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Expression of Functional TLR4 Confers Proinflammatory Responsiveness to Trypanosoma cruzi Glycoinositolphospholipids and Higher Resistance to Infection with T. cruzi

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TLRs function as pattern recognition receptors in mammals and play an essential role in the recognition of microbial components. We found that the injection of glycoinositolphospholipids (GIPLs) from Trypanosoma cruzi into the peritoneal cavity of mice induced neutrophil recruitment in a TLR4-dependent manner: the injection of GIPL in the TLR4-deficient strain of mice (C57BL/10ScCr) caused no inflammatory response. In contrast, in TLR2 knockout mice, neutrophil chemotraction did not differ significantly from that seen in wild-type controls. GIPL-induced neutrophil attraction and MIP-2 production were also severely affected in TLR4-mutant C3H/HeJ mice. The role of TLR4 was confirmed in vitro by testing genetically engineered mutants derived from TLR2-deficient Chinese hamster ovary (CHO)-K1 fibroblasts that were transfected with CD14 (CHO/CD14). Wild-type CHO/CD14 cells express the hamster TLR4 molecule and the mutant line, in addition, expresses a nonfunctional form of MD-2. In comparison to wild-type cells, mutant CHO/CD14 cells failed to respond to GIPLs, indicating a necessity for a functional TLR4/MD-2 complex in GIPL-induced NF-κB activation. Finally, we found that TLR4-mutant mice were hypersusceptible to T. cruzi infection, as evidenced by a higher parasitemia and earlier mortality. These results demonstrate that natural resistance to T. cruzi is TLR4 dependent, most likely due to TLR4 recognition of their GIPLs. The Journal of Immunology, 2004, 173: 5688–5696.

The initial recognition of invading pathogens is mediated mainly by the TLR family of type I transmembrane receptors. These germline-encoded receptors are defined by the presence of a Toll/IL-1R domain in their cytoplasmic region, and by leucine-rich repeats in the extracellular domain (reviewed in Ref. 1). TLRs are evolutionarily conserved and their orthologues are found in plants, insects, and mammals. To date, 13 TLRs have been reported in mammals, at least 10 of which are clearly expressed in human cells. TLR signaling induces the production of proinflammatory cytokines and up-regulates the expression of costimulatory molecules, linking innate to adaptive immune responses (2). Moreover, TLRs are expressed differently among diverse cell types and appear to respond to distinct ligands. A unique pathogen-associated molecular pattern (PAMP) ligand has been ascribed to date for some TLRs, such as flagellin for TLR5 (3) and CpG DNA for TLR9 (4). In contrast, several PAMPs appear to be dependent on TLR4 or TLR2 expression. Hence, TLR4 is a critical signal transducer for LPS (5), and is also triggered by Taxol (6) and by the F protein of the respiratory syncytial virus (7), among others. TLR2 expression is essential for the response to the peptidoglycan preparations from Gram-positive bacteria, lipoproteins from spirochetes and Gram-negative organisms (8, 9), macrophage-activating lipopeptide-2 (10), and zymosan (11), in addition to other microbial products.

Trypanosoma cruzi is the protozoan parasite causative of Chagas’ disease, endemic in Central and South America. The surface of trypanosomatid parasites contains large amounts of glycoinositolphospholipids (GIPLs), which occur either as GPI anchors for glycoproteins and polysaccharides, or as free GIPLs that contain the identical core structure of GPI (12). The oligosaccharide sequences and lipid structures of the major GIPLs purified from different T. cruzi strains have been determined (13–15). These studies have shown heterogeneity in the glycan and lipid composition of

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4 Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; KO, knockout; GIPL, glycoinositolphospholipid; CHO, Chinese hamster ovary; HSP, heat shock protein.
GIPLs, both between different strains of the parasite, as well as within a single strain.

