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Glycoinositolphospholipids from *Trypanosoma cruzi* induce B cell hyper-responsiveness *in vivo*

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The surface of the protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, is covered by a dense glycolipid layer, composed mainly by a structurally related family of glycoinositolphospholipids (GIPLs). In the present study we evaluated the *in vivo* effects of the GIPL on B cell function and immunoglobulin (Ig) secretion. We observed that GIPL injection led to a sustained increase in circulating IgM levels. B cells from GIPL injected mice showed higher response when activated *in vitro* with either LPS or dextran-conjugated anti-IgD antibodies or purified cytokines. GIPL purified from *T. cruzi* also showed an adjuvant effect, since this glycophospholipid boosted a polysaccharide-(TNP-Ficoll) induced IgG response. Taken together, our data indicate that *T. cruzi*-derived GIPL could be at least partially responsible for the remarkable B cell activation observed during *T. cruzi* acute infection *in vivo*.

Keywords: **glycoinositolphospholipids (GIPLs), B lymphocytes, immunoglobulin, *Trypanosoma cruzi***

Abbreviations: **GPI, glycosylphosphatidylinositol; GIPL, glycoinositolphospholipid; Ig, immunoglobulin; Ins, inositol; LPS, lipopolysaccharide; PI, phosphoinositol.**

Introduction

The surface of trypanosomatids is covered by glycoconjugates that are bound to the membrane through glycosylphosphatidylinositol (GPI) anchors. These glycoconjugates are composed by a lipid intramembrane portion, the GPI core containing Man α(1-4) GlcN α(1-6) $InsPo_4$ and a terminal segment that contains a protein, a polysaccharide or an oligosaccharide sequence [1]. GPI-anchored oligosaccharides (named glycoinositolphospholipids, GIPLs) are abundant on *T. cruzi* surface. These molecules contain a glycan domain linked through a non-*N*-acetylated glucosamine residue to an inositolphospho-ceramide [2]. Two series of closely related GIPL structures are expressed on the surface of strains of *T. cruzi* [3]. In the G strain (see Scheme 1), the glycan moiety of the major GIPL has a tetramannose sequence where the nonreducing mannose is substituted by β-galactofuranosyl residue, and the third mannose distal to inositol and the non-acetylated glucosamine are substituted by ethanolaminephosphate and 2-aminoethylphosphonate, respectively [3]. This PI-oligosaccharide is linked to a *N*-lignoceroyldihydrosphingosine [2].

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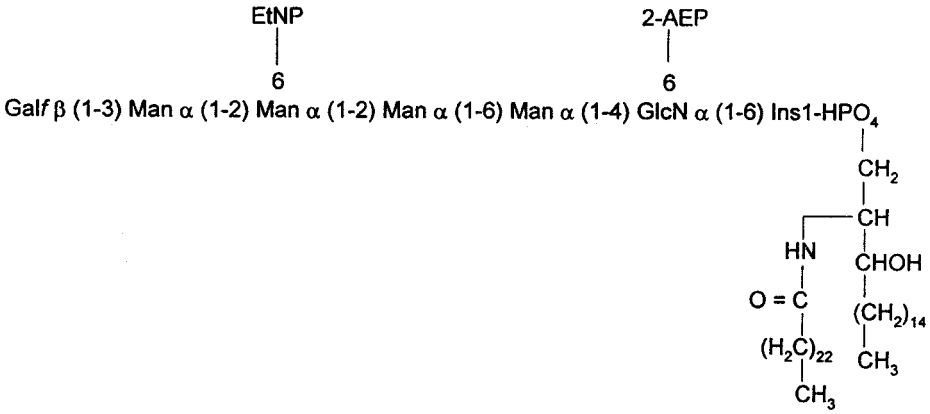
T. cruzi infection is associated with a polyclonal B cell activation. This alteration in B cell function is detected early during infection and can be observed even during the chronic phase [4,5]. This polyclonal B cell activation observed during murine experimental *T. cruzi*-induced infection is regulated by T cells [6,7] and natural killer cells (Arruda-Hinds *et al.*, unpublished results). Even though a polyclonal B cell activation is detected during infection, B cell function is inadequate since immunosuppression and the generation of auto-antibodies are observed [8,9]. Several studies have described that during the acute phase of *T. cruzi* infection there is a decrease in the response to both *T. cruzi* antigens and to antigens not related to the parasite. This hyporesponsiveness is reversible since it was shown that infected animals are non-responsive to *T. cruzi* antigens during acute infection but have normal response during the chronic phase of infection [10]. B cells isolated from acutely infected mice show spontaneous proliferation, increased production of IgM, and have higher expression of the Fas antigen, an activation profile that was suggested to represent a mechanism that controls B cell expansion [11]. Studies analyzing blood cells from infected chronic patients have shown increased levels of activated T cells and $CD5^+$ B cells in the circulation [12]. These activated cells persist despite the absence of detectable circulating parasites [13].



