Glycosylphosphatidylinositol-Anchored Mucin-Like Glycoproteins from Trypanosoma cruzi Bind to CD1d but Do Not Elicit Dominant Innate or Adaptive Immune Responses Via the CD1d/NKT Cell Pathway


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It has been proposed that self and protozoa-derived GPI anchors are natural ligands of CD1d. In this study, we investigated the ability of GPI anchors from *Trypanosoma cruzi* to bind to CD1d and mediate activation of NKT cells. We observed that GPI-anchored mucin-like glycoproteins (GPI mucins), glycoinositolphospholipids (GIPLs), and their phosphatidylinositol moieties bind to rCD1d and inhibit the stimulation of a NKT hybridoma by the α-galactosylceramide-CD1 complex. However, these GPI anchors and related structures were unable to activate NKT cells in vitro or in vivo. We found that high titers of Ab anti-GPI mucins, but not anti-GIPLs, were detected in sera from wild-type as well as in TAP1−/− and TAP2−/− mice after immunization. However, T-dependent anti-GPI mucin Ab isotypes, such as IgG1, IgG2a, IgG2b, and IgG3, were absent on MHC class II−/−, but were conserved in CD1d−/− and TAP1−/− mice. Furthermore, we found that CD1d−/− mice presented a robust cytokine as well as anti-GPI mucins and anti-GIPL Ab responses, upon infection with *T. cruzi* parasites. These results indicate that, despite binding to CD1d, GPI mucins and related structures expressed by *T. cruzi* appear not to evoke dominant CD1d-restricted immune responses in vivo. In contrast, MHC class II is critical for the production of the major Ig G isotypes against GPI mucins from *T. cruzi* parasites. The Journal of Immunology, 2002; 169: 3926–3933.

CD1 is a family of cell surface proteins that has been implicated in Ag presentation (1, 2). CD1 is non-MHC encoded, but shares features with both MHC class I, such as structural organization and β2-microglobulin association (3, 4), and MHC class II, such as endosomal trafficking (5, 6). As expected, given its specialization for lipid Ag binding, CD1 is functionally independent of the TAP peptide transporters (7, 8).

CD1 proteins are nonpolymorphic and comprise five isotypes. CD1a, -b, -c, and -e constitute group 1, found in humans but not in rodents, and CD1d constitutes group 2, which is conserved in rodents and humans. Group 1 isotypes can present mycobacterial lipids to CD4− and CD8− (double negative (DN)3), CD8+ or CD4+ T cell lines or clones expressing apparently diverse TCRs (9–12). CD1d presents α-galactosylceramide (α-GalCer) to DN or CD4−, CD8− NK1.1+ NKT cells, which express an invariant, germline-encoded TCR α-chain (Vα14-Jα18 or Ja281 by older nomenclature in mice, and Vα24-Jα18 or JaQ by older nomenclature in humans) paired with restricted Vβ-chains (mainly Vβ8 in mice and Vβ11 in humans) (13–16). NKT cells promptly release IL-4 and IFN-γ upon TCR engagement and without prior sensitization (17). They exert regulatory functions in tumor rejection (18, 19) and autoimmune diseases such as type 1 diabetes (20, 21). They also regulate various infectious conditions, such as LPS-induced shock (22) and *Plasmodium yoelii* and *Listeria monocytogenes* infections (23, 24).

Although the importance of CD1d and NKT cells is well established, the origin and identity of their natural ligands remain unknown. α-GalCer, a glycolipid extracted from marine sponges (25), binds to CD1d and strongly stimulates mouse and human NKT cells (26–29). However, α-GalCer may not be the natural ligand for CD1d because ceramides with α-linked dextro sugars have not been found in mammalian cells. A candidate natural ligand might be self GPI anchors (30). In fact, it has been suggested that GPI anchors of *P. falciparum* and *Trypanosoma brucei* induce Ab production in a CD1d-dependent manner (31). However, evidence for the role of GPI is controversial, because Ab responses to *P. falciparum* were found to be MHC class II rather than CD1d restricted by two other groups (32, 33).