The GIPL used in the present study is purified from epimastigote forms of the G strain of T. cruzi. Its major form contains a unit of galactofuranose at the nonreducing end linked to a mannotraose main chain. The mannotraose chain, in turn, has an ethanamine-phosphate substituent on the O-6 position of the third mannose, and is linked (1→4) to a glucosaminyl unit substituted at O-6 by a phosphate ester of 2-aminoethylphosphonic acid. This glycan is then attached glycosidically to an inositol-phosphorylhydroceramide, that is predominantly an N-lignocerylphinganine (14, 15). Evidence suggests the occurrence of GIPLs in the infective trypomastigote and intracellular amastigote forms of T. cruzi (16–18). GIPLs are also present on other protozoan parasites, but not in higher eukaryotic cells (12). In the past few years, different research groups, including our own, have demonstrated the immunostimulatory and regulatory activities of GIPL anchors present in the membrane of distinct pathogenic protozoan parasites such as Plasmodium falciparum, Leishmania donovani, and T. cruzi (19–22). However, in most cases, the putative host receptors for these molecules have not been determined. Recently, it has been shown that GIPL anchors of mucin-like glycoproteins purified from trypomastigotes of the Y strain of the protozoan parasite, T. cruzi, trigger cytokine and NO release by macrophages in a TLR2-dependent manner (23). However, when mice with a targeted deletion of iflr2 were experimentally infected, the genetic defect had no major impact on parasitemia or mortality (24). In contrast, MyD88 knockout (KO) mice are more susceptible to infection with T. cruzi (24), suggesting that other TLR(s) and/or receptor(s) that depend on this adapter molecule (e.g., IL-1R or IL-1R) is (are) involved in host innate immune responses to T. cruzi.

We report here the necessity of TLR4 expression for the in vivo proinflammatory responses to T. cruzi GIPL, as well as for the in vitro activation of NF-κB. Moreover, we show that TLR4-mutant mice, such as the C3H/HeN strain, are defective in their ability to control T. cruzi parasite replication in the early stages of infection, and hence display an earlier mortality when compared with C3H/HeJ mice.

Materials and Methods

Isolation and purification of T. cruzi GIPLs

The isolation and purification of GIPL has been previously described in detail (13, 15). Briefly, epimastigotes of T. cruzi (G strain) were grown in BHI-hemin medium supplemented with 5% FCS. Cells were harvested during the early stationary growth phase (5 days at 26°C with shaking), washed three times with 0.9% NaCl, and stored at −70°C. Cells were thawed and extracted three times with cold water, centrifuged (7000 × g, 10 min) and the remaining cell pellet was extracted with 45% aqueous phenol at 75°C for 15 min. The aqueous layer from the phenol extraction was dialyzed, freeze-dried to remove trace amounts of solvent, redissolved in water, and applied to a column of Bio-Gel P-100. The excluded material was lyophilized and the GIPLs were extracted by chloroform/methanol/water (10:10:3). The purified GIPL was gamma-irradiated for sterilization and resuspended first in endotoxin-free water (Invitrogen Life Technologies, Grand Island, NY), and then in endotoxin-free PBS. The GIPL appeared on SDS-PAGE as a fast-moving, single molecular species. The virtual absence of contaminating peptide material was confirmed by the absence of peptide-derived signals in nuclear magnetic resonance spectroscopy and mass spectrometry analyses of the purified material (14, 15). The GIPL preparation tested negative for LPS content using a Limulus amebocyte lysate test, with a limit of detection of 0.125 endotoxin U/ml (E-Toxate; Sigma-Aldrich, St. Louis, MO).

Chinese hamster ovary (CHO) cell lines

The CHO reporter cell line (CHO/CD14, clone 3E10; Ref. 25) was maintained as an adherent monolayer in Ham’s F-12/DMEM (1:1; Invitrogen Life Technologies) supplemented with 5% FBS, at 37°C, 5% CO2, and antibiotics. The 3E10 cell line expresses endogenous TLR4 and has been stably transfected with human CD14 and a reporter construct containing the human CD25 structural gene under the control of the human E-selectin promoter. This promoter contains a NF-κB binding site; CD25 expression is dependent upon NF-κB translocation and activation in the cell nucleus (25). In addition to the LPS-responsive cell line described above, we also tested an LPS nonresponder cell line designated clone 7.19 (26). This cell line was derived from 3E10, and reports NF-κB activation via the surface expression of CD25, similarly to the other CHO lines described. The LPS-nonresponsive phenotype of the 7.19 cell line is due to a C95Y mutation in the MD-2 gene, and is entirely defective in signaling via TLR4 (27).

