

Research Paper

Mouse Macrophage Galactose-type Lectin (mMGL) is Critical for Host Resistance against *Trypanosoma cruzi* Infection

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Abstract

The C-type lectin receptor mMGL is expressed exclusively by myeloid antigen presenting cells (APC) such as dendritic cells (DC) and macrophages (M ϕ), and it mediates binding to glycoproteins carrying terminal galactose and α - or β -N-acetylgalactosamine (Gal/GalNAc) residues. *Trypanosoma cruzi* (*T. cruzi*) expresses large amounts of mucin (TcMUC)-like glycoproteins. Here, we show by lectin-blot that galactose moieties are also expressed on the surface of *T. cruzi*. Male mMGL knockout (-/-) and wild-type (WT) C57BL/6 mice were infected intraperitoneally with 10^4 *T. cruzi* trypomastigotes (Queretaro strain). Following *T. cruzi* infection, mMGL-/- mice developed higher parasitemia and higher mortality rates compared with WT mice. Although hearts from *T. cruzi*-infected WT mice presented few amastigote nests, mMGL-/- mice displayed higher numbers of amastigote nests. Compared with WT, M ϕ from mMGL-/- mice had low production of nitric oxide (NO), interleukin (IL)-12 and tumor necrosis factor (TNF)- α in response to soluble *T. cruzi* antigens (TcAg). Interestingly, upon *in vitro* *T. cruzi* infection, mMGL-/- M ϕ expressed lower levels of MHC-II and TLR-4 and harbored higher numbers of parasites, even when mMGL-/- M ϕ were previously primed with IFN- γ or LPS/IFN- γ . These data suggest that mMGL plays an important role during *T. cruzi* infection, is required for optimal M ϕ activation, and may synergize with TLR-4-induced pathways to produce TNF- α , IL-1 β and NO during the early phase of infection.

Key words: mMGL, *Trypanosoma cruzi*, Proinflammatory cytokines, C-Type lectin receptor, Macrophages receptors.

Introduction

Chagas disease, also known as American trypanosomiasis, is a disease caused by the protozoan parasite *Trypanosoma cruzi*. This parasitic infection affects approximately 7-12 million people in Latin America,

and 60-80 million more are at risk (1, 2). The natural transmission of the *T. cruzi* parasite to humans and other mammals depends on the triatomine insect belonging to the family (Reduviidae), known as the

kissing bug (3). Inside the host, the parasite is internalized in the cells of the innate immune system. After multiplication by binary fission, the amastigotes are transformed into blood trypomastigotes, which are released into the bloodstream by cell lysis. This stage can infect a wide range of host cells or be taken up by the insect vector through its mouthparts, closing the life cycle (4).

Once an individual has acquired the infection, a progressive disease develops. The acute phase is characterized by a high number of trypomastigotes in the blood as well as, fever and hepatomegaly. The chronic phase presents fewer parasites in the blood. Many patients infected with *T. cruzi* remain asymptomatic. However, 10-20 years after the initial infection, 5-10% of people develop anatomical and functional abnormalities in the esophagus and colon, while approximately 30% develop myocarditis, leading to heart failure or sudden death (4).

The outcome of the immune response depends on the capacity of Antigen Presenting Cells (APCs) to sensing foreign molecular configurations, also known as pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs) expressed on the surface of macrophages (M ϕ) or dendritic cells (DC). Activation of PRRs leads to intracellular signals that activate innate immunity and orchestrate the development of an acquired immune response, which is necessary for protection against re-infection (5, 6). Some of these highly specialized receptors include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (6).

In phagocytic cells from both mouse and human, the molecule glycosylphosphatidylinositol (GPI) anchors to *T. cruzi* trypomastigotes, and is recognized as a potent activator of TLR2 (7-10). The glycoinositol-phospholipids (GIPLs) and unmethylated CpG motifs on DNA that are present in all stages of the *T. cruzi* life cycle are recognized by TLR4 (11, 12) and TLR9 (13), respectively. For activation of TLR2 and TLR9, the nonsaturated fatty acid chains and periodate-sensitive components from the GPI anchor covalently linked to mucin-like glycoproteins are required to trigger the production of inflammatory cytokines by APCs (11, 14-17).

In contrast to TLRs, CLRs recognize and internalize specific carbohydrates by lectin-glycan interactions. It has been suggested that CLRs work as mediators of microbial recognition and initiators of immune responses (5). The most important molecules from the CLR family include macrophage galactose type C-lectin (MGL), specific for mannose or fucose dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), the mannose receptor (MR), DEC205, and Dectin-1 (18). Some

of these receptors may function as adhesion, signaling or antigen-uptake receptors (19-21). Moreover, CLRs have been shown to contribute to the loading of endocytosed antigens on MHC class I or class II, thereby facilitating effective antigen-specific CD4⁺ and CD8⁺ T-cell responses (22, 23).

The CLRs are able to trigger distinct signaling pathways that modulate APC functions through the expression of specific molecules and cytokines. In most cases, CLRs promote antigen presentation and determine the polarization of T cells (24-26). However, most evidence about how CLRs shape the immune response and trigger signaling pathways has emerged using viral and bacterial pathogens, fungi or peptides (18). There is no clear evidence that parasites interact with CLRs and activate a specific signaling pathway.