The surface of *T. cruzi*, like that of other protozoa, contains abundant GPI-related structures. *T. cruzi* expresses ~10^7 copies/
cell comprising GPI-anchored mucin-like glycoproteins (GPI muci-
kins) and glycosinositolphospholipids (GIPILs) that together coat a si-
ficant extension (60–80%) of parasite plasma membrane (34, 35).
The α-galactosyl terminal residues of O-linked oligosacchar-
rides on GPI mucins represent the major targets for the lytic human
Ab response against T. cruzi (36–38). The GIPILs are exotic GPI
structures with no attached proteins and a conserved glycan core
whose galactofuranose residues appear to be highly immunogenic
(39). In this study, we investigated the ability of GPI mucins and
GIPILs from T. cruzi to bind CD1d and activate NKT cells. We also
tested the possibility that CD1d or NKT cells could promote the
production of Abs against glycolipid Ags in vivo. We observed that
GPI mucins, GIPILs, and their phosphatidylidylinositol (PI) moi-
eties bound to CD1d, but were unable to activate NKT cells. Fur-
thermore, in the vivo Ab responses against GPI mucins or GIPILs
were found to be independent of CD1d and NKT cells. Rather,
MHC class II appeared to be crucial for class-switching to Ab
against-GPI mucins of the IgG1, IgG2, and IgG3 isotypes. Together,
our results indicate that during infection with T. cruzi, GPI mucins
and GIPILs elicit innate and adaptive immune responses in a CD1/
NKT cell-independent manner.

Materials and Methods

Mice
Wild-type (WT) and I-Aβ⁻/⁻ C57BL/6 mice were obtained from The
Jackson Laboratory (Bar Harbor, ME), TAP1⁻/⁻ mice were obtained from
H. Ploegh (Harvard University, Boston, MA) and backcrossed to C57BL/6
for nine generations (40), and C57BL/6.CD1d⁻/⁻ mice were produced in
our laboratory and checked in a routine basis with a fluorescein-labeled
anti-CD1d mAb (41). All mice were raised in a specific pathogen-free
barrier environment and kept according to institutional animal care and
use guidelines. C57BL/6 mice used for infection were obtained and main-
tained from Centro de Pesquisas René Rachou, Oswaldo Cruz Foundation
(Fiocruz, Belo Horizonte, MG, Brazil). In experiments for T. cruzi infec-
tion, 8- to 10-wk-old male and female C57BL/6 and C57BL/6 mice were
used.

Purification of glycoconjugates
GPI mucins and GIPILs were purified as described (37, 42). In brief, par-
sisate pellets containing ~1 × 10^10 tryomastigotes or epimastigotes (Y
strain) were freeze dried and sequentially delipidated with chloroform:
methanol partition, followed by butanol/water partition. The extracts were
purified by hydrophobic-interaction chromatography in octyl-Sepharose
column (Amersham Pharmacia Biotech, Uppsala, Sweden) eluted with pro-
panol gradient (5–60%). The GPI mucins were detected by Western blot-
ing, chemiluminescent ELISA (CL-ELISA) using anti-α-galactosyl Abs
(37, 42), and silver staining of SDS-PAGE gels (43). GIPILs were identified by
electrospray ionization-mass spectrometry (ES-MS) analysis.

Isolation of PI moiety
T. cruzi GPI mucins and GIPILs were deaminated, as previously described
(42, 44). In brief, gparasite mucins were extracted with water-saturated butanol
(91% 1-butanol-9), dried, and redissolved in 0.1 M sodium acetate buffer,
PH 4.0, and deaminated by three additions of 0.5 M sodium nitrite at 60°C.
The samples were mixed with 9% butanol, and the released PIs were re-
covered by butanolic extractions. Epimastigote GPI moieties were submitted to
the same protocol without pre-extraction with butanol.

Mass spectrometry analysis
GPI anchors, released from GPI mucins by proteinase K treatment (42),
and GIPILs were analyzed by ES-MS. Spectra were obtained in a Finnigan
LCQDeca ion-trap mass spectrometer (Finnigan: ThermoQuest, San Jose,
CA). Samples were dissolved in 50% propan-1-ol, containing 10 mM am-
nonium acetate and 0.1% formic acid, and introduced into the electrospray source by injection through a 30-μm (internal diameter) fused silica cap-
illary at a flow rate of 5 μl/min. Electrospray capillary voltage was set to
36–46 V, and temperature to 200°C. Spectra were acquired in negative mode at 3 scans over a mass range of m/z 200-2000. Collision-induced
dissociation ES-MS of parent ions was conducted at a relative collisional
energy of 25–35% (2.5–3.5 V). Source parameters were optimized using
previously well-characterized T. cruzi GIPILs (42).