Mice

C3H/HeJ, C57BL/6, and C57BL/10ScCr mice were from the Universidade Federal Fluminense (Rio de Janeiro, Brazil); C3H/HePas mice were from Instituto de Ciências Biológicas, Universidade de São Paulo (São Paulo, Brazil), and C3H/HeN and C57BL/10 mice were obtained from the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). TLR2-KO mice were previously described (9), as well as 129 × C57BL/6 control mice, and were maintained at the Centro de Pesquisas René Rachou (Belo Horizonte, Brazil). Mice used for the experiments were sex and age matched.

Reagents and treatments

Mice were injected with 2.5 mg of Salmonella typhimurium LPS per kilogram (Difco, Detroit, MI). GIPL (in the indicated doses), or vehicle (PBS). Peritoneal fluid was obtained by peritoneal lavage, injecting 3 ml of cold PBS, centrifuged at 200 × g for 10 min for cell removal, and stored at −70°C. Mice were bled from the retro-orbital plexus using a Pasteur pipette (Sigma-Aldrich, St. Louis, MO) at the indicated times after LPS or GIPL challenge. Serum was collected and stored at −70°C. Other reagents were as follows: polymyxin B (Bedford Laboratories, Bedford, OH) and Pam3Cys-Ser-Lys (EC Microcollections, Tübingen, Germany).

Flow cytometry

Analytical flow cytometry was conducted with a FACS Calibur (BD Biosciences, San Jose, CA) and the data were processed with CellQuest software (BD Biosciences) as previously described (28). Cells from the peritoneal cavity were collected by injecting 3–5 ml of cold PBS, and after washing, 106 cells were resuspended in FACS buffer (PBS containing 1% BSA and 0.01% sodium azide; Sigma-Aldrich). FcR were blocked with anti-CD16/32 (clone 2.4G2) and 5% normal mouse sera. Cells were stained with PE-conjugated anti-GR-1 and FITC-conjugated anti-Mac-1 (CD11b) at 4°C for 30 min. CHO reporter cells were plated at a density of 1 × 106 cells/well in 24-well tissue culture dishes. The following day, either TNF-α, LPS, or purified GIPL were added as indicated, in a total volume of 0.25 ml of medium per well, for 18 h. The cells were then harvested with trypsin-EDTA and washed once with medium and again with FACS buffer. Subsequently, the cells were counted, and 1 × 105 cells were stained with PE-labeled anti-CD25 (mouse mAb to human CD25). All Abs were from BD Pharmingen. After washing twice with cold FACS buffer, cells were resuspended in the same buffer containing 0.5 µg/ml propidium iodide. Dead cells were excluded by appropriate gating of forward and side scatter and by propidium iodide staining. The fold induction of the expression of CD25 was calculated by dividing the median fluorescence from stimulated cells by the median fluorescence from corresponding unstimulated control cells (incubated with the same volume of PBS).

Chemokine and cytokine assays

TNF-α, MIP-2, and IL-10 levels in the peritoneal lavage and serum were analyzed with specific ELISA, as specified by the manufacturer (R&D Systems, Minneapolis, MN). All assays were done in duplicate. The sensitivities of TNF-α, MIP-2, and IL-10 ELISA were 15, 31.25, and 15 pg/ml, respectively.

Experimental infection with T. cruzi

Male C3H/HeJ and C3H/HeN (6–12 wk of age) were infected by the i.p. route with blood-form trypomastigotes of the Y strain of T. cruzi (29). Parasitemia levels were determined by directly visualizing and counting parasites in diluted tail vein blood using a hemocytometer. Mice were monitored daily for parasite-associated death.