Scheme 1. Structure of the majors glycoinositoiphospholipid (GIPL) from the G strain of *T. cruzi*. 2-AEP, 2-aminoethylphosphonate; EtNP, ethanolaminephosphate; Gal*f*, galactofuranose; GlcN, glucosamine; Man, mannose; Ins, inositol.

Recently, the whole *T. cruzi*-derived GIPL or its isolated moieties were shown to induce several modifications on lymphoid cell function *in vitro*. The ceramide lipid moiety of *T. cruzi* GIPL induced apoptosis of murine macrophages in the presence of IFN-gamma [14]. Also, it was described to inhibit polyclonal T cell activation [15]. The GIPL was also shown to raise cytoplasmic calcium levels and to increase anti-CD3-induced IL-2 secretion by T cell hybridomas [16]. The proposed roles of the glycan portion include the stimulation of IgM secretion by B cells in the absence of co-stimuli and the increase in B cell response induced by either surface immunoglobulin (Ig) ligation or cytokines [17]. The B cell stimulatory effect of the GIPL is mediated mainly by its oligosaccharide moiety [17]. The effect of the GIPL on B cells is both direct and modulated by natural killer activation induced by this glycoconjugate [18].

Molecules derived from different microorganisms are described to stimulate B cells through the induction of accessory signals necessary for B cell activation [19]. Our previous data strongly suggest that the *T. cruzi*-derived GIPL could have important immunopotentiating effect. However, our studies did not directly characterize whether the GIPL could mimic the *in vivo* alterations on B cell function that were observed during experimental *T. cruzi* infection and whether this molecule would modify *in vivo* B cell response to unrelated antigens. To address these issues, in the present study we investigated the effect of *in vivo* treatment with *T. cruzi* GIPL and its oligosaccharide moiety on B cell function and its effect upon B cell response to unrelated antigens. Our findings suggested that the GIPL can be one of the *T. cruzi*-derived products that could modify B cell function during infection.

Material and methods

Mice

Male and female BALB/c mice (6 to 8 weeks of age) were obtained from the animal facility of the Instituto de Microbiologia from Universidade Federal do Rio de Janeiro. The animals were bred and housed in the animal facility of the Instituto de Microbiologia according to institutional policies for animal care and usage.

Antibodies and other reagents

The dextran conjugated anti-IgD antibody (anti-IgD-dextran) was prepared by the conjugation of the $H\delta^a/1$ anti-IgD monoclonal antibody [20] to high molecular weight dextran as previously described [21]. The conjugate used in the described experiments had an average of six molecules of anti-IgD antibody per dextran molecule and was kindly provided by Dr. James J. Mond (Uniformed Services University, Bethesda, MD). Murine recombinant IL-4 (specific activity 2×10^7 U/mg) was obtained from Pharmingen (San Diego, CA). LPS W, extracted from *Escherichia coli* 0111:B4, was obtained from Difco Laboratories Inc. (Detroit, MI).

