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Activation of Toll-Like Receptor-2 by Glycosylphosphatidylinositol Anchors from a Protozoan Parasite¹

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Glycosylphosphatidylinositol (GPI) anchors and glycoinositolphospholipids (GIPLs) from parasitic protozoa have been shown to exert a wide variety of effects on cells of the host innate immune system. However, the receptor(s) that are triggered by these protozoan glycolipids has not been identified. Here we present evidence that *Trypanosoma cruzi*-derived GPI anchors and GIPLs trigger CD25 expression on Chinese hamster ovary-K1 cells transfected with CD14 and Toll-like receptor-2 (TLR-2), but not wild-type (TLR-2-deficient) Chinese hamster ovary cells. The protozoan-derived GPI anchors and GIPLs containing alkylacylglycerol and saturated fatty acid chains or ceramide were found to be active in a concentration range of 100 nM to 1 μ M. More importantly, the GPI anchors purified from *T. cruzi* trypomastigotes, which contain a longer glycan core and unsaturated fatty acids in the sn-2 position of the alkylacylglycerol component, triggered TLR-2 at subnanomolar concentrations. We performed experiments with macrophages from TLR-2 knockout and TLR-4 knockout mice, and found that TLR-2 expression appears to be essential for induction of IL-12, TNF- α , and NO by GPI anchors derived from *T. cruzi* trypomastigotes. Thus, highly purified GPI anchors from *T. cruzi* parasites are potent activators of TLR-2 from both mouse and human origin. The activation of TLR-2 may initiate host innate defense mechanisms and inflammatory response during protozoan infection, and may provide new strategies for immune intervention during protozoan infections. *The Journal of Immunology*, 2001, 167: 416–423.

Toll-like receptors (TLRs)³ have been identified as ancient receptors that confer specificity to the host innate immune system allowing the recognition of 'pathogen-associated molecular patterns' (1–3), including bacterial glycolipids such as LPS from Gram-negative bacteria (4), peptidoglycan from Gram-positive bacteria (5), and lipopeptides from diverse species of bacteria including *Mycobacterium tuberculosis* (6), *Mycoplasma fermentans*, *Treponema pallidum*, and *Borrelia burgdorferi* (5, 7).

Cells of the macrophage lineage exposed to these microbial components synthesize high levels of pro-inflammatory cytokines, including IL-12 and TNF- α , two mediators that appear to be important for the initiation of IFN- γ synthesis by NK cells (8). Recognition of bacterial glycolipids by IFN- γ -exposed phagocytic leukocytes is also responsible for the rapid triggering of a variety of protective immune processes, including the synthesis of reactive oxygen and nitrogen intermediates (9). Animals that have not yet developed adaptive immune responses to pathogens depend upon these processes for survival. Similar cytokine circuits and effector mechanisms appear to be involved in resistance to early infection by various protozoan parasites (8, 10), which are common etiologic agents of medical and veterinary diseases.

Glycosylphosphatidylinositol (GPI) anchors are abundant molecules in the membrane of parasitic protozoa (11). Recent studies have documented the immunostimulatory and regulatory activity of protozoan-derived GPI anchors and related structures (12). Among the biological properties of these protozoan GPI anchors is their ability to elicit the synthesis of pro-inflammatory cytokines as well as NO by host macrophages (12–19), similar in many respects to the LPS of Gram-negative bacteria. In contrast, purified glycoinositolphospholipids (GIPLs) and lipophosphoglycan from protozoan parasites suppress several functions of the host immune system, especially at higher concentrations (20–24).

Trypanosoma cruzi is the causative agent of Chagas' disease, a parasitic infection of enormous importance in Central and South America. To better understand the early stimulation of the innate immune system by parasitic protozoa, we have focused on the identification and chemical characterization of *T. cruzi* products that trigger the pro-inflammatory and effector functions of macrophages (17, 19). These studies indicate that GPI anchors purified from mucin-like glycoproteins (tGPI-mucin) of *T. cruzi* trypomastigotes (tGPI) play an essential role in triggering various macrophage functions. In addition, our data show that the potent

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³ Abbreviations used in this paper: TLR, Toll-like receptor; CHO, Chinese hamster ovary; eGIPL, epimastigote derived glycoinositolphospholipids; eGPI-mucin, GPI-anchored mucin-like glycoproteins derived from *T. cruzi* epimastigotes; GIPLs, glycoinositolphospholipids; GPI, glycosylphosphatidylinositol; MALP, macrophage-activating lipopeptide; tGPI, GPI anchor purified from tGPI-mucin; tGPI-mucin, GPI-anchored mucin-like glycoproteins derived from *T. cruzi* trypomastigotes; KO, knockout; PI, phosphatidylinositol; CER, ceramide; AAG, alkylacylglycerol; Osp A, outer surface protein A.

pro-inflammatory activity of tGPI is dependent on its fine structure, which apart from its longer glycan core includes an unsaturated fatty acid at the sn-2 position of the alkylacylglycerolipid component (17, 19).

Despite the growing evidence implicating GPI structures from parasitic protozoa in the induction of cytokine synthesis as well as in effector functions by macrophages, not much is known about the receptor(s) and signaling pathways that are triggered by these GPI anchors. Nevertheless, different studies indicate a similarity of signaling pathways, gene expression, and functions displayed by macrophages exposed to either purified tGPI anchors or tGPI-mucin (12, 17–19, 25) and bacterial glycolipids/lipopeptides (4–7). Recent studies demonstrate that TLR-2 or TLR-4 are responsible for triggering various functions in macrophages exposed to Gram-positive, *Mycoplasma* spp. and *M. tuberculosis* (5–7, 26) or Gram-negative bacteria (4, 26), respectively. Here, we tested the ability of *T. cruzi*-derived GPI anchors and GIPLs to trigger TLR-2 and TLR-4, and studied the role of such receptors in the ability of parasitic glycolipids to elicit IL-12, TNF- α , and NO by murine macrophages. Our results show that protozoan-derived GPI anchors and GIPLs preferentially activate TLR-2, which is largely responsible for the initiation of the various macrophage functions induced by these protozoan-derived glycolipids.