Statistical analysis

Statistical analyses were performed with StatView software (Abacus Concepts, Berkeley, CA). Data were compared using a two-tailed Student t test and are expressed as mean ± SEM. Data were considered statistically significant if p values were <0.05. Statistical analyses of survival curves were done using the Mann-Whitney U test.
Results

Neutrophil infiltration is induced by GIPL injection in the peritoneal cavity

We first investigated the capacity of GIPL to induce the infiltration of inflammatory cells in the peritoneal cavity of mice. For this, cells obtained from the peritoneal lavage 3 h after the i.p. injection of GIPL were stained with anti-Gr-1/PE and anti-Mac-1/FITC mAbs and analyzed by flow cytometry. Gr-1 and Mac-1 molecules are expressed by both neutrophils and monocytes, although the level of expression differs by these distinct cell types (28). Three hours after the i.p. injection of 50 μg of GIPL, C57BL/6 mice had an augmented percentage of neutrophils (Gr-1<sup>high</sup>/Mac-1<sup>int</sup> population) to nearly 15% of the gated cells (Fig. 1A). In contrast, PBS-treated mice typically had below 1% PMN. These results were validated microscopically by visual morphological characterization after staining with May-Grünwald-Giemsa (not shown). Total leukocyte counts did not differ significantly between GIPL- and PBS-injected mice (data not shown). Fig. 1B demonstrates the dose-response relationship of GIPL-induced neutrophils 3 h after injection.

Neutrophil attraction induced by GIPL in the peritoneal cavity is TLR4, but not TLR2 dependent

The inflammatory response to GIPL was then investigated in C57BL/10ScCr mice. This strain contains a large chromosomal deletion in the region of the tlr4 genomic locus and, therefore, is defective in the response to LPS (5, 30). Fig. 2A illustrates the dramatic decrease in the neutrophilia induced by GIPL injection observed in C57BL/10ScCr mice, compared with the response generated in the wild-type strain, C57BL/10. In contrast, TLR2 KO responded to GIPL, as shown in Fig. 2B. Although in some experiments, the neutrophil attraction induced by GIPL in TLR2-null mice was lower than the response elicited in wild-type mice, this difference was not statistically significant. The C57BL/10ScCr mouse strain also has a point mutation in the IL-12Rβ2 gene (31, 32), and hence, we also tested the capacity of GIPL to induce neutrophil attraction and MIP-2 production in an LPS-responsive (C3H/HePas) and an LPS-hyporesponsive (C3H/HeJ) strain. C3H/HeJ mice harbor a missense point mutation within the Toll/IL-1R domain of TLR4 that results in the substitution of histidine for a proline and renders C3H/HeJ mice hyporesponsive to LPS (5). As shown in Fig. 3A, chemoattraction of Gr-1<sup>high</sup>/Mac-1<sup>int</sup> cells was detected 3 h after the i.p. injection of GIPL in the wild-type C3H/HePas strain, but not in the TLR4-mutant C3H/HeJ mice. Similarly, GIPL induced detectable MIP-2 levels in the peritoneal lavage of C3H/HePas, but not in C3H/HeJ mice (Fig. 3B). The possibility that LPS or another immunologically active microbial contaminant contributes in some way to the observed response was considered. The virtual absence of contaminating peptidic or lipiddic material in the GIPL used was confirmed by the absence of contaminant-derived signals in nuclear magnetic resonance analysis of the purified material (not shown). Moreover, endotoxin activity in our GIPL preparations was undetectable when tested with a commercial Limulus amebocyte lysate test (data not shown).