B cell purification

Suspensions of single spleen cells were washed three times with RPMI 1640 (Sigma Chemical Co., St. Louis, MO) plus 10% FCS (ICN Biomedicals Inc., Costa Mesa, CA) and treated with a cocktail of culture supernatants of anti-T cell antibodies (anti-Thy-1, anti-CD4 and anti-CD8) [21] for 30 min on ice. This was followed by treatment with Low Tox-M rabbit complement (Cedarlane, Ontario, Canada) in the presence of tissue culture fluid containing the anti-rat κ chain mAb MAR 18.5 [22] at 37°C for 45 min. B cell fractionation was performed as previously described [23,24]. Briefly, discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden) were used to separate large (low density) B cells from small (high density) B cells. Gradients consisting of 70, 65, 60 and 50% Percoll (with densities of 1.086, 1.0815, 1.074, and 1.062 g/ml, respectively) were prepared. A suspension of splenic B cells obtained as described above was layered on the cold Percoll gradients and spun at $1900\times$ *g* for 15 min. The high density (small) cells were collected from the 70–65%

interface, and the low density (large) cells were collected from the 60–50% Percoll interface. Flow cytometry analysis of the B cell preparations showed an 85 to 90% $B220^+$ cell population and a contamination with less than 3% residual ($CD3^+$) T cells.

Measurement of Ig levels in the culture supernatants

B cells were cultured for 5 days in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), L-glutamine (2 mM), 2-mercaptoethanol (50 μM), non-essential aminoacids (100 μM), sodium pyruvate (1 mM) and gentamicin (50 μg/ml) [complete RPMI medium], in a final volume of 200 μl in flat bottom 96-well trays (Costar, Cambridge, MA). Quantification of IgM was performed by a modification of a previously described Ig ELISA [25]. Unlabeled and alkaline phosphatase-labeled affinity purified goat anti-mouse IgM (Southern Biotechnology Associates Inc. Birmingham, AL) were used. A color product was generated from cleavage of *p*-nitrophenolphosphate (Sigma Chemical Co., St. Louis, MO). The optical density of the product was measured in a 550 Microplate Reader (BIORAD, Hercules, CA). OD units were converted to IgM concentrations by extrapolation from standard curves determined in each assay by using purified myeloma proteins of known concentrations (ICN Biomedicals Inc., Costa Mesa, CA). Each assay system showed no significant cross-reactivity or interference from the presence of other isotypes found in the culture supernatants. Supernatants were obtained from triplicate cultures.

Proliferation assay

B cells were cultured for either 24 or 48 h in a final volume of 200 μl in complete RPMI medium in flat bottom 96-well trays (Costar, Cambridge, MA). Tritiated thymidine (1 μCi) (ICN Biomedicals Inc. Costa Mesa, CA) with a specific activity of 20 Ci/mmol was added to the cultures in the final 18 h of incubation and cultures were harvested with a PHD Cell Harvester (Cambridge Technology, Watertown, MA) onto glass fiber filters. Specific incorporation of tritiated thymidine was analyzed by liquid scintillation spectroscopy and results are expressed as the arithmetic mean of triplicate cultures.

In vivo experiments and measurement of serum anti-TNP-Ficoll antibodies

Mice were injected i.v. through the tail vein with the indicated doses of GIPL or TNP-ficoll diluted in phosphate buffered saline. Mice were bled at the indicated times. Serum was obtained and kept frozen until use. Serum anti-TNP antibody levels were titered with a solid phase microtiter plate ELISA using polyvinyl chloride round bottom Falcon Microtest III microtiter plates (Becton Dickinson, Oxnard, CA) as previously described [26]. Plates were read at OD_{405} with a 550 Microplate Reader (BIORAD, Hercules, CA). The titer point for each experiment was the serum dilution giving an OD reading midway on the linear portion of the titration curve.