Materials and Methods

T. cruzi- and parasite-derived glycolipids

Green monkey kidney-derived LLC-MK₂ cells (American Type Culture Collection, Manassas, VA) were grown at 37°C in DMEM (Life Technologies, Grand Island, NY) containing 5% heat-inactivated FBS, 40 μ g of gentamicin sulfate/ml, 0.3% sodium bicarbonate in a 5% CO₂ atmosphere. Trypomastigote forms of *T. cruzi* (Y strain) were obtained from the supernatant of LLC-MK₂ cells 6–8 days after infection with 10 trypomastigotes per cell. Epimastigote forms, from either Y or CL strain, were grown at 28°C in cell-free liver infusion tryptose medium supplemented with 10% FBS. Live parasites were collected from either tissue or liquid culture, washed three times in ice-cold PBS, and used in the assays with transfected Chinese hamster ovary (CHO) cells or kept at –70°C for purification of GPI-mucins, GPI anchors, or GIPLs. The epimastigote- and trypomastigote-derived GPI-mucins and GIPLs were purified by sequential organic solvent extraction and hydrophobic interaction chromatography as described elsewhere (19, 27). The following glycolipids were purified from *T. cruzi* parasites and used in the experiments presented here: tGPI-mucin, derived from tissue culture trypomastigotes of Y strain of *T. cruzi* containing a 1-*O*-(C16:0)-2-*O*-alkyl-(C18:1 or C18:2) acylglycerol; eGPI-mucin, derived from epimastigotes of Y strain of *T. cruzi* containing a 1-*O*-(C16:0)-2-*O*-alkyl-(C16:0) acylglycerol; eGIPL Y CER, derived from epimastigotes of Y strain of *T. cruzi* containing a ceramide (C24:0) fatty acid-(C18:0) sphinganine; eGIPL CL AAG, derived from epimastigotes of CL strain of *T. cruzi* containing a (C16:0)-2-*O*-alkyl-(C16:0) acylglycerol; and eGIPL CL CER, derived from epimastigotes of CL strain of *T. cruzi* containing a ceramide (C24:0) fatty acid-(C18:0) sphinganine. These GPI-anchored molecules also vary according to their glycan core composition and ethanolaminephosphate and ethanolaminephosphonate head groups. Detailed compositional and structural analyses of each of the GPI-anchored preparations are described in Almeida et al. (19). Nitrous acid deamination was used to produce the phosphatidylinositol (PI) and glycan core of tGPI or tGPI-mucins, as previously described (19). Edman microsequencing (five cycles) and positive-ion mode matrix-assisted laser desorption ionization time-of-flight mass spectrometry (19), positive-ion mode electrospray mass spectrometry, and *Mycoplasma* spp. detection ELISA tests were used to exclude the possibility that *T. cruzi*-derived glycolipids were contaminated with *Mycoplasma* spp.-derived lipopeptides. These results indicated that *T. cruzi*-derived glycolipids were free of *Mycoplasma* spp. lipopeptides. Furthermore, *N*-acetylation abolished NO-, TNF- α -, and IL-12-inducing activity of mycoplasma macrophage-activating lipopeptide (MALP), but had no significant effect on bioactivity of the tGPI-mucin (Ref. 28; data not shown).

Bacteria and bacteria-derived glycolipids/lipopeptides

The UV killing of *Staphylococcus aureus* (ATCC 12.692) and *Escherichia coli* (HB101) followed the same procedure. Bacteria were grown overnight

in 200 ml of Luria Broth, centrifuged for 30 min at 1200 \times g, and resuspended in 20 ml PBS. Bacterial density was resolved by limiting dilution of washed bacteria, determining the CFUs. A UV germicide lamp (G15T8; General Electric, Fairfield, CT) was used at 10 cm of an open petri dish with 2 ml of bacteria for 20 min, and the bacteria were stored at –20°C until use. LPS from *E. coli* serotype O55:B5 prepared by the Westphal method was obtained from Sigma (St. Louis, MO). Outer surface protein A (Osp A) was purified from *B. burgdorferi* (5), and synthetic MALP from *M. fermentans* was obtained as previously described (29).

CHO cell lines

The CHO reporter cell lines (CHO/CD14, CHO/CD14/TLR-2, and CHO/CD14/TLR-4) (5) were maintained as adherent monolayers in Ham's F-12/DMEM supplemented with 5% FBS, at 37°C, 5% CO₂, and antibiotics. All of the cell lines are derived from clone 3E10, a CHO/CD14 cell line that has been stably transfected with a reporter construct containing the structural gene for CD25 under the control of the human E-selectin promoter. This promoter contains a NF- κ B binding site; CD25 expression is completely dependent upon NF- κ B translocation to the cell nucleus (30). Cells expressing TLRs were constructed by stable transfection of the CHO/CD14 reporter cell line with the cDNA for human TLR-2 or TLR-4 as described (5). In addition to the LPS responsive cell lines described above, we also tested an LPS nonresponder cell line (30) designated clone 7.19 as well as a clonal line derived from this mutant that was transfected with CD14 and TLR-2 (7.19/CD14/TLR-2). This cell line was derived from 3E10, and reports NF- κ B activation via the surface expression of CD25, similarly to the other CHO lines described. The LPS nonresponsive phenotype of the 7.19 cell lines appears to be due to a mutation in the MD-2 gene, and thus is defective in signaling via TLR-4 (30).