Competition for lipid binding to rCD1d
Binding to CD1d was determined using a competition assay, as described
(45), with some modifications. Purified mouse rCD1d molecules were
coated at 5 μg/ml in PBS overnight at 4°C on 96-well tissue culture plates.
After washing three times with PBS, CD1d-coated wells were incubated with
various concentrations of competitors in PBS at room temperature for
18 h before addition of 0.5 μl of PBS (1 μM; a gift from Y. Koezuka,
Pharmaceutical Research Laboratory, Kirin Brewery, Takasaki, Japan) for
3 h at room temperature. Both competitors and α-GalCer were sonicated
before use. After washing the plates, 5 × 10^4 DN32D3 cells (Vai4-1r18/
Vβ8 NKT hybridoma) (15) per well were added in 1:1 EHA/ RPMI mix-
ture (Biofluids, Rockville, MD) supplemented with 50 μM 2-ME, penicil-
in-streptomycin-gentamicin, glutamine (endoplasmic reticulum (ER)
medium), and 5% heat-inactivated FCS (ER-5), and incubated for 18 h at
37°C. Supernatants were harvested, and IL-2 was released and measured by
CTLL assay, as previously described (46).

Macrophage stimulation
Murine peritoneal thioglycolate-elicited macrophages were harvested and
cultured, as described elsewhere (43, 47). WT or CD1d⁻/⁻ C57BL/6 peri-
toneal exudate cells were harvested in cold FCS-free DMEM (Life Tech-
nologies, Grand Island, NY), centrifuged, and resuspended in DMEM sup-
pplemented with 5% heat-inactivated FCS and 40 μg/ml gentamicin to
a final concentration of 2 × 10^6 cells/ml. Cells were incubated for adherence
to 96-well plates for 3 h, and unattached cells were washed away. Mac-
rophages were primed overnight with IFN-γ (25 U/ml; Pharmingen) and
were subsequently incubated with tryomastigote GPI mucins. Superna-
tants were collected after 24 and 48 h for TNF-α and NO measurements,
respectively (43, 47). Tryomastigote GPI mucins were quantified using ELISA
Development System mouse TNF-α, catalogue DY410; R&D Sys-
tems, Minneapolis, MN), and NO was measured using Griess reagents (48).

Immunication with GPI mucins and GIPILs
AgS (0.8 nmol GPI mucins or 0.8 nmol GIPILs) were diluted in saline and
mixed 1:1 with Alum (2% Alhydrogel 2%; Accurate Chemical and Sci-
entific, Westbury, NY) by vortexing for 3 min and incubated for 2 h at room
temperature before injection. One hundred microliters of the mixture was
injected into the footpad and tail-base sides of C57BL/6 WT and mutant
mice. After 2 wk, mice were boosted with the same dose of Ag mixed with
Alum. At the end of the third week, mice were killed, and sera, as well as
intragland and popliteal lymph nodes, were harvested for Ab detection and T
cell purification assays.

T cell enrichment and proliferation
Murine lymph node cells were harvested in HBSS (Life Technologies, Grand
Island, NY) containing 5% FCS, and the cell suspensions were passed
through nylon wool columns for T cell enrichment, as described (49). A
total of 3 × 10^4 T cells and 1 × 10^2 2000 rad-irradiated splenocytes
was cultured at 200 μl final volume in ER medium supplemented with 10%
FCS (ER-10) at 37°C for 3 days before pulsing with 0.5 μCi/well [3H]thy-
midine for 8–18 h.

Infection with T. cruzi
Mice were infected i.p. with 5000 blood-form tryomastigotes Y strain of
T. cruzi. Parasitemia levels were evaluated by counting parasites in 5 μl
blood from the tail vein. Mortality was evaluated by daily inspection of the
cages.