Extraction of GIPL and isolation of PI-oligosaccharides

T. cruzi epimastigotes were grown in BHI medium (DIFCO Lab. Inc., Detroit, MI) as previously described [3]. The harvested cells (approximately 10^{11}) were extracted at 80°C with 45% aqueous phenol. The aqueous layer was dialyzed, freeze dried, and applied to a column (2 cm × 100 cm) of Bio gel P-60. The excluded material was lyophilized and the GIPL was recovered by extraction with chloroform/methanol/water (10:10:3). The extract was evaporated to dryness, dissolved in water and lyophilized. The dry material was diluted in water and added to the cultures after gamma-irradiation on a ^{60}Co source. The intact GIPL appeared on SDS-PAGE as a fast moving carbohydrate and lipid positive band of apparent molecular mass of 2.08 kDa. Approximately 3 μg of GIPL (1.4 nmoles) was obtained from 4.5×10^7 *T. cruzi* cells [3]. The PI-oligosaccharides were isolated from the intact GIPL by alkaline hydrolysis (KOH 1 M, 72 h, at 37°C). After neutralization with acetic acid, nonpolar material (ceramide domain) was removed by chloroform extraction. The aqueous layer was passed through a column of Dowex 50W-X8 (H^+) resin, and the PI-oligosaccharides were eluted with water and desalted in a column of TSK HW 40. The absence of contaminating peptide material was confirmed by nuclear magnetic resonance analysis of purified PI-oligosaccharides [3]. The PI-oligosaccharides has a calculate molecular weigh of 1.23 kDa [3]. Analysis of hydrolysis product of the GIPL revealed no contamination with bacterial LPS. The presence of 2-keto-3-deoxyoctulosonic acid (KDO), a characteristic product of the hydrolysis of LPS, was not detected in material analyzed by high pH anion exchange chromatography using an electrochemical detector. Also, LPS per-*O*-trimethylsilylated derivatives were not detected in gas liquid chromatography. The sensitivity of pulse amperometric detection is at least 0.05 nmoles and that of gas chromatography is around 2 pmoles.

Statistical analysis

Statistical analysis was performed by the Student's *t* Test.

Results

Treatment with *T. cruzi* whole GIPL or with its oligosaccharide domain increases circulating IgM levels and B cell activation *in vivo*

Injection of different doses of this glycoconjugate increased serum IgM levels (Figure 1A). A detectable response was induced after the injection of either 100 or 200 μg/animal and the 200 μg dose was used throughout the study. Increased IgM levels could be observed even twenty one days after GIPL injection (Figure 1B). Injection of either the GIPL or its