The MGL molecule is a member of the CLR family. Human MGL (hMGL) is a type II C-Type lectin, which is selectively expressed in APCs such as immature dendritic cells (iDC) and M ϕ and is overexpressed in subsets of DCs with tolerogenic functions and alternatively activated M ϕ . MGL recognizes Gal/GalNAc residues of N- and O-glycans carried by glycoproteins and /or glycosphingolipids and it promotes endocytosis (27).

It is well-known that endogenous functions for MGLs include pattern recognition of tumor antigens, foreign glycoproteins derived from helminth parasites, and cleaning of apoptotic cell embryos (28). The mechanisms or pathways used for internalizing carbohydrates by MGL have not been fully described, but has been reported that phagocytic cells are able to endocytose antigens through MGL, which they are transported along the endosomal-lysosomal pathway and presented in MHC-II molecule (29). Mice have two homologous copies of hMGL, MGL1 and MGL2, while humans and rats have only one copy (30, 31). Mouse MGL (mMGL) is a transmembrane glycoprotein of 42KD that recognizes Gal/GalNAc and Lewis X and Lewis A structures (30, 32).

In this work, we investigated the role of mMGL in immune response to an acute *T. cruzi* infection using mMGL knockout mice (mMGL^{-/-}). Our results demonstrate that mMGL plays an important role in *T. cruzi* infection and may be useful to prevent *T. cruzi* invasion and to induce inflammatory cytokines and NO production by macrophages.

Materials and Methods

***Trypanosoma cruzi* lysate antigen.** Epimastigotes of *T. cruzi* that had been maintained at 28°C by sequential culture in a liver infusion tryptose medium (LIT) with 25 mg/l of hemin supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U of penicillin/streptomycin were isolated,

washed three times in PBS, and centrifuged at 20,000 g for 15 min (33). Protease inhibitors were added (0.1 to 2 µg/ml aprotinin, 0.5 to 2 mM EDTA, 1 to 5 mM phenylmethyl fluoride, 1 µg/ml pepstatin, 50 µg/ml TLCK (a-p-tosyl-L-Lysine chloromethyl Ketone) (Sigma, St. Louis, MO), and parasites were sonicated six times for 10 s each at 50 W using a sonic Dismembrator 300 (Fisher). Parasite destruction was confirmed using a microscope. Parasite lysates were then centrifuged at 20,000 g for 30 min to separate the soluble fraction, which was stored at -70°C until use. Total protein content was determined in the soluble fraction (34).

SDS-PAGE and Lectin-Blotting. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and lectin blotting were performed by standard techniques. Briefly, antigen extracts in non-reducing sample buffer were boiled for 5 min at 95°C and separated on 12% polyacrylamide gels at a concentration of 40 µg/well. Separated proteins were transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ, USA) using a Western blotting unit (Bio-Rad). The membrane was blocked overnight at 4°C with 2% (w/v) bovine serum albumin in PBS pH 7.2, washed thoroughly with PBS/Tween 0.1% and incubated with *Artocarpus integrifolia* lectin (Jacalin)-Peroxidase (Sigma-Aldrich), a lectin specific to Gal residues, for 3 h. After washing, bound peroxidase on the membrane was developed with 1: 1000 PBS/ H and diaminobenzidine at a concentration of 2 mg/ml.

Mice. Eight to ten week-old male C57BL/6 mice were purchased from Harlan (México City, Mexico). The generation of mMGL-deficient mice from C57BL/6 mice by a disruption in mMGL exons 2 and 3 has been previously described (35). mMGL^{-/-} mice on a C57BL/6 background were donated by Glycomics Consortium. All mice were genotyped by PCR. Briefly, total DNA was extracted from the tails of the mice (36), and DNA amplification was performed using 1 µg of DNA, oligo(dT) and specific primers for 35 cycles of 30 sec at 65°C. Oligos for the *mMGL* gene were (5'-ATGTCATGACTCAGGATC-3' and 5'-CTTGGTCCCAGATCCGTATC-3'), and for the neomycine gene (Neo), 5'-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3' and 3'-AAGAACTCGTCAAGAAGGCGATAGAAGGCG-5'. A PCR fragment of 633 bp to mMGL and 492 bp to Neo were visualized to identify WT or mMGL^{-/-} mice, respectively. Wild type C57BL/6 purchased from Harlan (México) were used as control mice. All animal studies were performed according to the guidelines for the Care and Use of Laboratory Animals, as adopted by the U.S. National Institutes of Health and the Mexican Regulation of Animal Care and maintenance

(NOM-062-ZOO-1999, 2001).

Parasites and experimental infections. The Mexican *T. cruzi* TBAR/MX/0000/Queretaro strain belonging to DTU TcI was used in this work. Experimental infections of male mMGL^{-/-} and WT mice were induced by intraperitoneal (i.p.) injection with 10⁴ blood trypomastigotes that were obtained from previously infected mice, counted, and suspended in 100 µl of sterile phosphate-buffered saline (PBS). Parasitemia was determined every week by using hemocytometer counts of parasites in the blood diluted 1:10 in PBS with 3.8% sodium citrate.