Chemiluminescent ELISA
Serum Abs to GPI mucins or GIPILs were assayed by CL-ELISA. In brief,
microtiter black or white (Maxisorb Nuc 96 FluoroNunc Plate; catalog
237018 and 436110, respectively; Nunc, Albertslund, Denmark) 96-well
plates were coated with 6 pmol GPI mucins or GIPILs per well diluted in
50 μl PBS for 18 h at 4°C. The wells were washed three times with PBS-
0.05% Tween 20 (PBS-T) and blocked with PBS-1% BSA for 2 h at 37°C.
After washing with PBS-T, serially diluted serum samples in PBS-1% BSA
were added to the wells and incubated for 1 h at 37°C. The plates were then
washed three times with PBS-T, and biotinylated goat anti-mouse IgG +
IgM (H + L) (1:50,000), IgM (1:20,000) (Jackson ImmunoResearch Labor-
atories, West Grove, PA), IgG1 (1:10,000), IgG2a (1:10,000), IgG2b
(1:2,000), or IgG3 (1:2,500) (0.5 mg/ml; Southern Biotechnology Associ-
ates, Birmingham, AL) was added for 1 h at 37°C, followed by streptavi-
din-peroxidase (1:5,000; Southern Biotechnology Associates). The wells
were washed four times with PBS-T and once with carbonate buffer, pH
9.6. ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ) 100
μl/well was added, and the plates were immediately read using a Luminoimeter (program BB Lux, Wallac 1450 Microbeta Plus Liquid Scintillation Counter; PerkinElmer, Wellesley, MA).

Cytokines and Ab produced in infected mice
Murine spleen cells from infected and noninfected C57BL/6 or CD1d<sup>−/−</sup> mice were obtained on day 8 after infection, as previously described (50), and cultured at 5 × 10<sup>6</sup> cells/ml/well, in 24-well plates, with RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Supernatants were harvested after 48 or 72 h at 37°C, and the levels of cytokines and Abs was measured by ELISA kits (R&D Systems). Mice were bled on days 0, 8, and 14 after infection, and the level of serum cytokines and Abs was measured by conventional and CL-ELISA, respectively, as described above.

Statistical analysis
Data are presented as means ± SEM. Statistical differences were determined by one-way ANOVA, followed by t test to evaluate differences between the experimental vs the control groups. The p values were determined using Student’s t test and considered significant if <0.05.

Results

Structural analysis of GPI anchors and GIPLs
Mucin-derived GPIs and free GIPLs purified from epimastigote forms of <i>T. cruzi</i> yielded highly pure samples. The results presented in Fig. 1 show the negative ion mass spectra of GPI anchors and GIPLs (lower panel). The former presented a major doubly charged ([M-2H]<sup>2−</sup>) pseudomolecular ion species at m/z 917.1 (M = 1836.2), which could be assigned to a GPI species containing four hexoses, glucosamine (GlcN), ethanolaminephosphate, 2-aminoethylphosphonate (AEP), and phosphatidylinositol (myo-inositol-phosphate-C<sub>16:0</sub>-alkyl-C<sub>18:1</sub>/C<sub>18:2</sub>-acylglycerol). The former presented a major doubly charged ([M-2H]<sup>2−</sup>) pseudomolecular ion species were observed for the GIPL preparation at m/z 1066.1 (M = 2134.2), which could be assigned to a compound containing six hexoses, GlcN, AEP, and myo-inositol-phosphoceramide (C24:0-fatty acid-C18:0-sphinganine). These two preparations were used in the different experiments presented in this study. Both mucin-derived GPIs and GIPLs, upon nitrous deamination, released phosphatidylinositol alkylacylglycerol (GPI-derived PI) and inositol-phosphoceramide (GIPL-derived PI), generating major ion species at m/z 795.6 and 892.8, respectively. These parent ion species were further fragmented to confirm the proposed assignments given above (data not shown).

GPI anchors derived from GPI mucins of trypomastigote form of <i>T. cruzi</i> were also purified and characterized by ES-MS, which revealed the composition (Hex<sub>4</sub>-GlcN)(ethanolaminephosphate-C<sub>16:0</sub>-alkyl-C16:0-O-acetylglycerol). This material was utilized in experiments using inflammatory macrophages.