Flow cytometry analysis

CHO reporter cells were plated at a density of 1 \times 10⁵ cells/well in 24-well tissue culture dishes. The following day, either bacteria, live protozoa, or purified glycolipids were added as indicated, in a total volume of 0.25 ml of medium/well, for 18 h. The cells were then harvested with trypsin-EDTA and washed once with medium and again with PBS. Subsequently, the cells were counted and 1 \times 10⁵ cells stained with PE-labeled anti-CD25 (mouse mAb to human CD25, R-PE conjugate; Caltag Laboratories, Burlingame, CA) 1:200 in PBS, on ice, in the dark, for 30 min. After labeling, the cells were washed twice with 1 mM sodium azide in PBS, resuspended in 1 mM sodium azide in PBS, and examined by flow cytometry (BD Biosciences, San Jose, CA) for the expression of surface CD25 as described (5). Analyses were performed using CellQuest software (BD Biosciences).

Inflammatory and bone marrow macrophages

Wild-type, TLR-2 knockout (KO), and TLR-4 KO mice (4, 26) were inoculated i.p. with 2 ml of 3% thioglycollate and, 4 days later, the elicited peritoneal exudate cells were harvested in cold serum-free DMEM. The medium used in the macrophage cultures (MacMed) consisted of DMEM supplemented with 40 μ g of gentamicin/ml and 5% heat-inactivated FCS. Bone marrow was washed from the femur of 8- to 12-wk-old wild-type or TLR-2 KO mice with DMEM supplemented with gentamicin. Cells were washed, resuspended in marrow culture medium, and plated at 10–15 \times 10⁶ cells/10 ml/plate on 100-mm nontissue culture-treated petri dishes. Marrow culture medium consisted of DMEM supplemented with 10% FCS and gentamicin, plus 30% supernatant from confluent cultures of L929 fibroblasts, as a source of macrophage-CSF. After 5–7 days of culture at 37°C, 5% CO₂, plates were gently washed to remove nonadherent cells. Plates contained 3–5 \times 10⁶ macrophages, >95% pure.

Peritoneal and bone marrow-derived macrophages were resuspended in MacMed at 2 \times 10⁶/ml, and 100- μ l aliquots were dispensed into wells of a 96-well plate. Cells were allowed to adhere at 37°C and 5% CO₂ for 3 h, and were then washed once with serum-free DMEM and 150 μ l of MacMed was added to each well in the presence or absence of 50 U/ml IFN- γ and incubated overnight at 37°C and 5% CO₂. Different macrophage-stimulating preparations were added to the macrophage cultures in a final volume of 200 μ l/well. Aliquots of the supernatant (50 and 100 μ l) were collected after 24 and 48 h of culture for nitrite, TNF- α , and IL-12 (p70 or p40, as indicated) measurements, respectively (17, 19). The concentration of nitrite was determined by the Griess reaction (31). Levels of TNF- α and IL-12 (p70 or p40, as indicated) in the supernatants were measured by a commercially available ELISA kit (Duoset; R&D Systems, Minneapolis, MN).

Results

Live *T. cruzi* trypomastigotes trigger NF- κ B-dependent expression of CD25 in CHO cells transfected with TLR-2

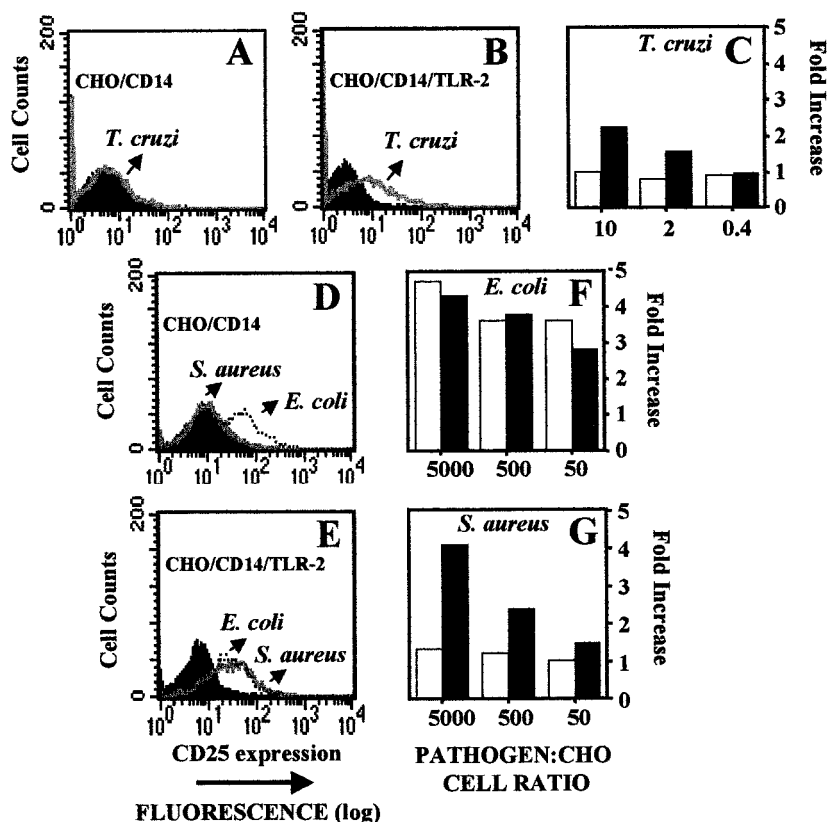
CHO reporter cell lines that were stably transfected with CD14 alone (CHO/CD14), TLR-2 and CD14 (CHO/CD14/TLR-2), or TLR-4 and CD14 (CHO/CD14/TLR-4) were exposed for 24 h to live *T. cruzi* trypomastigotes (17), and NF- κ B activation was assessed by measuring the expression of CD25 by flow cytometry (5, 30). No increase in the induction of CD25 expression by CHO/CD14 cells exposed to *T. cruzi* parasites was observed (Fig. 1A). In contrast, live *T. cruzi* trypomastigotes enhanced the CD25 expression in the CHO/CD14/TLR-2 cell line (Fig. 1B), indicating that TLR-2 expression had led to the activation of NF- κ B. Activation of NF- κ B was dependent on the parasite-to-CHO/CD14/TLR-2 cell ratio (Fig. 1C), the maximum effect being reached with 10 parasites per cell. This concentration of parasites saturated the response; we observed similar levels of activation when the CHO/CD14/TLR-2 cells were exposed to 50 trypomastigotes per cell (data not shown). Mock parasite pellets obtained from LLC-MK₂ cell supernatants were unable to trigger CD25 expression on the CHO/CD14/TLR-2 cell line (data not shown). As positive controls, we stimulated CHO/CD14 and CHO/CD14/TLR-2 cells with either UV-killed *E. coli* or *S. aureus* (5). Data in Fig. 1, D and E, show that *E. coli* induced expression of CD25 in both CHO/CD14 and CHO/CD14/TLR-2 cells. In contrast, *S. aureus* induced expression of CD25 only on the surface of CHO/CD14/TLR-2 (Fig. 1E), but not on CHO/CD14 cells (Fig. 1D). The maximal CD25 expression on CHO cells was obtained at a bacteria-to-cell ratio of 500:1 (Fig. 1F) and 5000:1 (Fig. 1G) for *E. coli* and *S. aureus*, respectively. We conclude that live *T. cruzi* trypomastigotes were capable of triggering TLR-2.