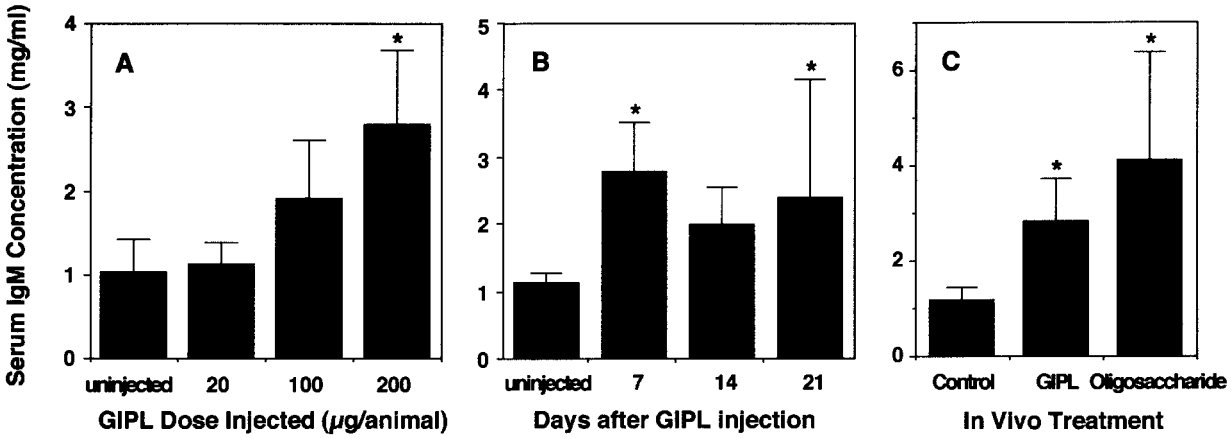


Figure 1. GIPL purified from the G strain of *T. cruzi* increases serum IgM levels. **(A)** Groups of five mice were injected i.v. with either the indicated doses of GIPL (purified from the *T. cruzi* G strain) or with phosphate buffered saline. Serum was obtained from mice bled 7 days after injection and was pooled before IgM measurement. Data represent the mean of IgM levels obtained in three independent experiments. **(B)** Sera were obtained 7, 14 or 21 days after injection of 200 μg of *T. cruzi* GIPL; control mice were injected with phosphate buffered saline. **(C)** Mice, 8 per group, were injected i.v. with the *T. cruzi* GIPL (200 μg), the GIPL-derived oligosaccharide moiety (70 μg) or phosphate buffered saline. Mice were bled 5 days after treatment. Serum IgM levels were measured by ELISA. IgM concentration was determined by isotype specific ELISA. Data are represented as mean ± SD. *Statistical significant values $p \leq 0.05$.

isolated oligosaccharide moiety showed stimulatory effects (Figure 1C). The oligosaccharide moiety comprises 60% of the molecular mass of the total GIPL molecule [3] and is responsible for most of the induction of Ig secretion *in vitro* [17]. Therefore we used a lower dose of the oligosaccharide in the *in vivo* experiments (Figure 1C). Control mice injected with either high molecular dextran or phosphatidylcholine had no increase in the IgM circulating levels. Dextran-treated mice showed an average of 1.35 mg/ml of serum IgM, while phosphatidylcholine-treated mice presented around 1.37 mg/ml of IgM in serum.

Since B cells obtained from GIPL-treated mice appeared to be activated, we investigated their spontaneous proliferation when analyzed *ex-vivo*. We also tested the response of those cells to either a non mitogenic dose of a classical murine B cell activator (bacterial LPS) or to a mitogenic type 2 activator, anti-IgD antibody conjugated to dextran [27]. As can be seen in Table 1, cells obtained from either GIPL- or oligosaccharide-treated mice proliferated spontaneously *in vitro* after a 24 or 48 h incubation. These cells showed an eight times higher response to a non mitogenic dose of LPS, but responded normally to anti-IgD-dextran (Table 1). Despite this increased B cell responsiveness *in vitro*, we did not detect any increase in the relative B cell number in spleen, even though the total splenic cell number of GIPL-treated mice was slightly increased in some experiments (data not shown).

B cells obtained from GIPL or oligosaccharide-treated mice secrete high levels of Ig when activated *in vitro*

Our initial studies suggested that after a first contact with GIPL, B cells become highly responsive to different stimuli. Accessory cells can modulate B cell responses to GIPL [18]. To diminish the interference of accessory cells in our system, we also tested

Table 1. Proliferation of B cells obtained from GIPL- and oligosaccharide-treated mice

	B cell proliferation (tritiated thymidine incorporation)[b]		
Treatment[a]	*Control mice*	*GIPL- Injected*	*Oligo-injected*
No stimulus 24 h	184	567	1638
No stimulus 48 h	598	1482	3741
LPS 48 h	686	5633	1285
anti-IgD-dextran + IL4 48 h	40 325	52 388	49 817

[a] Mice were injected with GIPL (200 μg/animal), GIPL-derived oligosaccharide (70 μg/animal) or phosphate buffered saline (control mice). High density splenic B cells were obtained 5 days later. Cultures were set using 2×10^5 cells in 200 μl. LPS was used at 0.02 μg/ml, anti-IgD-dextran antibody was used at 10 ng/ml and IL-4 was added at 125 U/ml.
[b] Data shown represent the mean value of tritiated thymidine incorporation (cpm) of cultures set in triplicate. Standard deviation was below 10% and is omitted for simplicity.