Histopathology. Non-infected and *T. cruzi* infected hearts from mMGL^{-/-} and WT mice were fixed overnight in formaldehyde and embedded in paraffin blocks, after which 5-µ-thick transverse sections were mounted on slides, stained with hematoxylin and eosin (H&E), and scored as previously described (37). The presence of inflammatory cells was scored as (0) - absent/none, (1) - focal or mild with ≤1 foci, (2) - moderate with ≥2 inflammatory foci, (3) - extensive with generalized coalescing of inflammatory foci or disseminated inflammation and (4) - severe with dif-fused inflammation, interstitial edema, and loss of tissue integrity. The foci of pseudocysts (Tc nests) were scored as (0) absent, (1) 0-1 foci, (2) 1-5 foci, and (3) 5 foci. Using an Olympus BX51 microscope (Olympus American, Melville, NY) equipped with a digital video camera, 4 mice per group with 10-slides per mouse were evaluated for each group.

Isolation of macrophages and activation. Peritoneal exudate cells (PECs) were obtained from the peritoneal cavity at 0, 21, 28 and 35 days post *T. cruzi* infection of mMGL^{-/-} and WT mice under sterile conditions using 10 ml of ice-cold Hank's balanced salt solution (Microlab, México). Following two washes with Hank's solution, red blood cells were lysed by resuspending the cells in Boyle's solution. After two washes, viable cells were counted by trypan blue exclusion (routinely over 95%) with a Neubauer hemocytometer. PECs were adjusted to 5x10⁶ cells/ml in DMEM medium supplemented with 10% fetal calf serum (FCS), 100 U of penicillin/streptomycin, and 2 mM glutamine (all from Gibco-BRL, Grand Island, NY) and were cultured in 24-well plates (Costar, Cambridge, MA, USA). After 2 hours at 37°C and 5% CO₂, non-adherent cells were removed by washing with warm supplemented DMEM medium. Adherent cells (Mφ) were removed from the plate by washing with 5 mM EDTA in warm PBS and were then read-justed to 1x10⁶ cells/ml. Viability was checked again at this point (>90%), and samples were analyzed by FACS using the macrophage marker F4/80 (Mφ pu-rity was estimated as >85%). One milliliter of Mφ was plated on 12-well plates (Costar), left untreated or

stimulated with LPS (0.5 µg/ml; Escherichia coli 0111:B4 Sigma-Aldrich), or Poly:IC (25 µg/ml; Polyinosinic-polycytidylic acid potassium salt), or 25 µg/ml of total *T. cruzi* antigen (TcAg). Mφ were incubated for 48 hours at 37°C and 5% CO₂. Supernatants were collected for quantification of cytokine production. Cytokine levels (IL-12, IFN-γ, TNF-α, IL-10, IL-4, and IL-13) were measured using the sandwich ELISA method according to the manufacturer's instructions (Preprotech, México).

Reverse transcriptase-PCR. Total RNA was extracted from Mφ obtained as described above from untreated or *in vitro* *T. cruzi* infected (21 days after infection) WT or mMGL^{-/-} mice, using the TRIzol reagent (Sigma). cDNA was prepared using a first strand synthesis superscript II kit (Invitrogen) from 5 µg of total RNA. cDNA samples were standardized based on the content of the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) cDNA. The primers for GAPDH were F-CTC ATG ACC ACA GTC CAT GC and R-CAC ATT GGG GGT AGG AAC AC (201 bp). The primers for Arginase-1 were F-CAG AAG AAT GGA AGA GTC AG and R-CAG ATA TGC AGG GAG TCA CC (250 bp). The primers for Ym1 were F-TCA CAG GTC TGG CAA TTC TTC TG and R-TTT GTC CTT AGG AGG GCT TCC TC (436 bp). The primers for TNF-α were F-GGC AGG TCT ACT TTG GAG TCA TTG C and R-ACA TTC GAG GCT CCA GTG AAT TCG (307 bp). The primers for inducible nitric oxide synthase were F-CTG GAG GAG CTC CTG CCT CAT G and R-GCA GCA TCC CCT CTG ATG GTG (449 bp). The primers for IL-1β were F-GAG TGT GGA TCC CAA GCA AT and R-CTC AGT GCA GGC TAT GAC CA (500 bp). The primers for TLR-4 were ACC TGG CTG GTT TAC ACG TC and R-CTG CCA GAG ACA TTG CAG AA (201 bp). The primers for TLR-3 were F-CCC CCT TTG AAC TCC TCT TC and R-TTT CGG CTT CTT TTG ATG CT. The primers for TLR-2 were F-AAG AGG AAG CCC AAG AAA GC and R-CGA TGG AAT CGA TGA TGT TG (199 bp). Polymerase chain reaction (PCR) was performed in a total volume of 50 µL in PCR buffer in the presence of 10 mM dNTPs, 15 pM each primer, and 1.5 U of kappa TaqDNA polymerase (kapabiosystems Boston M.A, USA) using a XP-cycler (Bioer, Switzerland). After 35 cycles of amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

***In vitro* T. cruzi infection and flow cytometry analysis of macrophages.** One milliliter (1x10⁶) of Mφ obtained as described above from WT or mMGL^{-/-} mice was plated, and infected *in vitro* with epimastigotes of *T. cruzi* (ratio 1: 10) for two hours. Following infection, parasites were washed off. Mφ were

incubated at 4°C for 15 minutes in blocking buffer and 2% FCS, 10 µg/ml anti-CD16/32 in FACS buffer (PBS supplemented with 2mM EDTA and 0.5% BSA), followed by staining for 20 minutes on ice with the antibodies (Ab) of interest at the appropriate dilution as determined by titration. Abs included APC-conjugated anti-F4/80, FITC-conjugated MHC-II and PE-conjugated TLR-4 as well as appropriate isotype control Abs (all Abs from Biolegend, San Diego, CA, USA).