Highly purified GPI anchors from *T. cruzi* trypomastigotes are potent activators of TLR-2

The CHO cells transfected with human CD14 alone or human CD14 and TLR-2 were also exposed to tGPI-mucin (17, 19) or LPS, and NF- κ B activation was evaluated by measuring the expression of the CD25 reporter transgene by flow cytometry (5, 30). CD25 expression was enhanced in both CHO/CD14 (Fig. 2A) and CHO/CD14/TLR-2 (Fig. 2B) exposed to LPS, because both lines of CHO cells express endogenous TLR-4. In contrast, tGPI-mucin induced expression of CD25 on CHO/CD14/TLR-2 (Fig. 2B), but not on CHO/CD14 (Fig. 2A) cells. Fig. 2C shows the titration of LPS activity in CHO/CD14 cells. It is noteworthy that maximal expression of CD25 in CHO/CD14/TLR-2 cells exposed to tGPI-mucin (Fig. 2D) was observed at 10 nM, but even at subnanomolar concentrations (i.e., 0.1 nM), a significant increase in the expression of CD25 was achieved. These data indicate that the tGPI-mucin is among the most potent TLR-2 agonists thus far described (5–7).

Other studies, using different protozoan GPI anchors have suggested distinct activities of the diacylglycerol (*Trypanosoma brucei*) (16) or dimyristoylglycerol (*Plasmodium falciparum*) (32) components, compared with the glycosylinositolphosphate moiety of GPI anchors isolated from these parasites (16, 32). Our previous results suggested that the tGPI is responsible for the ability of tGPI-mucin to induce cytokine and NO synthesis by murine macrophages and that both the sn-2 unsaturated acyl chain and periodate-sensitive units from tGPI are essential for their activity on macrophages (17, 19). In the experiments shown in Fig. 3, A and B, we confirmed that the ability of tGPI-mucin to activate NF- κ B via TLR-2 was mostly recovered using a highly purified tGPI preparation. Removal of the lipid moiety from the tGPI (Fig. 3B) or tGPI-mucin (data not shown) by nitrous acid deamination (19)

FIGURE 1. TLR-2 mediates cellular activation upon exposure to live *T. cruzi* trypomastigotes. CHO/CD14 (A) or CHO/CD14/TLR-2 (B) cells were left untreated (in black) or exposed to live trypomastigote at parasite: CHO cell ratio of 10:1 (gray lines) and the expression of the reporter transgene (CD25) was measured 18 h later. C, Parasite dose effect, as indicated by parasite: CHO cell ratio on expression of CD25 in CHO/CD14 (open columns) and CHO/CD14/TLR-2 (filled columns). CHO/CD14 (D) or CHO/CD14/TLR-2 (E) cells were left untreated (in black) or exposed to UV killed *S. aureus* (gray lines) or to UV killed *E. coli* (dotted lines) at 10^4 bacteria/cell. F (*E. coli*) and G (*S. aureus*). Bacteria dose effect on fold increase of CD25 expression on CHO/CD14 (□) and on CHO/CD14/TLR-2 cells (■). The expression of the reporter transgene (surface CD25) was measured by flow cytometry. The fold increase on expression of CD25 was calculated by dividing the median fluorescence from stimulated by the median fluorescence from corresponding unstimulated control cells. The data presented reflect at least three independent experiments.



