, even, bone marrow (Fig. 6B). These results indicate that, although at low frequencies, peritoneal cavity lodges a population of antibody-secreting cells which seems to have an enhanced ability to secrete antibodies, and consequently, may contribute to the pool of circulating antibodies produced during *T. cruzi* infection.

To further elucidate a possible role of peritoneal antibody-secreting cells in the resistance to *T. cruzi* infection, we analyzed if the antibodies secreted by peritoneal B cells from

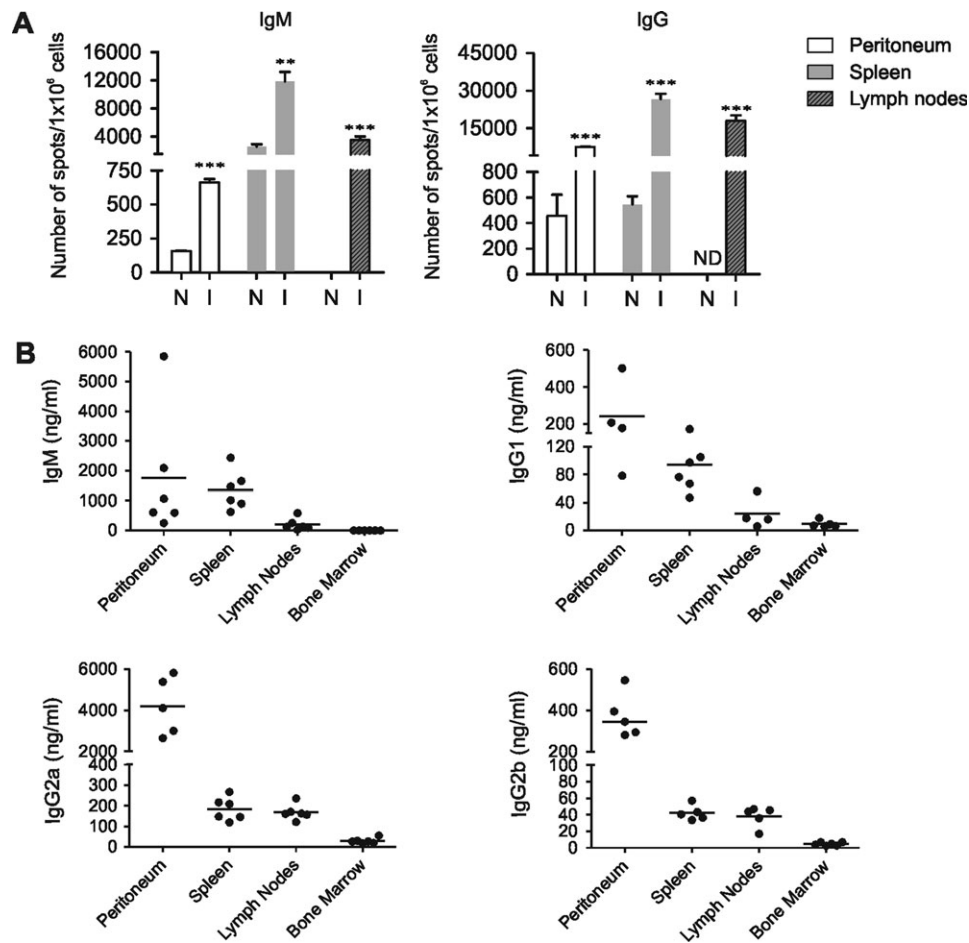


Fig. 6. Antibody-secreting plasma cells are at low frequency in peritoneal cavity of *T. cruzi*-infected mice but secrete high amounts of unspecific Igs. (A) Frequency of IgM or IgG-secreting cells in peritoneum (white bars), spleen (gray bars) or lymph nodes (gray striped bars) from *T. cruzi*-infected mice determined by ELISpot. Results are expressed as the mean number of spots/1 × 10⁶ cells ± SD of three to four different well counts. ***P* < 0.01, ****P* < 0.001. Data are representative of three to five independent experiments. (B) Concentration of IgM, IgG1, IgG2a and IgG2b determined by ELISA in the cell supernatants from peritoneum, lymph nodes, spleen and bone marrow obtained from *T. cruzi*-infected mice (15 days p.i., *n* = 6) cultured during 48 h with medium. Results are expressed as mean concentration of duplicates ± SD. ND, not detectable.

T. cruzi-infected mice were parasite specific. By ELISA and immunofluorescence, we determined that peritoneal B-lineage cells from *T. cruzi*-infected mice produced unexpectedly low levels of parasite-specific IgM and IgG that were detected only at days 18 and 32 p.i. (Fig. 7 and data not shown). Therefore, peritoneal plasma cells arising during *T. cruzi* infection would be an important source of antibodies but with reduced specificity for parasite antigens.