For infection analysis; 1x10⁶ Mφ obtained from WT or mMGL^{-/-} mice were plated, and infected *in vitro* with epimastigotes of *T. cruzi* (ratio 1: 10) for two hours. For infection analysis, epimastigotes of *T. cruzi* were washed in PBS and resuspended at 1x10⁷/ml in 5 mM carboxyfluorescein succinimidyl ester (CFSE) in serum-free DMEM for 15 min at 37°C. Following infection, parasites were washed off, and Mφ infected and not infected were incubated in DMEM medium supplemented with 10% FCS for 2, 6, 12 or 24 hours at 37°C and 5% CO₂.

For infection analysis on activated Mφ, 1x10⁶ thioglycollate-elicited Mφ were left untreated or treated for 24 h with IFN-γ or IFN-γ/LPS. Cells were washed and infected with CFSE labeled epimastigotes of *T. cruzi* for 2 h (10:1 parasite to Mφ ratio), after this the parasites were washed off.

All cells described in this section were washed 3x in FACS buffer and fixed in 0.8% paraformaldehyde before acquisition and analysis (BD FACStation and FlowJo software).

Statistical analysis. Comparisons between WT and mMGL^{-/-} groups were made by using Student's unpaired *t* test. P values of < 0.05 were considered significant. For survival assays, a log-rank test was used with the Graph Pad computer program (Graph Pad 6, San Diego, CA).

Results

Intact carbohydrates on total antigens of *T. cruzi* are rich in glycoproteins bearing galactose residues. Given that *T. cruzi* parasites are rich in carbohydrates (38), we developed a PAGE (Fig. 1A) and lectin-blot analysis (Fig. 1B) to determine whether the *T. cruzi* Queretaro strain contained glycoproteins bearing terminal Gal and GalNAc sugars, which are recognized by mMGL with high affinity. According to the lectin-blot analysis (Fig. 1B) the total soluble antigens of *T. cruzi* were highly recognized by Jacalin lectin, indicating the presence of Gal residues, similar to other reports with different *T. cruzi* strains (39).

mMGL^{-/-} mice develop high parasitemia levels and increased mortality to *T. cruzi* infection. To investigate the role of mMGL in immunity to *T. cruzi*, mMGL^{-/-} and WT mice were i.p. infected with 10⁴

metacyclic trypomastigotes of *T. cruzi*, and parasitemia was monitored weekly. *T. cruzi*-infected mMGL^{-/-} mice developed significantly higher blood parasitemia levels than similarly infected WT mice, which contained nearly four-fold fewer parasites in their blood by days 21 to 35 post infection (pi) (Fig. 2A, $P < 0.05$). Both groups developed blood parasitemia on day 14 pi, but on day 28 pi, mMGL^{-/-} mice displayed significantly greater levels of parasitemia, which peaked at day 28 pi and was positively detected until day 42 pi (Fig. 2A). In contrast, the maximum peak of parasitemia in WT mice was observed on day 21 pi, and these mice controlled the infection by day 42. Furthermore, mMGL^{-/-} mice succumbed to *T. cruzi* infection as early as day 23 pi, and only 37% of them survived to day 42, whereas 80% of the WT mice survived throughout the infection (Fig. 2B).

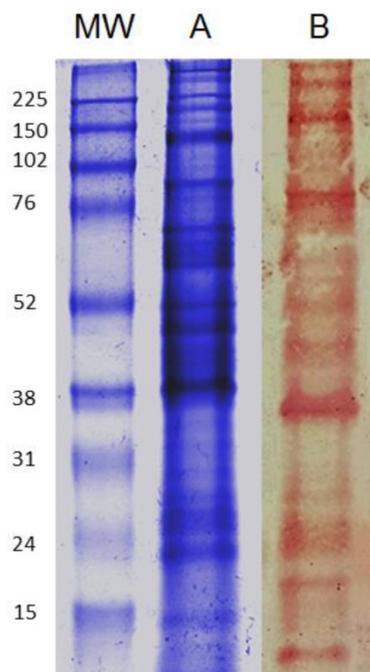


Figure 1. Total *T. cruzi* antigen is rich in glycoproteins bearing galactose (Gal) and N-acetylgalactosamine (GALNAc). TcAg were separated by SDS-PAGE and transferred to NC sheets that were used to detect N-linked glycans with horseradish peroxidase (HRP)-conjugated Jacalin. *T. cruzi* antigen lectin blood, SDS-page A); Jacalin-Blot B). Antigens were from different infected mice. MW indicates the molecular weight markers in kilodaltons (kDa).