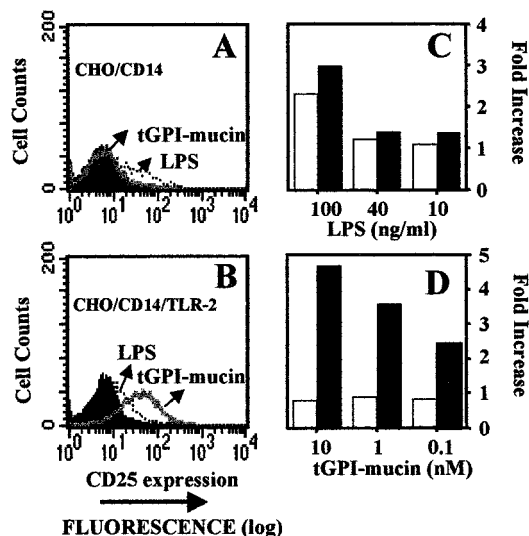


FIGURE 2. TLR-2 expression imparts CHO cell responsiveness to tGPI-mucin. CHO/CD14 (A) or CHO/CD14/TLR-2 (B) cells were exposed to medium (in black), to tGPI-mucin at 10 nM (gray lines), or to LPS at 100 ng/ml (dotted lines). The cells were subjected to flow cytometric analysis for the CD25 transgene expression. The CHO/CD14 (□) or CHO/CD14/TLR-2 (■) cells were exposed to different concentrations of LPS (C) or tGPI-mucin (D), and the expression of the gene reporter was measured by flow cytometry. The fold increase on expression of CD25 was calculated by dividing the median fluorescence from stimulated cells by the median fluorescence from unstimulated control cells. The data presented are typical of the results from three independent experiments.

completely abolished the activity of tGPI anchors in CHO/CD14/TLR-2 cells. The resulting PI also failed to elicit expression of the CD25 reporter transgene in these cells, even at 100 nM (Fig. 3B). These results indicate that both the lipid tail and the glycan moiety of the tGPI are necessary for triggering TLR-2, which is in agreement with our previous studies indicating that the cytokine and/or NO-inducing activity could not be dissociated (19).

Highly purified GPI anchors and GIPLs from *T. cruzi* epimastigotes are less potent activators of TLR-2

Recently, we compared the bioactivity of 12 different preparations of GPI anchors or GIPLs derived from either trypomastigote or

epimastigote developmental stages of *T. cruzi*. All these preparations were highly purified and had defined structures as determined by electrospray mass spectrometry and electrospray-mass spectrometry-collision-induced dissociation/mass spectrometry (ES-MS-(CID)-/MS) (19). We found that the unsaturated fatty acids at the sn-2 position of the alkylacylglycerol is likely to be essential for the extremely potent bioactivity of tGPI-mucin or tGPI on murine inflammatory macrophages. We also tested different GPI-mucins (eGPI-mucins) and GIPLs (eGIPLs) from the epimastigote stage of *T. cruzi* for their ability to induce the expression of CD25 by CHO/CD14/TLR-2. The eGPI-mucins and eGIPLs differ in their fine structure from tGPI in that they contain a lipid moiety with either ceramide or alkylacylglycerol containing only saturated fatty acid chains. Furthermore, they contain a shorter glycan moiety when compared with the tGPI one (19). As shown in Fig. 3C, eGPI-mucin or eGIPL activated NF- κ B-mediated CD25 expression in CHO/CD14/TLR-2 cells, but only at micromolar concentrations. The bioactivity of eGPI-mucin and eGIPLs on CHO/CD14/TLR-2 cells was higher than that previously observed in murine inflammatory macrophages (Refs. 17–19 and Fig. 3C). This could be explained in part by the high degree of expression of TLR-2 in the transfected CHO cells, which may be higher than macrophages. Together, these findings are consistent with our previous study (17–19) and indicate that in general eGPI-mucin or eGIPL are at least 100- to 1000-fold less active in triggering the TLR-2 than the tGPI-mucin or tGPI.

GPI anchors and GIPLs derived from both trypomastigotes and epimastigotes trigger NF- κ B-dependent expression of CD25 in CHO cells transfected with TLR-2, but containing defective TLR-4 signaling

CHO cells express endogenous TLR-4, and low levels of activation were observed in CHO cells transfected with CD14, but not with TLR-2, when *T. cruzi*-derived tGPI anchors were used at high concentrations (i.e., 0.1–1 μ M). It was important to test the ability of different GPI/GIPL preparations in triggering CD25 expression in CHO cells with a defective TLR-4 signaling complex (clone 7.19, a mutant LPS nonresponder cell line that is defective in the expression of MD-2; Ref. 30) because of the possibility that small amounts of contaminating endotoxin in our preparations might confound interpretation of the results. As shown in Fig. 4, A–E, clones of 7.19 transfected with CD14 only or CD14 plus TLR-2