Functional TLR4, but not TLR2, expression is required for NF-κB activation by GIPL in vitro

The necessity of functional TLR4 expression for GIPL-induced signaling was further investigated in vitro, using a previously described reporter cell line in a CHO background (25, 26) that reports NF-κB activation via the surface expression of CD25. Fig. 4A shows the profile of CD25 expression by flow cytometry of the
wild-type CHO/CD14 cell line (clone 3E10) after activation with GIPL. Although CHO/CD14 cells respond to picogram-per-milliliter quantities of LPS, they are deficient in TLR2 expression due to a point mutation in the extracellular domain of this molecule. A second CHO/CD14 cell line, clone 7.19, has been characterized as LPS nonresponsive due to a point mutation in the TLR4 coreceptor, MD-2 (26). Clone 7.19 is thus defective in signaling via TLR-4 (27). As shown in Fig. 4B, clone 7.19 did not respond to GIPL nor to Pam3Cys (a TLR2 ligand), although the CD25 reporter gene was induced in response to TNF-α/H9251. In contrast, GIPL-induced CD25 expression could be detected in the LPS-responsive 3E10 cell line, which expresses endogenous TLR4, but lacks TLR2 (Fig. 4B).

Finally, we found that polymyxin B did not inhibit NF-κB activation induced by GIPL in 3E10 cells (Fig. 4C), supporting the conclusion that the observed response was not due to endotoxin contamination in the GIPL preparation.

The kinetics of neutrophil attraction and of TNF-α, MIP-2, and IL-10 induction by GIPL

Next, we determined the kinetics of neutrophil appearance after i.p. injection of either GIPL or LPS (Fig. 5). Again, we performed these experiments on a mass-per-animal basis, because both GIPLs and LPS are a heterogeneous collection of closely related molecules, and molar conversions are impossible to perform. A 100-μg injection of GIPL induced the rapid attraction of neutrophils that was detectable 1 h after i.p. injection and peaked at 4 h. By 12 h after injection, the percentage of neutrophils declined to near base-line levels. In comparison, the response to 50 μg of LPS was delayed, as might have been expected based upon previously published data (33). High levels of neutrophils, approaching the effect of GIPLs, were detected 12 h after LPS injection. But rather than...
decline at this time point, the percentage of PMN in the peritoneal cavity of LPS-challenged animals increased slowly over the 24-h study period.

We analyzed the kinetics of the induction of proinflammatory and anti-inflammatory cytokines like TNF-α and IL-10, and of the production of the neutrophil attractant chemokine MIP-2 under similar conditions. TNF-α is a key mediator of inflammatory responses, released primarily from stimulated macrophages and mastocytes, that can further induce other cytokines and chemokines from a variety of cell types. One hour after the injection of 100 µg of GIPL or 50 µg of LPS, similar increase in TNF-α levels were observed (Fig. 6A). However, although TNF-α was still detectable 4 h after LPS treatment, it was no longer found in the lavage fluid of mice that received GIPL. In contrast to the similar levels of TNF-α observed in the peritoneal cavity, a major distinction between the effects of GIPL and LPS was noted in TNF-α induction in the serum. As shown in Fig. 6B, the mean of the TNF-α levels detected in the serum was 1.28 ng/ml 1 h after i.p. challenge, and declined steadily thereafter. In dramatic contrast, mice injected with GIPL had 30-fold less TNF-α at the same time point. Additional kinetic studies analyzing TNF-α induction in the serum at 20, 40, and 60 min confirmed low but persistent GIPL-induced TNF-α, which peaked 1 h after the treatment (data not shown).

MIP-2 is a member of the CXC chemokine subfamily, and is structurally and functionally related to the human chemokine IL-8 (34, 35). The GIPL-induced MIP-2 levels in the peritoneum were comparable to MIP-2 levels observed in response to LPS after the first hour of treatment (Fig. 6C), whereas, similarly to the release of TNF-α, serum MIP-2 induction was much lower in GIPL-injected mice in comparison to LPS-treated animals (Fig. 6D). Moreover, MIP-2 induced by LPS remained at high levels in the peritoneum fluid until 4 h after treatment, whereas it was no longer detected at this time in GIPL-injected mice (Fig. 6C).

IL-10 is a multifunctional cytokine, whose main function appears to be to resolve inflammatory responses (36). Therefore, with the aim of further understanding the control of neutrophil infiltration caused by GIPL, the presence of IL-10 in the peritoneal cavity was also analyzed. Fig. 6E shows that IL-10 could be detected only shortly after GIPL injection, and its levels were significantly lower than in the LPS-induced response. Similar to MIP-2, IL-10 was still detected at high levels 4 h after LPS, but not GIPL treatment.