Competition assay for lipid binding to CD1d
Binding of GPI mucins, GIPLs, and PIs was tested in microwell plates coated with soluble CD1d. Binding was detected as a decreased IL-2 release by DN32D3 hybridoma cells upon addition of α-GalCer, their cognate CD1d-binding ligand. The results were expressed as percentage of inhibition of the IL-2 induced by α-GalCer alone in the absence of competitor. The GPI mucins and GIPL structures tested caused nearly 100% inhibition of DN32D3 activation by α-GalCer even at 3:1 and 1:1 competitor/α-GalCer molar ratio (Fig. 2). The PI moieties derived from GPI mucins or GIPLs also competed with α-GalCer, although not as well, perhaps because their solubility was inferior to that of the glycoconjugates. We further titrated the inhibitor down in some experiments and found reduction in the range of 30–50% of inhibition for both GPI mucin and GIPL at 1:0.5 ratio. The PI portions of both molecules

**FIGURE 1.** ES-MS profile of <i>T. cruzi</i>-derived GPI anchors. Purified epimastigote mucin-derived GPI (upper) and GIPL (lower) samples were analyzed by negative ion mode ES-MS. Left, ES-MS spectra; right, proposed assignment for the major ion species. Proposed compositional assignments (42) corresponding to the major [M-2H]<sup>2−</sup> pseudomolecular ions in both panels are indicated. EtNP, ethanolaminephosphate; InsP, myo-inositol-phosphate; AAG, alkylacylglycerol.
molecules on macrophages was CD1d dependent. WT and
investigated whether the previously described activity of these
ned. Because GPI mucins bind to CD1d, we
of receptors required for macrophage activation by GPI mucins
GIPLs from
Toll-like receptor 2 on macrophage activation by GPI mucins and
phages (42, 43, 47). We have recently shown the involvement of
Trypomastigote-derived GPI mucins are able to activate macro-
CD1d expression is not necessary for macrophage activation by
GPI mucins
Trypomastigote-derived GPI mucins are able to activate macro-
phages (42, 43, 47). We have recently shown the involvement of
Toll-like receptor 2 on macrophage activation by GPI mucins and
GIPLs from T. cruzi parasites (51, 52). However, the complete set
of receptors required for macrophage activation by GPI mucins
still remains to be defined. Because GPI mucins bind to CD1d, we
investigated whether the previously described activity of these
molecules on macrophages was CD1d dependent. WT and
CD1d−/− macrophages primed with IFN-γ were stimulated with
trypomastigote-derived GPI mucins and TNF-α and NO production
evaluated in the culture supernatants after 24 and 48 h, re-
spectively (Fig. 3). Although IFN-γ priming is not necessary for
TNF-α and NO release, it was used in this study because it causes a
significant increase in the production of these mediators, 2- and
4- to 6-fold increase, respectively (43, 47). Identical results were
obtained for both CD1d-deficient and CD1d-sufficient macro-
phages, demonstrating that CD1d expression is not required for
GPI mucin activity on macrophages. Similar results were observed
with LPS (data not shown).

FIGURE 2. T. cruzi-derived GPIs compete with α-GalCer for binding
to CD1d. Plate-bound CD1d (5 μg/ml) was incubated with different molar
ratios of competitor per α-GalCer (1 nmol/ml), as indicated. T. cruzi-de-
derived GPIs were incubated onto CD1d for 18 h, followed by 3 h with
α-GalCer. After washing, DN32D3 cells were added for 18 h, and the
supernatant was harvested for evaluation of IL-2 release with CTLL assay.
The competition is expressed as percentage of inhibition of IL-2 release
obtained with DN32D3 incubated on plate-bound CD1d loaded with
α-GalCer alone. The background values for IL-2 for unstimulated DN32D3
cells were in the range of 0.02 U/ml. The DN32D3 cells stimulated with
α-GalCer-CD1d complexes released 4.83 U/ml. Upper panel, GPI mucins (□)
and GPI-derived PI (●). Lower panel, GIPLs (○) and GIPL-derived PI (●).
Asterisk indicates no significant inhibitory activity as compared with NKT
hybridoma cells stimulated with α-GalCer in the absence of competitors.