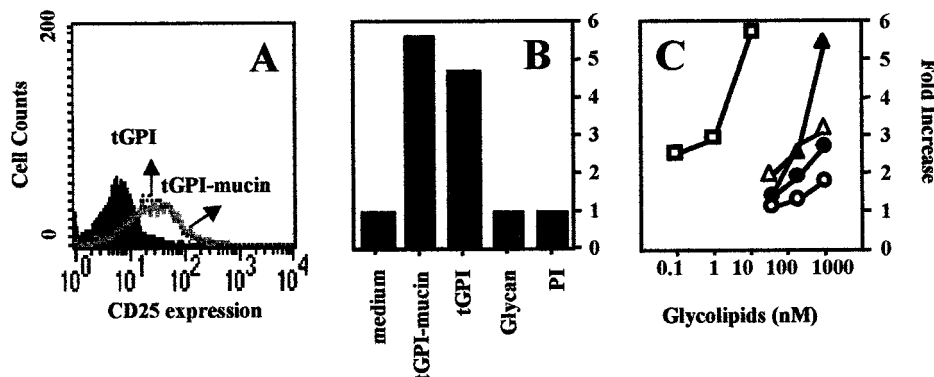
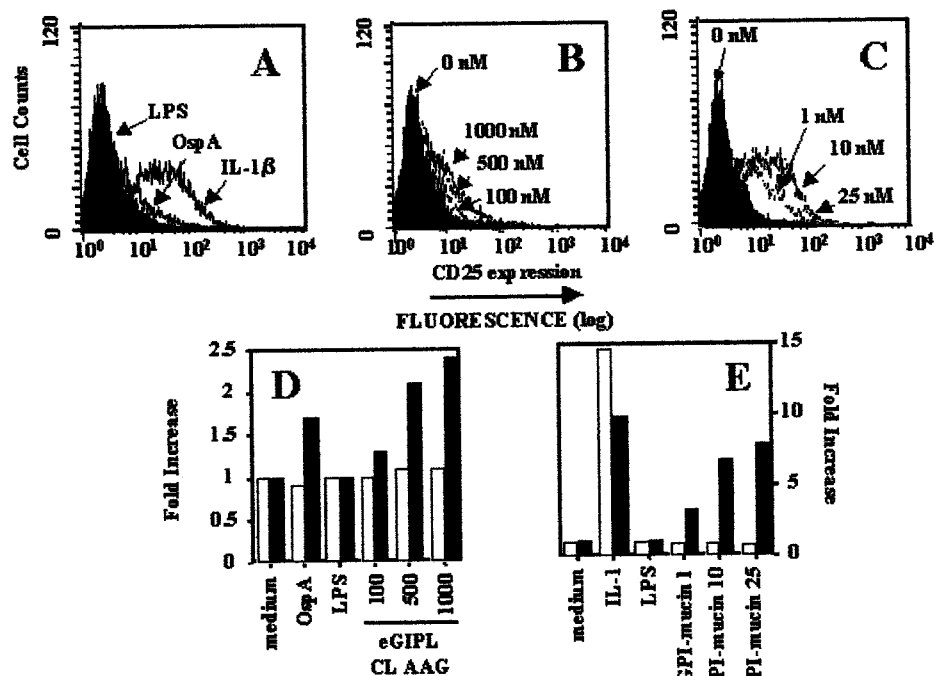


FIGURE 3. TLR-2 mediates cellular activation upon exposure to *T. cruzi*-derived GPI anchors and GIPLs. A, CHO/CD14/TLR-2 reporter cells were exposed to medium (in black), to 10 nM of tGPI-mucin (gray lines), or to 10 nM of highly purified tGPI (dotted lines). Again, cellular activation was analyzed by flow cytometry. B, CHO/CD14/TLR-2 were exposed to medium, tGPI-mucin (10 nM), tGPI (10 nM), the glycan (100 nM), or to PI (100 nM) portions of tGPI, and CD25 expression was measured by flow cytometry. C, CHO/CD14/TLR-2 cells were exposed to various concentrations of different *T. cruzi*-derived glycoconjugates: tGPI-mucin (□), eGPI-mucin (○), eGIPL Y CER (▲), eGIPL CL AAG (●), and eGIPL CL CER (△). Expression of CD25 was evaluated 18 h later. The y-axis of both B and C indicated fold increase obtained by dividing the median fluorescence of activated cells by median fluorescence of unstimulated cells. The data presented here reflect the results of three independent experiments.

FIGURE 4. GPI anchors and GIPLs activate LPS nonresponder CHO/CD14 cells transfected with TLR-2 (clone 7.19). 7.19/CD14/TLR-2 cells were stimulated with IL-1 β , LPS, or outer surface protein A (A), different concentrations of eGIPL CL AAG (B), or tGPI-mucin (C), and expression of CD25 was evaluated 18 h later. The fold increase values of CD25 expression on 7.19 cells transfected with CD14 only (\square) or CD14 and TLR-2 (\blacksquare) exposed to different stimuli are presented in D and E. The y-axis of D and E indicated fold increase obtained by dividing the median fluorescence of activated cells by median fluorescence of unstimulated cells. The data presented here reflect the results of three independent experiments.



were highly refractory to LPS stimulation. These findings are in agreement with previous studies showing that TLR-4, but not TLR-2, is responsible for the CHO cell activation by highly purified LPS (4, 33). In contrast, the 7.19 transfected with CD14 and TLR-2, but not with CD14 only, were responsive to tGPI-mucins, eGIPLs, or to the lipopeptide, OspA (5). These results show that TLR-2 is sufficient for responsiveness to these lipids, and does not require a functional TLR-4/MD-2 signal transduction complex to mediate NF- κ B activation in transfected CHO cells exposed to GPI anchors or GIPLs derived from *T. cruzi* parasites. Consistent with the results presented in Fig. 3, tGPI-mucin was more active than eGPI-mucin (data not shown) or GIPLs in activating 7.19/CD14/TLR-2.

TLR-2 is essential for the pro-inflammatory activity of T. cruzi trypomastigote-derived GPI anchors on macrophages

We have shown previously that tGPI-mucin or tGPI induces high levels of NO and IL-12 in IFN- γ -primed inflammatory macrophages (17, 19). In contrast, the induction of high TNF- α levels by protozoan glycolipids occurs in either IFN- γ -primed or unprimed inflammatory macrophages. Our previous studies also show that although the receptors triggered by tGPI-mucin and LPS are functionally similar (12, 25), they are not identical, because macrophages from TLR-4 mutant C3H/HeJ mice can mount a major cytokine and NO response upon stimulation with tGPI-mucin or tGPI (17–19). Therefore, we tested the involvement of TLR-2 and TLR-4 on cytokine induction by tGPI-mucin and tGPI in murine inflammatory macrophages. Macrophages from TLR-4 KO or TLR-2 KO mice were exposed to the prototypical TLR-4 and TLR-2 “ligands” LPS (4) and MALP (7), respectively. The data presented in Fig. 5 confirm the results obtained with CHO/CD14/TLR-2 cells, showing that IFN- γ -primed inflammatory macrophages from TLR-2 KO mice did not produce NO, TNF- α , and IL-12 upon stimulation with either tGPI-mucin or tGPI. The inflammatory macrophages from TLR-4 KO were still responsive to the *T. cruzi* glycolipids, producing levels of NO, TNF- α , or IL-12 comparable to macrophages from wild-type mice. In contrast, macrophages from TLR-4 KO mice, but not from TLR-2 KO mice,