**FIGURE 5.** Kinetics of neutrophil attraction induced by GIPL or LPS i.p. injection. C57BL/6 mice were injected i.p. with PBS (□), 100 µg of GIPL (●), or 50 µg of LPS (▲). After the indicated time points, peritoneal lavage was performed, and the collected cells were stained as in Fig. 1. Data are expressed as the mean ± SEM of the percentages of Gr-1highMac-1int cells in three individually analyzed mice at each time point.

**FIGURE 6.** Kinetics of TNF-α, MIP-2, and IL-10 expression induced by GIPL or LPS treatment. Groups of three C57BL/6 mice were injected i.p. with PBS (□), 100 µg of GIPL (●), or 50 µg of LPS (▲). Peritoneal lavage fluid (A, C, and E) and serum (B and D) were collected at the indicated time points, and TNF-α, MIP-2, and IL-10 levels were measured by ELISA in duplicate. Each data point is expressed as the mean ± SEM and represents the result obtained from individually analyzed mice.

**TLR4-mutant C3H/HeJ mice are more susceptible to infection with T. cruzi Y strain**

Classical genetic studies previously established that the resistance to *T. cruzi* is governed by multiple genetic factors, including H-2-linked gene(s) (37, 38). Inbred strains may vary from highly resistant to highly susceptible, as reflected by parasitemia levels and survival time. Following these criteria, C3H strains have been classified as “susceptible,” although we could not find in the literature any systematic study comparing C3H/HeJ, which bear a functionally important point mutation in *Tlr4*, to other C3H strains carrying the wild-type *Tlr4* allele. Therefore, we compared C3H/HeJ and C3H/HeN strains in terms of parasitemia and mortality after infection with 2 × 10⁶ blood-trypomastigote forms of the Y strain. Although subtle differences between the G strain (the strain used for the GIPL isolation and the previous experiments in this report) and the Y strain exist, the lipid moiety in the GIPL extracted from the epimastigote forms of both strains of *T. cruzi* is predominantly ceramide (15), and their GIPLs are virtually indistinguishable from one another. Furthermore, nearly identical results have been reported with GIPLs derived from either strain in previous in vitro studies on cell activation (39, 40).

As shown in Fig. 7A, the in vivo response to *T. cruzi* infection in C3H mice is bimodal; a first peak of parasitemia is followed 5–10 days later by a second peak. As the animals appear to be recovering from the second peak (at least based upon the counts of blood trypomastigotes), they typically succumb to the disease.
contrast to the wild-type mice, the number of trypomastigotes in the infected C3H/HeJ mice rose far more dramatically after the first peak and no second recovery phase was observed. In fact, 100% of C3H/HeJ mice were dead by day 16 of infection (Fig. 7C), whereas the wild-type mice typically survived an additional 3–7 days. When the entire survival curves were analyzed by the Mann-Whitney U test, the difference in the survival curves was clearly statistically significant (p < 0.01). These results indicate a role for TLR4 signaling in the control of T. cruzi infection in vivo.

Discussion
In the present study, we describe the in vivo proinflammatory properties of GIPL molecules derived from T. cruzi and found a requirement for TLR4 expression, but not TLR2 expression for a functional response. The responses to GIPLs that we documented included GIPL-induced neutrophilia and chemokine induction after systemic injection. Moreover, in vitro assays using TLR2-deficient or MD-2-mutant CHO reporter cell lines (25, 26), confirmed the necessity of functional TLR4 expression for GIPL-induced NF-κB activation.

Although LPS is the best studied of the putative TLR4 ligands, other PAMPs with a TLR4-dependent proinflammatory activity have been reported. These include liopteichoic acid (9), the F protein of respiratory syncytial virus (7), bacterial heat shock proteins (HSP) (41), Taxol (6), and several endogenous ligands, such as human HSP70 (42), fibronectin (43), and oligosaccharides derived from hyaluronate (44). Although there are some data suggesting that LPS might contaminate HSP preparations (45, 46), and hence, the reports that HSPs activate TLR4 have been controversial, the question nevertheless remains: how could such structurally different molecules be engaged specifically by a single germinal protein receptor? One possibility is that TLR4 is a key signaling element that participates as a component of a larger signaling unit together with other receptor components, in which ligand specificity resides. In fact, to date, saturable direct binding of LPS to TLR4 (or of any of the above-mentioned TLR4 “ligands”) has not been demonstrated. Indeed, the entire Toll literature is notable for the paucity of good ligand-binding studies.