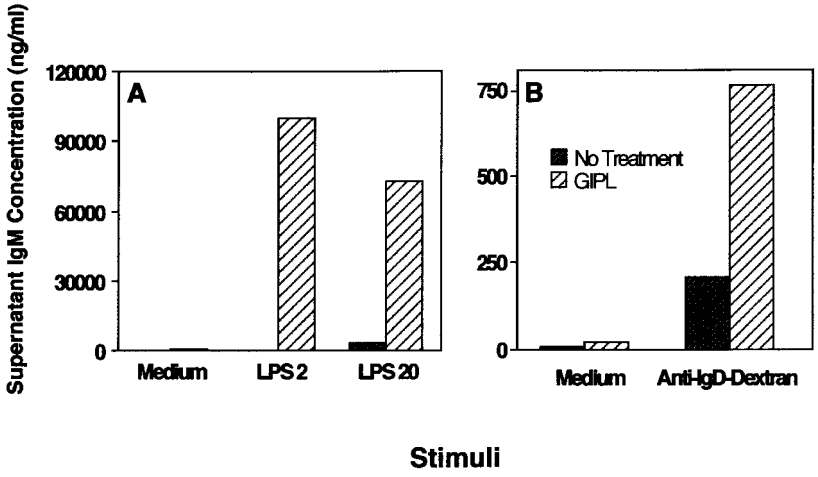


Figure 2. Injection of GIPL primes B cells for enhanced IgM responses *in vitro*. Splenic high density B cells were obtained 3 days after i.v. injection of 200 μg of the *T. cruzi* G strain GIPL. High density cells (10^5 in 200 μl cultures) were left unstimulated or stimulated for 5 days with LPS (used in μg/ml at the indicated doses) or anti-IgD-dextran (used at 10 ng/ml). Control cultures were left unstimulated. IgM levels of pooled supernatants run in triplicate were measured by ELISA. Data shown are representative of 5 to 4 independent experiments, respectively.

the response of highly purified B lymphocytes. Cells obtained three days after *in vivo* treatment with GIPL, showed a large IgM secretory response to LPS when compared to control cells (Figure 2A). Increased responses could be detected even when we used B cells obtained 10 days after treatment with GIPL (data not shown). Purified B cells from GIPL-treated mice also showed a higher IgM response to anti-IgD-dextran (Figure 2B). Control B cells obtained from mice injected with either dextran or phosphatidylcholine did not show higher responses to B cell activators *in vitro* (data not shown). B cells from mice injected with GIPL-derived oligosaccharide showed increased IgM responses to LPS (Figure 3).

In vivo treatment with GIPL have immunopotentiating effect

B cell function is altered during *T. cruzi* infection since there is a significant decrease in the response to different antigens during the early phases of infection [28]. In order to test if *in vivo* treatment with the GIPL would induce similar changes in B cell function, we investigated the response of GIPL-treated mice to an immunization with the type 2 antigen TNP-ficoll. Concomitant injection of the GIPL did not affect the IgM response to TNP-ficoll (Figure 4A), but boosted the IgG1 response (Figure 4B). IgG2a and IgG3 anti-TNP responses were also increased in GIPL-treated mice (Figure 4C). Increased IgG response could be detected even after 21 days of immunization (data not shown).

Discussion

The present study demonstrates an *in vivo* immunostimulatory effect of the GIPL purified from *T. cruzi*. This molecule is present at relatively high amounts ($l0^7$ GIPL molecules per epimastigote) in the surface of this protozoan and has potent effect upon B cells *in vitro* [17]. Here, we observed that *in vivo* contact with the GIPL primes B cells for enhanced responsiveness *in vitro*, even when suboptimal doses of B cell activators are used. Increased responses were observed regardless of the B cell activator employed. Normal B cells do not respond to anti-IgD-dextran *in vitro* in the absence of cytokines [27]. However, in our system, B cells purified from GIPL-treated mice became hyperresponsive, even to this weak stimulus. Our data showed an extremely low response of normal B cells to all B cell activators used. It is important to emphasize that cultures described in both Figures 2 and 3 were carried out for 5 days. After this culture period, normal B cells