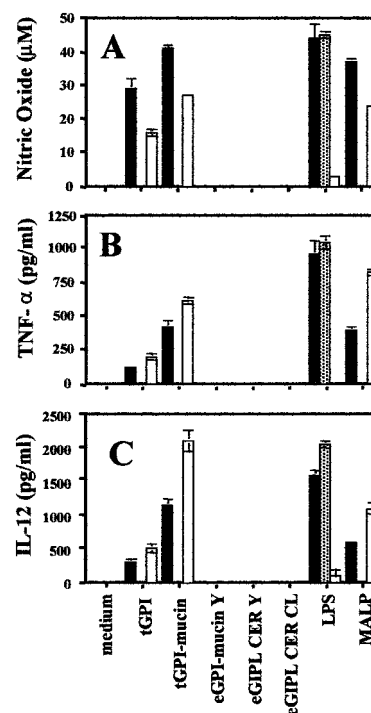


FIGURE 5. Inflammatory macrophages derived from TLR-2-KO mice were unresponsive to tGPI-mucin or tGPI. Peritoneal macrophages from wild-type (\blacksquare), TLR-2 KO (\square), or TLR-4 KO mice (\square) were isolated 4 days after i.p. thioglycollate injection. The cells were exposed to medium, to tGPI (10 nM), tGPI-mucin (10 nM), eGPI-mucin Y (100 nM), eGIPL CER Y (100 nM), eGIPL CER CL (100 nM), LPS (100 ng/ml), or to MALP (100 nM), in the presence of IFN- γ (50 IU/ml), and supernatants were harvested 24 h later to measure nitrite (A), TNF- α (B), and IL-12 (p70) (C). The results shown here are representative of one of two experiments performed.

were unresponsive to LPS. As previously reported (17–19), eGPI-mucin and eGPIs at 100 nM were unable to elicit NO, TNF- α , or IL-12 synthesis by macrophages from either wild-type or TLR-2 KO mice. Consistently, eGPI-mucins and eGPIs (17–19) as well as GPI anchors from *T. brucei* (16) and *P. falciparum* (13) elicited NO and TNF- α synthesis by murine inflammatory macrophages only at micromolar concentrations. As shown in Table I, although less responsive than inflammatory macrophages, bone marrow macrophages also produced significant amounts of NO, TNF- α , and IL-12 upon stimulation with tGPI-mucin. We observed TNF- α synthesis by either unprimed or IFN- γ -primed macrophages exposed to tGPI-mucin. In essence, the results with macrophages derived from TLR-2 KO and TLR-4 KO mice show that TLR-2 is not only a major receptor triggered by GPI anchors from parasitic protozoa, but is also essential for the macrophage response to tGPI or tGPI-mucin.

Discussion

The ability of *T. cruzi* trypomastigotes to signal host cells is well established as an important strategy for infection of the vertebrate host (34). For instance, trypomastigotes trigger the calcium influx in nonprofessional phagocytic cells, triggering lysosome recruitment, and enhancing susceptibility of host cells to infection with *T. cruzi* (35). Similarly, *T. cruzi* parasites stimulate a TGF- β signaling pathway that also enhances host cell susceptibility to infection (36). In contrast, induction of a NF- κ B-dependent mechanism by the parasite results in enhancement of resistance to *T. cruzi* infection in nonimmune host cells (37). Independent studies have shown the ability of parasite molecules to trigger different activities in cells from host innate immune system, which are also important determinants of host resistance to *T. cruzi* infection (38–40). In fact, an early study demonstrated that host resistance/susceptibility to infection is, at least in part, determined at the very early stages of infection, before the development of acquired immunity (41). Therefore, the combinatory effect of these different signaling pathways triggered by *T. cruzi* parasites in nonimmune cells and/or cells from innate immune system may have important consequences in different aspects of *T. cruzi* infection such as load of tissue parasitism, tissue tropism, and the pathogenesis of Chagas' disease.

Studies using infection with different parasitic protozoa, including *T. cruzi*, have demonstrated the importance on the early IL-12-induced T cell-independent IFN- γ synthesis on host protection, before the development of parasite-specific immune responses (8, 10, 40). The nature of protozoan-stimulatory molecule(s) that trigger the cells from host innate immune system is still not entirely resolved, although recent reports (17–19, 42–45) have reflected serious efforts to address this question. The GPI anchors and GPIs are excellent candidates for important parasitic molecules that initiate the recognition of protozoan parasites by the host innate immune system (13, 19, 21, 25, 42, 43). Several studies indicate that GPI anchors derived from *T. cruzi* trypomastigotes have the ability to trigger the synthesis of pro-inflammatory cytokines by cells of monocytic lineage (17, 19), as previously reported for *P. falciparum* (13) and *T. brucei* (14, 16). Despite the growing evidence implicating GPI structures from parasitic protozoa in the induction of cytokine synthesis by macrophages, not much is known about the counterpart receptor(s) and signaling pathways that are triggered by protozoan GPI-anchors. Our recent study (46) demonstrated that like LPS, tGPI-mucin or tGPI are capable of triggering phosphorylation of extracellular signal-related kinases-1 and -2, stress-activated protein kinase kinase-1/mitogen-activated protein kinase kinase-4, and stress-activated protein kinase-2. Furthermore, using different specific inhibitors for mitogen-activated protein kinase and NF- κ B, we found that similar IC₅₀ values are required to inhibit cytokine synthesis induced by LPS and tGPI-mucins (or tGPI). However, macrophages from LPS-hyporesponsive mice were still responsive to tGPI-mucins. Together, these results suggest that although functionally similar, the receptors used by LPS and tGPI-mucin are not the same.