This concept does not exclude the possibility that diverse TLR4 “ligands” differ in subtle ways in their binding to the TLR4. LPS might represent a special case not shared with other ligands, because of its dependence on the presence of MD-2 to initiate signal transduction (47). However, the data reported herein suggest that GIPLs and LPS function immunologically in a nearly identical manner. MD-2 is a non-membrane-spanning molecule that associates with TLR4 and to which LPS binding has been definitively demonstrated (48, 49). The absence of response to GIPLs in TLR4-deficient mice and in the MD-2-mutant CHO reporter cell line indicate that GIPLs share with LPS the necessity of forming a TLR4-MD-2 complex for signaling. Although a detailed study of the structural and physicochemical similarities between LPS lipid A and the T. cruzi GIPLs have not been performed, several pieces of evidence suggest that similarities might exist. For example, we previously mapped GIPL activities to its ceramide domain, mainly N-lignoceroyldihydrosphingosine (39, 40). Structural similarities between ceramide and a region of lipid A have been known for many years (50). Defective responses to ceramide in strains of mice with TLR4 lesions, such as the C3H/HeJ and C57BL/10ScN strains, have been reported (51, 52).
To further understand the mechanisms by which the GIPL molecule act as an inflammatory agent, we compared the GIPL-induced proinflammatory effects with the response elicited by the prototype TLR4-dependent signaling molecule, LPS. The kinetics of neutrophil attraction as well as cytokines (TNF-α and IL-10) and chemokine (MIP-2) in vivo production induced by GIPL and LPS were performed. Interestingly, although MIP-2 and TNF-α levels in the peritoneal cavity were comparable at the first hour after GIPL and LPS injection, these were detected at much lower levels in the serum of GIPL-treated mice, compared with LPS-treated animals at the same time point. Moreover, whereas no MIP-2 was detected in the peritoneal cavity of GIPL-injected mice after 4 h, MIP-2 levels were sustained for at least 4 h after LPS injection, and could still be detected in the serum and peritoneal cavity at 12- and 24-h time points, respectively. This fact may explain the delay in neutrophil attraction observed in LPS treatment, because it has recently been proposed that the ratio of local- to-systemic chemokine concentrations regulates neutrophil recruitment (53). Also, IL-10 production, which attained higher levels and lasted for a longer period in LPS-injected mice, might contribute to the relative delay in neutrophil attraction. In this context, it is worth noting that the inhibition of IL-10 bioactivity in vivo has been reported to increase levels of serum TNF-α and MIP-2 and, therefore, augment lung PMN leukocyte influx during endotoxemia (54).

The observed differences between the LPS- and the GIPL-induced kinetics of neutrophil attraction and cytokine production may be due to differences in the binding of the two molecules for a common receptor. A rapid ligand “off time,” for example, could result in the intense but brief activation signal observed with GIPs. Alternatively, differences in the composition of the signaling complex might account for these kinetic issues, as discussed above. In contrast, GIPs and LPS may attain different circulation levels and clearance rates after i.p. injection.

Although not statistically significant, there appears to be a trend in the reduction of the GIPL-induced neutrophil attraction to the peritoneal cavity of TLR2-KO mice. We have not yet resolved whether this result is biologically significant, and if so, whether it could be due to a minor TLR2-signaling component present in the GIPL preparation (15), or to a more complex signal interaction between TLR4 and TLR2. Interestingly, like the Gram-negative bacteria which activate the innate system through different TLRs (LPS triggering TLR4 and lipoproteins triggering TLR2 activation pathways, for example), T. cruzi may use different molecules of the GPI-family to activate TLR2 and TLR4 signaling pathways. Although similarities in both the signal transduction pathways and the genes that are activated exist, TLR2 and TLR4 agonists activate different patterns of gene expression (55).