***T. cruzi*-infected mMGL^{-/-} mice develop severe heart pathology.** The heart is one of the main target organs affected by *T. cruzi* infection, therefore we evaluated whether the higher mortality of mMGL^{-/-} mice was associated with heart damage. We found that on day 21 pi, the hearts of WT mice showed low tissue parasitism with moderate inflammatory mononuclear cell infiltration and no major histopathological signs of lesions (Fig. 3A at 21 and 28 days post infection -dpi and 3B). In contrast, heart histopathology sections from infected mMGL^{-/-} mice showed an

intense inflammatory reaction (Fig. 3A at 21 and 28 dpi and 3B), with a marked increase in amastigote nests (Fig. 3C) accompanied by severe heart injury due to large necrotic lesions. These results suggest that mMGL^{-/-} mice succumb to the classical pathophysiology of the infection, which is typical of the severe acute phase of experimental Chagas' disease.

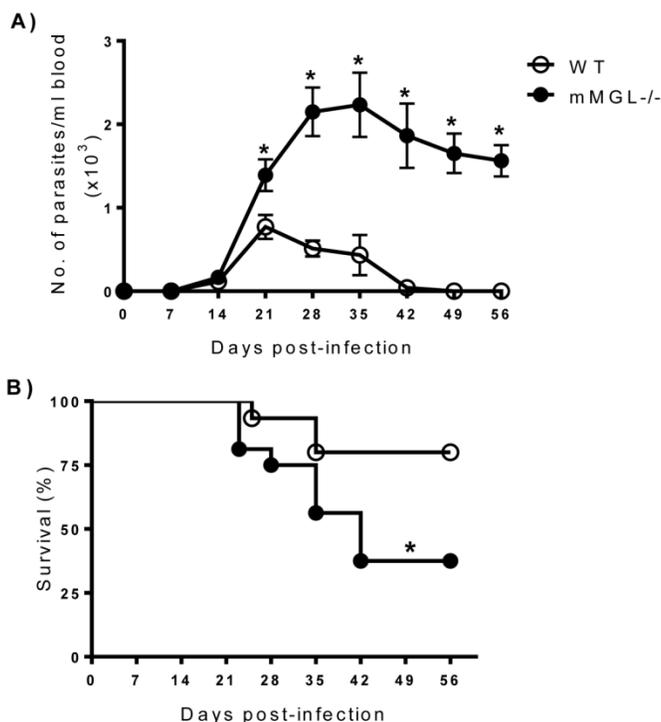


Figure 2. mMGL^{-/-} mice succumb to acute *T. cruzi* infection. WT and mMGL^{-/-} mice were infected with 10^4 *T. cruzi* parasites. Parasitemia A), data shown represent the mean \pm SE of at least 21 mice per data point corresponding to three independent experiments (7 mice per experiment). Survival rate B), data shown represent the mean \pm SE of 15 mice corresponding to three independent experiments (5 mice per experiment). Both experimental groups were monitored every week until seven weeks p.i. * $P < 0.05$ with respect to WT. Student's t test, and log rank test for parasite load and survival were used respectively (Graph Pad Prism 6).

mMGL^{-/-} M ϕ from *T. cruzi* infected-mice have impaired pro-inflammatory cytokine expression. Given that the inflammatory immune response is a well-defined mechanism to control early *T. cruzi* dissemination (16, 40), we decided investigate the pro-inflammatory profile of M ϕ obtained from mMGL^{-/-} and WT mice during acute *T. cruzi* infection. Peritoneal M ϕ from uninfected and *T. cruzi* infected mice at 21 and 35 dpi were left untreated or treated for 48 hours with LPS, POLY:IC or TcAg, and supernatants were recovered for cytokine detection by ELISA. As expected, uninfected M ϕ (day 0) from mMGL^{-/-} or WT mice showed similar levels of NO, TNF- α , IL-12 and IL-10 in response to all stimuli (Fig. 4A-D). As the infection progressed, mMGL^{-/-} M ϕ left untreated exhibited lower levels of NO at 21 and 35 dpi (Fig. 4A) and lower levels of TNF- α , IL-12 and

IL-10 at day 21 post-infection, compared with their similar WT Mφ (Figure 4A).