T cell response to GPI mucins

Next, we asked whether T. cruzi-derived GPI structures could
stimulate T cell proliferative responses in vivo. WT mice were
immunized with GPI mucins or GIPLs s.c., and cell suspensions of
lymph node were separated by nylon column. T cell-enriched prepar-
ations were cultured in vitro with irradiated spleen cells from
unimmunized mice and stimulated with GPI structures. T cells
from GPI mucin-immunized mice responded well to Ag restimu-
lation in vitro, whereas those from GIPLs did not (Fig. 4, upper panel).
To characterize the Ag presentation pathway involved in
the response to GPI mucins, we immunized WT, CD1d−/−, or
MHC class II−/− mice. There was a marked decrease in T cell
proliferation in MHC II-deficient mice, whereas CD1d−/− mice
responded normally (Fig. 4, lower left panel).

We also tested the ability of the NKT cell hybridoma DN32D3,
which expresses the canonical Vα14-Jα18 Vβ8 TCR (16), to re-
spond to GPI structures presented by CD1d. CD1d complexed with
GPI mucins or GIPLs did not induce IL-2 release by DN32D3
cells; neither did the PI moieties of these glycolipids (data not
shown). In contrast, NKT cells exposed to α-GalCer-loaded CD1d
produced high levels of IL-2.

Ab response to GPI mucins

To characterize the MHC or CD1 Ag presentation pathway in-
volved in helper activity for Ab response in vivo, WT, CD1d−/−,
MHC class II−/−, or TAP−/− mice were immunized with GPI
mucins or GIPLs, and their serum Ab (IgM + IgG) titers were

measured by CL-ELISA. WT and mutant mice produced similar levels of Abs, although MHC class II$^{-/-}$ mice exhibited a reduction in total Ab production against GPI mucins (Fig. 5A, upper panel). In contrast, GIPL immunization did not elicit Ab production even in WT mice (Fig. 5A, lower panel). Fig. 5B shows that IgM was produced by all WT and mutant mice. IgG1, IgG2a, and IgG3 were normal in WT and CD1d$^{-/-}$ mice. IgM was produced by all WT and mutant mice. IgG1, IgG2a, and IgG3 were normal in WT and CD1d$^{-/-}$ mice. IgM was produced by all WT and mutant mice.

**Discussion**

NKT cells have been implicated as an important component influencing disease outcome during bacteria and protozoa infections (22–24). In the case of protozoan infections, it was observed that in the absence of CD4$^+$ T, in MHC class II-deficient mice, NK$^+$ CD4$^+$ T provided help for the development of CD8$^+$ T lymphocytes and resistance against Toxoplasma gondii infection (53). In the early stages of Leishmania major infection, CD4$^+$ NKT cells are markedly increased in resistant mice, but not in susceptible one (54). The number of NKT cells is also increased during experimental malaria infection and mediates some level of resistance against the liver stages of the parasite (25). Furthermore, the in vivo administration of α-GalCer to P. yoelii- or Plasmodium berghei-infected mice results in strong antimalaria activity mediated by IFN-γ-secreting NKT cells (55).

GPI anchors were shown to be natural ligands of CD1d (30), and are abundantly expressed in the surface of protozoan parasites (34, 35). Thus, it is tempting to speculate that GPI anchors and related structures may be the main targets for early NKT cell responses during acute infection with protozoan parasites. In fact, CD1d-restricted IL-4-secreting CD4$^+$ NKT cells specific for GPI anchors have been implicated in mediating Ab production against GPI-anchored proteins of P. falciparum or T. brucei (31). In this context, T. cruzi parasites arise as an interesting model, as they express large amounts of GPI-anchored proteins (mainly GPI mucins) and GIPLs on their surface. It is noteworthy that GPI mucins are heavily glycosylated (37, 38) and highly polymorphic (56), whereas GIPLs on their own are of glycolipid nature and have no protein covalently attached to them. Therefore, GPI mucins and GIPLs may not be suitable for presentation via the conventional pathways involving MHC class I and class II. Nevertheless, in