Discussion

In the present work, we showed that peritoneal B cell response to *T. cruzi* infection is characterized by a marked reduction in the number of CD19⁺ B cells. Although a similar observation was reported by Minoprio *et al.* (23), we characterized this phenomenon and determined that the disappearance of peritoneal B cells induced by *T. cruzi* infection is due to an enhanced differentiation into plasma cells. Moreover, we determined that the reduction in B-2 and B-1 perito-

neal B cell numbers showed different kinetics, starting at day 4 p.i. for B-2 cells and at day 12–15 p.i. for B-1 cells. The delayed disappearance of B-1 cells was surprising because this compartment is known to suffer modifications at early times of infection, before the onset of the adaptive immune response (6). In fact, during bacterial infections, B-1 cells respond after just 3–6 h p.i. with visible peritoneum changes such as the diminution of resident B-1 cells and the down-regulation of integrins to allow detachment from local matrix (39). This B-1 cell behavior is not observed during *T. cruzi* infection, where, in turn, peritoneal B-2 cells presented an innate-like behavior, responding earlier than peritoneal B-1 cells.

Electron microscopy studies confirmed the presence of cells with typical plasma cell morphology in the peritoneal cavity of *T. cruzi*-infected mice. Interestingly, also atypical plasma cells containing high number of cytoplasmatic Ig⁺ granules were detected within the peritoneal cavity from infected mice. These cells have been previously named

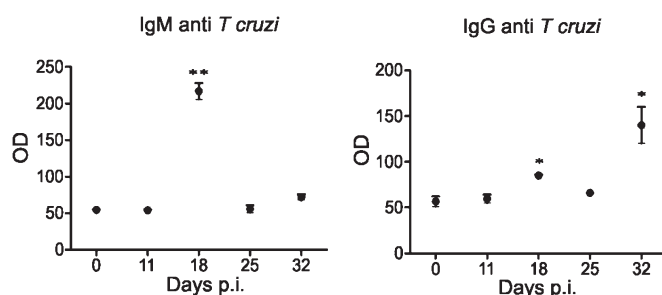


Fig. 7. Peritoneal B cells from *T. cruzi*-infected mice secrete low levels of parasite-specific antibodies. Levels of *T. cruzi*-specific IgM and IgG determined by ELISA in the supernatant of peritoneal cells from non-infected control (day 0; $n = 4$) or *T. cruzi*-infected mice (different days p.i.; $n = 6$) cultured during 48 h with medium. Results are expressed as OD mean \pm SD. * $P < 0.05$, ** $P < 0.01$. Data are representative of three to five independent experiments.

'Mott-like cells' since they resemble the so-called Mott cells, a pathologic state of plasma cells that contain intracellular inclusions of Igs (Russell bodies). These cells are frequent in lymphoid tissues of mice and humans suffering autoimmune diseases and in normal mice as a consequence of extreme antigenic load (40). Neither the genesis nor the significance of Mott-like cells in normal or autoimmune states is well understood; however, it has been described that B-1, but not B-2, cells can be induced *in vitro* to form Mott-like cells in the presence of LPS or IL-5 (41). Considering this, it is possible that the Mott-like cells detected in the peritoneal cavity of *T. cruzi*-infected mice are originated from B-1 cells whereas the conventional plasma cells develop from B-2 cells. Furthermore, although the specific role of Mott-like plasma cells in the experimental Chagas disease has not been elucidated, their association with autoimmune manifestations (41) suggests that these cells may be involved in the autoimmune responses observed during *T. cruzi* infection.

Many phenotypic and functional characteristics such as the loss of B-lineage surface markers (i.e. CD19, CD23, CD5 and MHC-II), the ability to secrete antibodies and the expression of prototypical transcription factors such as Blimp-1 and IRF-4 (35) confirmed that the intracellular IgM⁺CD19⁺ cells detected in the peritoneal cavity of infected mice are plasma cells. However, peritoneal antibody-secreting cells show some features that differ from those ascribed to 'conventional' splenic B-2-derived plasma cells (42). For example, peritoneal plasma cells from infected mice did not express the classical plasma cell marker, Syndecan-1, and express lower levels of Blimp-1 in comparison to splenic Syndecan-1⁺ plasma cells. It has been reported that antibody-secreting cells are heterogeneous as regards Blimp-1 and Syndecan-1 expression (43). Kallies *et al.* (43) showed that cells with low levels of Blimp-1 are able to secrete a similar concentration of IgM but higher concentrations of IgG2a and IgG3 in comparison to Syndecan-1⁺ Blimp-1^{hi} cells, suggesting that there is not always a correlation between the ability to secrete antibodies and the level of Blimp-1 expression. In addition, these cells remained for long periods in the peritoneal cavity even though the peritoneum has not been described as a plasma cell homing site. In fact, some controversial findings suggest

that peritoneal committed-plasmablasts abandon peritoneal cavity to differentiate in mesenteric lymph nodes and to finally home to intestinal lamina propria (44).