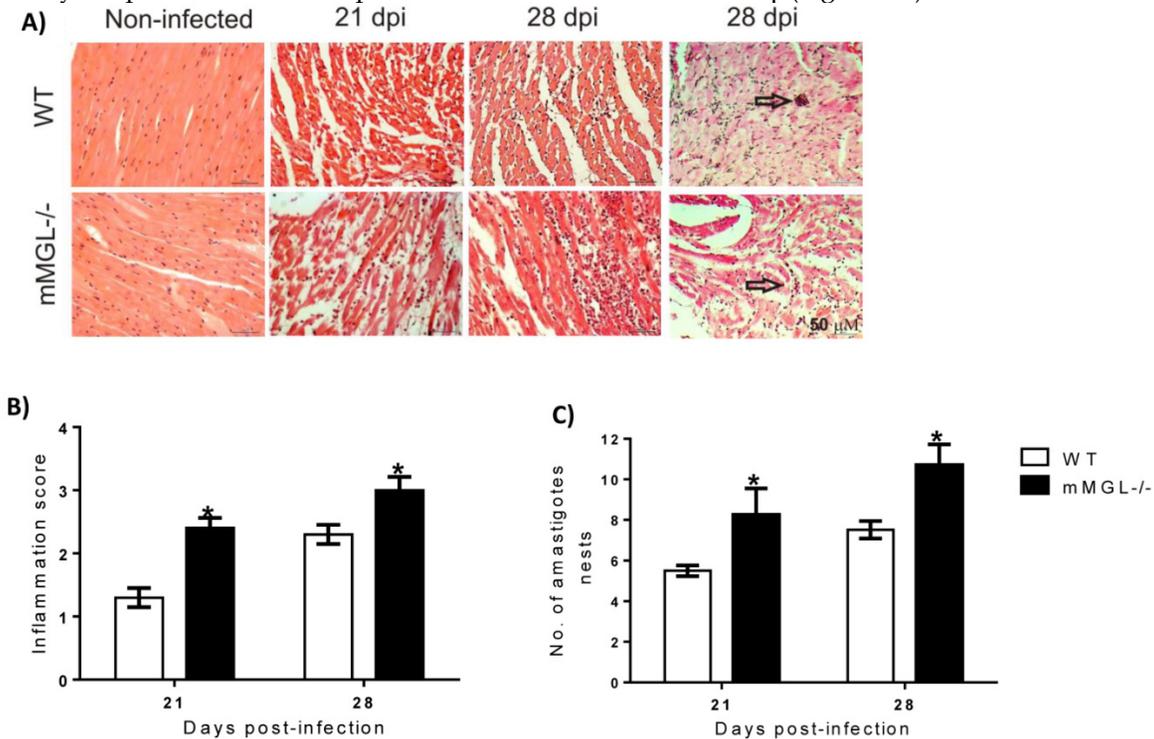


Figure 3. Hearts from mMGL^{-/-} mice display higher *T. cruzi* parasitism and inflammation compared with WT mice. Histology of hearts post-*T. cruzi* infection A). Quantitative scoring of heart inflammation from H&E-stained tissue sections B). Number of amastigote nests in 25 histopathological fields C). Representative H&E images of heart tissue sections shown myocyte fibers cut in longitudinal (blue: nuclear, pink: muscle/cytoplasm/keratin) (arrows indicate parasite nests) (magnification: 40X, Olympus BX51 microscope). Data are expressed as the means ±SE of the means (n 4 to 5 animals per group). Mice were killed at 0, 21 and 28 days p.i. Arrows point to parasite nests. *P<0.05 with respect to WT values obtained the same day. Student's t test was used (Graph Pad Prism 6).