**FIGURE 4.** GPI mucins promote T cell-specific response in vivo. Upper. Cell suspensions from lymph nodes of GPI mucin (■) or GIPL (□)-immunized or unimmunized (□) C57BL/6 WT mice were passed through nylon column. T cell-enriched preparations were stimulated in vitro with medium, 80 μg/ml GPI mucins, or 45 μg/ml GIPLs and irradiated spleen cells. Asterisk indicates that the value is statistically different from the unimmunized mice ($p < 0.05$). Lower left. Lymph node T cell-enriched preparations from immunized WT (□) and CD1d$^{-/-}$ (▲) mice were incubated with medium or GPI mucin. Cell proliferation was evaluated by $[^{3}H]$thymidine incorporation. Different letters mean statistically different results ($p < 0.05$). Lower right. NKT hybridomas were added to a CD1d-coated plate preincubated with PBS, α-GalCer, GPI mucins, and GIPLs. Cell activation was evaluated by IL-2 measurement in the supernatants by CTLL assay. Results are shown as average ± SD of three mice per group. The experiments were repeated twice with same number of animals/group, and all experiments yielded identical results. Asterisk indicates that the value is statistically different from other stimuli and unstimulated control as well ($p < 0.05$).

**FIGURE 5.** Ab response to GPI mucins is CD1d independent. A, WT (■) or CD1d$^{-/-}$ (▲), MHC II$^{-/-}$ (□), and TAP$^{-/-}$ (●) C57BL/6 mice were immunized and boosted s.c. with 0.8 nmol GPI mucins (upper panel) or GIPLs (lower panel). Antisera collected on day 21 were titrated and evaluated by CL-ELISA. Results are expressed on relative luminescence units (RLU). B, WT (□) or CD1d$^{-/-}$ (▲) or MHC II$^{-/-}$ (□) C57BL/6 mice were immunized and boosted s.c. with 0.8 nmol GIPLs. Sera collected on day 21 were tested by CL-ELISA using biotinylated anti-IgG + IgM (H + L), anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, or anti-IgG3 Abs. The results are expressed on RLU. Asterisks indicate statistical difference to WT and CD1d$^{-/-}$ mice ($p < 0.05$). Results are shown as average ± SD of three mice each group. The experiments were repeated twice with same number of animals/group, and all experiments yielded identical results.
of T. cruzi infection (35–39, 57). Indeed, α-GalCer analogs with shortened acyl chains or sphingosine lipid chains (27), but not β-GalCer or α-mannosylceramide (26), have their NKT cell-stimulatory activity preserved. Monosialoganglioside (GM1) and lipoarabinomannan, likewise, bind to CD1d, but do not stimulate DN32D3 hybridomas (58). The TCR antigenic specificity seems to be conferred by residues of the

**Table I. Cytokine levels in the sera and culture supernatants of spleen cells from C57BL/6 and CD1d−/− mice infected with T. cruzi**

<table>
<thead>
<tr>
<th>Cytokine (ng/ml)</th>
<th>C57BL/6</th>
<th>Day 0</th>
<th>Day 8</th>
<th>CD1d−/−</th>
<th>Day 0</th>
<th>Day 8</th>
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<td></td>
<td></td>
<td>0.36 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.45 ± 1.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.34 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.39 ± 1.18&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>IFN-γ</td>
<td>0.57 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<td>IL-10</td>
<td>0.49 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.03 ± 0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.14 ± 2.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01</td>
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<tr>
<td>IL-12</td>
<td>18.13 ± 8.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.12 ± 6.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.13 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<td>TNF-α</td>
<td>0.86 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.62 ± 0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.13 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<td>0.17 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.51 ± 1.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.06 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.00 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>IFN-γ supernatants</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.19 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<tr>
<td>IL-10 supernatants</td>
<td>0.92 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.90 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.91 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<tr>
<td>IL-12 supernatants</td>
<td>0.08 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.17 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<sup>a,b</sup> Different letters on the same line mean statistically different. nd, Indicates not detectable levels of a specific cytokine, and considered zero for the purpose of statistical analysis.
CDR3 loop encoded by the D segment, Jβ, or by N-region addition. In agreement with this, 24.8 hybridoma, which expresses Vα14-Jα281 associated with Vβ8.2/Jβ2.5, does not respond to α-GalCer, but is stimulated by phospholipids with several polar head groups (59). DN32D3 instead do not recognize those phospholipids. Different from 24.8 hybridoma, DN32D3 expresses Vα14-Jα281 associated with Vβ8.2/Jβ2.4. Thus, despite their limited diversity, we cannot rule out that another CD1d-restricted NKT cell population may be recognizing T. cruzi GPI structures in vivo. However, our data indicate that the major NKT cell subset, which bears Vα14-Jα18, does not recognize T. cruzi-derived GPI anchors and related structures (i.e., GPIPLs and PI) tested in this study.