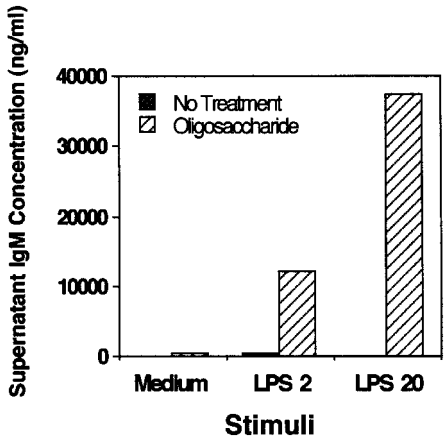


Figure 3. Injection of GIPL-derived oligossacharide primes B cells for enhanced IgM responses. Splenic high density B cells were obtained 5 days after injection of GIPL-derived oligosaccharide (70 μg/animal). The cells (10^5 in 200 μl cultures) were stimulated for 5 days with the indicated doses of LPS (in μg/ml). Control cultures were left unstimulated. IgM levels from a pool of supernatants ran in triplicate were determined by ELISA. Data shown are representative of 3 independent experiments.

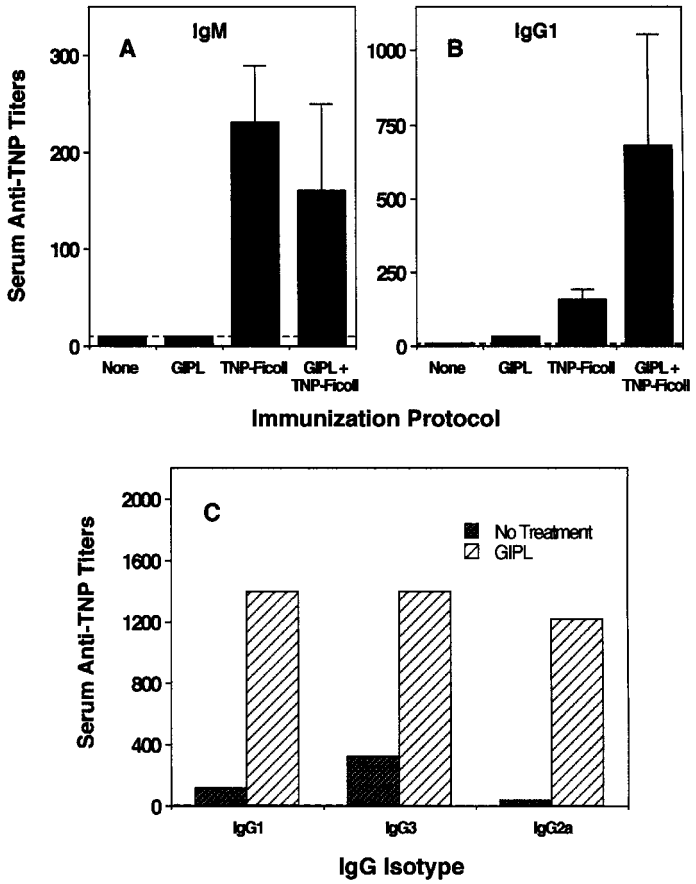


Figure 4. GIPL injection modulates antigen-specific Ig response. **(A, B)** Groups of five mice were injected i.v. with TNP-ficoll (50 μg/ animal) and GIPL purified from the G strain of *T. cruzi* (200 μg/ animal) either alone or in combination. Animals were bled 7 days after antigen injection and either IgM **(A)** or IgG1**(B)** anti-TNP titers were determined by ELISA. Data shown represent mean ± standard deviation for each group. **(C)** Groups of five mice were treated as described above. Anti-TNP IgG1, IgG2a and IgG3 titers from pooled sera were measured by ELISA. Phosphate buffered saline-injected mice showed undetectable anti-TNP antibodies (titers < 10). Dashed line indicates the limit below which anti-TNP antibodies were undetectable. Data shown in top and bottom figures were obtained in an independent experiment and are representative of 3 independent experiments. *Statistical significant values $p \leq 0.05$.

do not usually show a high IgM secretory activity yet. This could explain the low response these cells showed to either LPS or anti-IgD-dextran.