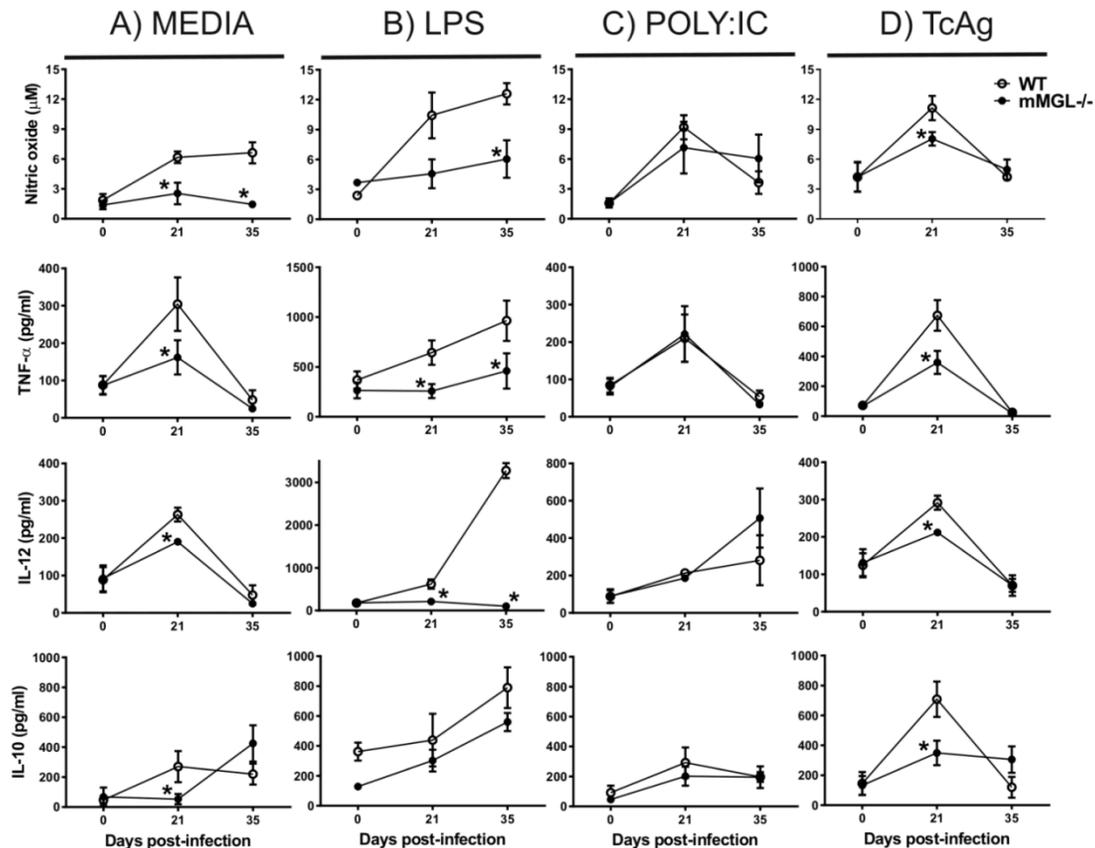


Figure 4. NO₂⁻, TNF-α, and IL-12 production are reduced in response to LPS or TcAg in mMGL^{-/-} Mφ compared with WT Mφ. Naive or infected Mφ from WT or mMGL^{-/-} mice were recovered after 21, 28 and 35 days post *T. cruzi* infection. Mφ were left untreated or treated for 48 hours with LPS (0.5 μg/ml), POLY: IC (25 μg/ml) or TcAg (25 μg/ml) as indicated.

Supernatants were recovered and the levels of IL-12 A), TNF- α B), IFN- γ C) were measured by ELISA sandwich. Results are shown as the means of replicate samples \pm SEM and are representative of three experiments. * $P < 0.05$ by Student's *t* test (GraphPad Prism 6).

LPS is considered a Th1 activating signal that favors inflammatory cytokine production by M ϕ (41). Here, we show that in response to LPS, mMGL-/- M ϕ from 21 or 35 days after infection displayed impaired production of NO, TNF- α and IL-12 but not IL-10 compared with WT M ϕ (Fig. 4B). In response to TcAg, mMGL-/- M ϕ produced lower levels of NO, IL-12 and IL-10 only at 21 dpi (Fig. 4D), whereas no differences were observed with POLY:IC stimulation (Fig. 4C). These results indicate that mMGL-/- M ϕ coming from *T. cruzi* infected animals may be less activated, or may display an alternative activation M ϕ (AAM ϕ) phenotype.

mMGL-/- M ϕ from 21 days post-*T. cruzi* infection display a defect in their activation more than a phenotype switch. Signals encountered by developing M ϕ during migration determine the development of highly divergent M ϕ phenotypes with specific functional properties at the site of inflammation or infection (42). Classically activated (CA)M ϕ , activated by Th1-type signals such as IFN- γ , produce high levels of nitric oxide (NO), enhancing antimicrobial and cytotoxic properties (43, 44). AAM ϕ are dependent on the products of activated Th2 cells, such as IL-4 and IL-13, and play important roles in allergy and the response to parasitic infection (45, 46). AAM ϕ express secretory lectin Ym-1 and Arginase-1 (Arg-1) over NO. Recently, mMGL has also been considered a marker for AAM ϕ elicited on M ϕ and DCs during infection with *Trypanosoma brucei* or *Taenia crassiceps* (47, 48). In contrast, CAM ϕ produce high levels of NO from inducible nitric oxide synthase (iNOS), and they do not express Ym1 and Arg-1(41).

In order to determine whether the impaired inflammatory cytokine production in mMGL-/- M ϕ was due to a phenotype switch from CAM ϕ to AAM ϕ , we analyzed the gene expression of Arg-1, Ym-1 and iNOS as M ϕ phenotype markers, TNF- α and IL-1 β as inflammatory markers, and TLR-2, TLR-3 and TLR-4 as activation markers. Peritoneal M ϕ recovered from mMGL-/- and WT mice 21 days after infection and M ϕ from non-infected mice were left untreated (media) or treated overnight with LPS, POLY:IC or TcAg for gene expression analysis by RT-PCR. We found that non-infected mMGL-/- M ϕ showed high Arg-1, Ym-1 and TLR3 expression in response to LPS (Fig. 5 B, C and H). High Arg-1 and TLR3 expression was observed in response to TcAg (Fig. 5 B, H), but similar mRNA levels of TNF- α , iNOS, IL-1 β , TLR-4 and TLR-2 in response to LPS compared with WT-M ϕ (Fig. 5 D, E, F, G and I). No or low mRNA expression of Ym-1, IL-1 β and TLR-4 was ob-

served in response to TcAg compared with WT-M ϕ (Fig. 5 C, F and G).