In this study, we investigated the ability of *T. cruzi* parasites to trigger TLR-2 and TLR-4. Our results show that different GPI anchors and GPIs derived from trypomastigote and epimastigote stages of *T. cruzi* present variable potency in activating NF- κ B-dependent CD25 expression in CHO cells transfected with both human CD14 and TLR-2. As shown here, most protozoan-derived GPI anchors had the ability to trigger TLR-2 function in the range of 0.1–1 μ M. In addition, our results show that tGPI anchors containing extra galactose residues in the glycan core and unsaturated

Table I. Levels of TNF- α , IL-12(p40), and nitrite in the supernatants from inflammatory and bone-marrow macrophages derived from wild-type or TLR-2 KO mice^a

	Wild Type		TLR-2 KO	
	Medium	tGPI-mucin	Medium	tGPI-mucin
Unprimed inflammatory macrophages				
TNF- α (pg/ml)	<25	795 \pm 158	<25	<25
IL-12(p40) (ng/ml)	1.2 \pm 1.0	4.6 \pm 1.0	<1.0	<1.0
Nitrite (μ M)	<5.0	6.9 \pm 1.4	<5.0	<5.0
IFN- γ -primed inflammatory macrophages				
TNF- α (pg/ml)	<25	1425 \pm 218	<25	<25
IL-12(p40) (ng/ml)	<1.0	22.3 \pm 6.0	<1.0	<1.0
Nitrite (μ M)	9.4 \pm 3.0	37 \pm 5.6	8.1 \pm 0.2	7.0 \pm 1.0
Unprimed bone marrow macrophages				
TNF- α (pg/ml)	<25	91 \pm 28	<25	<25
IL-12(p40) (ng/ml)	<1.0	3.2 \pm 1.0	<1.0	<1.0
Nitrite (μ M)	<5.0	<5.0	<5.0	<5.0
IFN- γ -primed bone marrow macrophages				
TNF- α (pg/ml)	<25	617 \pm 156	<25	<25
IL-12(p40) (ng/ml)	<1.0	6.7 \pm 1.0	<1.0	<1.0
Nitrite (μ M)	8.4 \pm 1.9	16.3 \pm 2.0	7.8 \pm 2.3	7.2 \pm 0.9

^a The cells were exposed to medium or to tGPI-mucin (10 nM), in the presence or absence of IFN- γ (50 IU/ml), and supernatants were harvested 24 and 48 h later to measure TNF- α and nitrite as well as IL-12(p40), respectively. The results shown here are representative of one of two experiments performed in triplicate.

fatty acids in the sn-2 position of the alkylacylglycerolipid component present maximal activity in the range of 1–10 nM. This activity was shown to be independent of TLR-4 and essential for induction of the pro-inflammatory cytokines (i.e., TNF- α and IL-12) as well as NO by murine macrophages.

The GPI-linked proteins are also ubiquitous on the plasma membrane of higher eukaryotic cells. Despite their diversity, all GPI anchors share a common core structure (11). Thus, our initial findings indicating that parasite GPI anchors are important in initiating host immune responses lead to the important question as to why mammalian GPI anchors do not ordinarily induce unrestrained autoimmunity. Mammalian cells typically express 10^5 copies of GPI anchors per cell, whereas parasitic protozoa express up to 1–10 million copies of GPI anchors (and related structures) per cell (11). In addition, as shown here, subtle changes in the GPI structure may confer an extreme potency in triggering TLR-2. Consequently, both the amounts as well as the fine structure of protozoan-derived GPI anchors may be important aspects in determining the activation of innate defense mechanisms in the vertebrate host.

It is well established that *T. cruzi* parasites are potent nonspecific stimulators of the host innate immune system (38–40). The data presented here suggest that tGPI activation of TLR-2 may directly initiate IL-12, TNF- α , and NO production in vivo, thus fostering host resistance during early infection with this parasite. Inflammation elicited by the parasite is thought to play a role on the genesis of cardiac and/or esophageal pathology observed in Chagas' disease (40, 47–49). In addition to TLR-2 and TLR-4, many other members of this receptor family have been identified (1–3). The distribution of these different members belonging to the family of TLRs appears to vary among immune and nonimmune host cells (50–52). Thus, it is possible that other TLRs may be triggered by parasite-derived molecules, and not only be important on macrophage parasite interaction, but also be involved in the interplay of parasites and nonimmune host cells.

Finally, TLRs appear to be highly conserved, being ancient receptors that confer a certain degree of specificity to the innate immune system in both insects and mammals (1–3). Therefore, it seems reasonable to argue that the interaction of TLRs and GPI anchors from parasitic protozoa may have the important role of determining the fate of parasitism both in invertebrate and vertebrate hosts, thus being a significant element in the protozoan life cycle. Further understanding of the interaction of GPI anchors and related structures with receptors from TLR family, both from invertebrate and vertebrate hosts, may be helpful to develop new prophylactic and therapeutic means to fight the debilitating and often fatal diseases caused by distinct protozoan parasites.

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