Next, we investigated the NKT cell involvement in T cell-mediated response to highly purified T. cruzi GPI mucins and GPIPLs in vivo. Different from previous studies, in this study we used a molecularly defined protocol, immunizing animals with highly purified molecules, instead of whole parasites, which reduces the risk of dubious results due to the cross-reactivity with other parasite molecules. We found that both proliferative and Ab responses were similar in WT and CD1d−/− mice immunized with GPI mucins. In contrast, both responses were largely reduced in MHC class II−/− mice. These results indicate that NKT cells are not required for in vivo response to GPI mucins, contrasting with data described elsewhere, suggesting that CD1d-NKT cell interactions are required for providing cognate help for IgG production anti-circumsporozoite protein of Plasmodium via GPI anchors (31). Our results rather indicate that IgG production to T. cruzi GPI mucins is mediated through classical MHC class II-CD4+ T cell interaction. Together, these findings suggest that the T cell epitope involved in providing help to B cells to respond to GPI mucins is a peptide instead of glycolipid. Similar results were reported by Molano et al. (32), who showed that MHC class II, rather than CD1d, is crucial for anti-circumsporozoite IgG responses during immunization with irradiated Plasmodium parasites.

Different studies have demonstrated the ability of T. cruzi-derived GPIPLs to act as polyclonal activators as well as adjuvants for B cell activation and Ab production (60, 61). Although anti-GIPL IgM and IgG Abs can be found in serum of patients with Chagas’ disease (39, 55), the ability of purified GPIPLs to elicit a specific Ab response is less documented. Unlike GPI mucins, immunization with GPIPLs was not successful in mice. We did not detect any GPIPL-specific Abs after immunization. It is possible that IgM anti-GIPL Abs found during infection with T. cruzi parasites both in humans and mice are a result of a T cell-independent production. Furthermore, the cross-reaction with T. cruzi glycoproteins could be used to explain the IgG production specific for GPIPLs observed during T. cruzi infection. In fact, the B cell epitope β-galactofuranose, the main Ab target in the GIPLs, is also shared by other parasite glycoproteins (39).

We also tested whether the macrophage activation by GPI mucins requires CD1d. In fact, recent data from our laboratory show that the activity of GPI mucins on macrophage involves Toll-like receptor 2 (51, 52). However, the complete requirement of receptors for macrophage activation has not been defined. Regardless, our results demonstrate that CD1d is not required for the macrophage activation by T. cruzi-derived GPI mucins, as indicated by the similar levels of TNF-α and nitrite in the supernatants of macrophages from WT and CD1d−/− stimulated with GPI mucins. Finally, a recent study has demonstrated a consistent enhancement of parasitemia in CD1d−/− and Jα18−/− mice infected with the CL strain of T. cruzi. However, no differences in terms of mortality were observed between the WT and knockout mice (62). When we used the Y strain of T. cruzi to infect WT and CD1d−/−, we did not observe any difference in terms of mortality either. No differences between WT and CD1d−/− were observed either in the levels of IgM, IgG1, or IgG2a responses against GPI mucins/GIPLs or in those of the cytokines produced by macrophages (i.e., TNF-α, IL-10, and IL-12) or NKT cells (i.e., IFN-γ and IL-4) in response to infection with T. cruzi. Thus, even if NKT cells of diverse TCR may be activated in vivo by T. cruzi, they seem to have a minor importance for the progress of the infection, as, in our case, neither parasitemia and mortality, nor cytokine and Ab production are altered in CD1d deficient.

In conclusion, we demonstrated in this study that T. cruzi-derived GPI-anchored structures compete for α-GalCer binding to CD1d, but this interaction is not important either for Ab production or for cellular responses to GPI mucins or GIPLs elicited during infection with T. cruzi. Rather, IgG production to GPI mucins seems to be mediated through classical MHC class II-CD4+ T cell interaction. Furthermore, our data indicate the CD1d and NKT cell pathway has no major role in host resistance to T. cruzi infection. However, the relevance of CD1 for response to GPIPLs or GPI anchors cannot be totally discarded. It is possible that other CD1 isoforms found in humans, but absent in mice, may present GPI anchors and related structures to T cells. Additional experiments to answer these questions are an important matter of future investigation.

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References


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