Different reports have established a key role for chemokines in guiding antibody-secreting cells to the bone marrow, inflamed tissues or mucosal sites (37, 45). CXCR4 expression is key for bone marrow homing but it is also involved in peritoneal B cell homing (1) while IFN- γ drives plasmablasts to inflamed tissue by increasing the expression of CXCR3 in differentiating plasmablasts and the secretion of CXCR3 ligands by cells present in inflamed tissues (37). Even though the expression of chemokine receptors not always correlates with cell migration, a plausible explanation for the confinement of peritoneal antibody-secreting cells from *T. cruzi*-infected mice to the peritoneal cavity should be found in their phenotype. In this regard, we observed that despite the high levels of IFN- γ secreted by peritoneal cells from *T. cruzi*-infected mice (Fig. 5A), the peritoneal plasma cells did not upregulate CXCR3 and remained in peritoneum. Furthermore, by maintaining the expression of CXCR4, the infection-induced peritoneal plasma cells would license these cells home to bone marrow; however, the lack of Syndecan-1 expression, required for bone marrow homing (1), will constrain these cells to remain in the peritoneal cavity.

Another phenotypic characteristic that may modulate the permanence of antibody-secreting cells in the peritoneal cavity of *T. cruzi*-infected mice is the absence of Fc γ RIIb upregulation. Fc γ RIIb is a low-affinity inhibitory receptor for the Fc portion of IgG (46) whose expression is normally up-regulated during B cell activation and plasma cell differentiation (34). Fc γ RIIb cross-linking by immunocomplexes or specific antibodies turns the cells susceptible to apoptosis in a process that controls plasma cell persistence and is required for tolerance. In this regard, systemic lupus erythematosus (SLE)-prone NZB or MRL mice, characterized by their increased number of plasma cells (47), fail to upregulate Fc γ RIIb in germinal center B cells. Consequently, plasmablasts have no detectable Fc γ RIIb and cannot be killed by this pathway (48). Similarly to SLE-prone mice, the peritoneal plasma cells from *T. cruzi*-infected mice expressed low levels of Fc γ RIIb in comparison with cells from normal mice and, therefore, would be resistant to this control mechanism. Considering this, it is possible that the phenotype of the infection-induced plasma cells may favor the persistence of autoreactive plasma cells and the autoimmune manifestations associated to this parasite infection.

Regarding the signals that induce the differentiation of peritoneal plasma cells during *T. cruzi* infection, we determined that soluble mediators secreted by peritoneal cells were not capable of inducing B cell differentiation *in vitro*. However, we cannot rule out that cytokines increased during the infection such as IL-10, IL-6 and IL-5, known to induce B-2 cell differentiation, may have a role *in vivo*, maintaining antibody-secreting cell survival (49). Although refractory to environmental soluble factors, peritoneal B-1 and B-2 cells, but not mature splenic B cells, differentiate into plasma cells *in vitro* in a T-independent fashion when cultured with the trypomastigote of *T. cruzi*. This differentiation is observed when the ratio parasite/host cell is higher than 0.5 (Fig. 5D and

data not shown). This result may explain why at the beginning of the infection the parasites did not trigger B-1 cell differentiation, but differentiation is observed at the peak of parasitemia when the parasite load is high (26).

Analyzing the contribution of different lymphoid tissues to the humoral response during *T. cruzi* infection, we determined that peritoneal cavity presented lower frequency of antibody-secreting plasma cells than spleen and lymph nodes. The apparent discrepancy between the strong reduction in the number of peritoneal CD19⁺ cells and the low frequency of antibody-secreting cells may be explained by the presence of Mott-like cells that exhibit a CD19^{low/-} phenotype compatible with terminally differentiated plasma cells which may be no detectable by ELISpot as consequence of their reported defects on Ig secretion (40). Interestingly, despite the low frequency of antibody-secreting plasma cells, peritoneal cells from *T. cruzi*-infected mice secreted higher amounts of antibodies than the other lymphoid tissues. This result underscores the enhanced ability of peritoneal cells from *T. cruzi*-infected mice to secrete Igs, reinforcing the idea that the population of peritoneal Syndecan-1⁻ plasma cells exhibit distinctive features in comparison to Syndecan-1⁺ plasma cells from spleen and lymph nodes.

In agreement with Reina-San-Martin *et al.* (50), we further established that the peritoneal B cell response observed during *T. cruzi* infection is scarcely specific for the invading pathogen. Despite this, it is likely that the peritoneal plasma cells induced during *T. cruzi* infection can release 'sticky' antibodies that could contribute to parasite control. This hypothesis is supported by reports showing that peritoneal B cells produce antibodies of broad specificity that, at very high concentration, are able to unspecifically stick to several microorganism providing protection (51).

Supplementary data

Supplementary Figures 1 and 2 are available at *International Immunology* Online.

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