In order to characterize the *in vivo* effect of the GIPL, we initially tested the levels of serum IgM. We observed that this glycoconjugate induced a detectable and sustained increase in circulating IgM levels. This effect resembles the increase in circulating Ig levels detected during *T. cruzi* infection [4]. Previous studies have shown that despite the polyclonal B cell activation, infection with *T. cruzi* induces a general decrease in antigen-specific B cell responses [28]. However, we observed here that treatment with *T. cruzi* GIPL actually induced an increase in B cell responsiveness. It was previously described that T cell function is modified after *T. cruzi* infection [29] and that infection-associated abnormal B cell response is controlled by T cells [6,7]. The GIPL was described to have a direct effect on B cells [18] and it is possible that this effect prevails despite the inhibitory effect of the GIPL on T cells [15].

We observed that *in vivo* treatment with GIPL increased B cell responses to TNP-Ficoll, a polysaccharide antigen. Polysaccharide-induced B cell response is T cell-independent [30] and this could explain the increased anti-TNP-Ficoll response despite the possible deficient T cell activation after GIPL treatment [15]. Polysaccharide-stimulated responses are increased by natural killer cell activation *in vivo* [31] and we have recently described that GIPL induces natural killer cell activation *in vitro*, and that this natural killer cell activation can potentiate B cell response [18]. Activation of accessory cells after GIPL injection can also be suggested in our system by the increase in the anti-TNP-Ficoll IgG response. *In vivo* Ig class switch is highly dependent on cytokines [32] and it was previously shown that natural killer cells are induced to secrete cytokines after immunization with polysaccharides [33].

The ability of microbial products to potentiate B cell response has been observed in different systems. Bacterial-derived products like porins [34] and lipoproteins [35] can act *in vitro* as immunopotentiating substances. Other molecules, like the unmethylated CpG dinucleotides in bacterial DNA stimulate a potent Ig secretory response to unrelated antigens after *in vivo* immunization [36]. Bacterial DNA also acts as

a potent *in vitro* B cell activator, since it induces B cell proliferation and polyclonal Ig secretion [37]. Protozoan-derived molecules also have immunopotentiating effects *in vivo*. The covalent binding of *Leishmania* HSP70 to unrelated antigens potentiates the production of Ig and cytokines [38].

Our studies did not characterize the mechanism by which the GIPL induces B cell activation *in vivo*. One possible mechanism would be its internalization by B cells. It was recently described that this glycoconjugate can be internalized by T cells and can stimulate intracellular T cell calcium increase [16]. This molecule could also be acting on B cells through the binding to oligosaccharide receptors on B cell surface. We have previously described that the *in vitro* effect of the GIPL on B cells is mainly mediated by its oligosaccharide domain [17]. Oligosaccharides derived from *Schistosoma mansoni* eggs induce both proliferation and cytokine secretion by $CD5^+$ B cells [39,40]. Finally, intact GIPL could also induce B cell activation through a direct action on the membrane lipid portion. The GIPL has a ceramide domain and it was suggested that this class of lipid induces signal transduction on different systems [41].

One important issue on the *in vivo* effect of the GIPL we observed in the present report is related to the concentration of the glycoconjugate injected *in vivo*. The GIPL we used was injected as a single purified molecule. Previous studies have shown that *T. cruzi* shows other molecules with important B cell activating properties. Both components from the cytosolic alkaline fraction and purified proteins obtained from *T. cruzi* were shown to promote the activation, proliferation and differentiation of normal murine B lymphocytes into antibody-secreting cells [42,43]. Also, it was recently described that a protein with proline racemace activity stimulates polyclonal B cells activation. This protein is both associated to the surface of infective forms of the protozoan and detected in the culture supernatant [44]. No studies have analyzed the synergistic effect of different molecules from *T. cruzi* on B cell activation yet. However, based on the already published studies, it is possible to hypothesize that, during a natural infection, different *T. cruzi* molecules could be acting synergistically to induce B cell activation.

Taken together, our data extend our previous findings on the immunopotentiating effect of the *T. cruzi*-derived GIPL on B cells. During the early phase *T. cruzi* infection there is a remarkable polyclonal B cell activation that can be detected even during late infection periods, when the protozoan is no longer detected in the circulation [5]. Our findings that the GIPL can induce an increase in circulating Ig concentration may suggest that this glycolipid could be modulating the increased Ig secretion during Chagas' disease.

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