The innate immune response triggered by the invasion of T. cruzi parasites undoubtedly influences many different aspects of a clinical infection, and hence plays an important role in the pathogenesis of Chagas’ disease. At the earliest stages of the infection, T. cruzi activates professional phagocytes to secrete chemokines and proinflammatory cytokines (56). However, little is known at present about parasite molecules that trigger the innate immune response at the onset of infection. GPI anchors of mucin-like glycoproteins, composed by sn-1-O-(C16:0)alkyl-2-O-acylglycerol purified from the trypomastigotes of the T. cruzi Y strain, are potent inducers of cytokine and NO release from IFN-γ-primed macrophages. Indeed, these molecules activate innate immunity in a TLR2-dependent way (21, 23). However, TLR2 ablation led only to a small difference in parasitemia, it did not significantly affect the survival of T. cruzi-infected mice (24). In contrast, MyD88-deficient mice were far more susceptible to infection than wild-type mice, as indicated by higher parasitemia levels and accelerated mortality (24). These results suggest that more TLRs than TLR2 are involved in the pathogenesis of the disease in mice.

In this work, we describe how GIPL from the G strain, whose lipid moiety is instead composed by a N-lignoceroylsphinganine, requires TLR4 expression for its in vivo proinflammatory activity, as well as for inducing the NF-κB activation in the CHO cell lines. To our knowledge, this is the first example of a protozoan-derived molecule to induce a TLR4-dependent response. Evidence suggests the occurrence of free GIPLs in infective trypomastigote and intracellular amastigote forms of T. cruzi, although they are present in smaller quantities in the blood trypomastigote forms relative to epimastigotes (16–18). Interestingly, the GPI anchor of mucin-like glycoproteins from the infective metacyclic trypomastigote stage has a similar composition of the glycoinositolphospholipid purified from epimastigote forms (eGIPL) G used in this study. It contains the same conserved Man₅GlcN glycan sequence, and the myo-inositol-phosphate-lipid moiety is predominantly (70%) formed by inositol-phosphocheramides (57). The presently described comparison between C3H/HeJ (TLR4 mutant) and C3H/HeN (wild-type) mice strains regardless resistance to T. cruzi infection, revealed a higher susceptibility to infection, both in terms of the course of parasitemia and in terms of mortality, in the TLR4-mutant strain. We propose that GIPL-triggered TLR4 signaling confers the higher resistance observed in the wild-type mice. Directly testing this hypothesis is currently not possible, however, due to the absence of a T. cruzi strain lacking GIPL expression. Nevertheless, by investigating whether the higher susceptibility of C3H/HeJ mice to infection with T. cruzi Y strain is also observed when other T. cruzi strains are used, and by correlating GIPL composition to innate resistance to T. cruzi infection, we should be able to approach a reasonable degree of certainty concerning the validity of this hypothesis.

No discussion of these results could be complete without some speculation concerning the possibility that the immunological response to T. cruzi infection elicited through TLR pathways might have a role in the progression of the disease toward its chronic phase. During this phase, which develops in ~35% of human patients and often occurs years after the infection, inflammatory infiltrates in the hearts and intestines of chagasic patients are observed. In patients with cardiomyopathy, parasite Ags and/or DNA could be found in the affected tissues. Paradoxically, parasitism is correlated relatively poorly with the degree of inflammation during the chronic phase. Although controversial, it has never been excluded that autoimmune events take part in this process (reviewed in Refs. 58–60). In fact, an increasing body of evidences indicates a connection between chronic inflammation and autoimmunity (61–63). Therefore, the study of the involvement of TLR4 and/or TLR2 signaling in experimental models of the chronic phase of the Chagas’ disease could be of considerable value in elucidating the pathophysiology of chronic chagasic cardiomyopathy, which remains one of the major causes of heart failure among younger individuals in Latin America today.

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