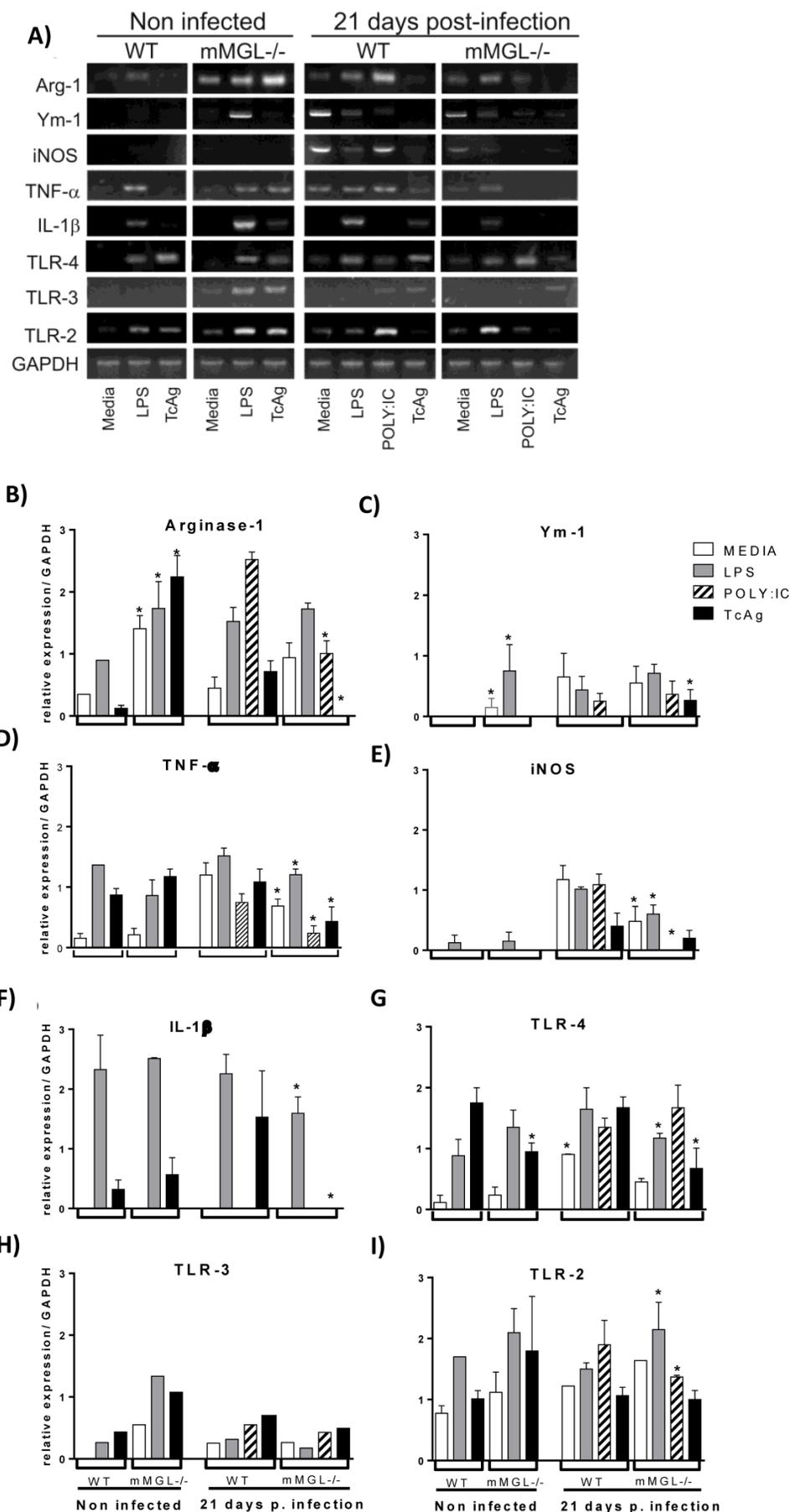
Untreated (media) mMGL-/- M ϕ from 21 dpi showed similar expression of Arg-1, Ym-1, IL-1 β , TLR3 and TLR2 (Fig. 5 B, C, F, H, and I -media) but low expression of iNOS, TNF- α and TLR-4 in these basal condition (Fig 5 D, E, G -media). In response to LPS, 21 day post-infection M ϕ showed similar mRNA expression of Arg-1 and Ym-1 (Fig 5B, C) but low mRNA expression of iNOS, TNF- α , IL-1 β and TLR-4 (Fig. 5 D, E, F, G). In response to POLY:IC, low mRNA expression of Arg-1, iNOS and TNF- α was observed (Fig. 5B, E, D, F and G -21 dpi). In response to TcAg, mMGL-/- M ϕ showed high Ym-1 expression (Fig. 5C -21 dpi), similar levels of iNOS, TLR3 and TLR-2 (Fig. 5E, H and I -21 dpi), and no or low levels of Arg-1, TNF- α , IL-1 β and TLR-4 compared with WT M ϕ (Fig. 5B, D, F, and G. -21 dpi).

mMGL-/- M ϕ display deficiencies in MHC-II and TLR-4 cell surface activation markers during *in vitro* infection with *T. cruzi*. In order to rule out the possibility that altered activation status could be present in mMGL-/- M ϕ , we studied the surface expression of MHC class II (MHC-II), co-stimulatory molecules CD80 (B7.1), CD86 (B7.2) and TLR-4. Peritoneal naïve M ϕ from WT or mMGL-/- mice were infected *in vitro* with trypomastigotes of *T. cruzi* at a ratio of 1:10 and marker expression was monitored in uninfected M ϕ (0 hpi) and *T. cruzi*-infected M ϕ at 2, 6, 12 and 24 hpi. Uninfected mMGL-/- M ϕ expressed similar levels of MHC-II (Fig. 6A, 0 hpi), CD80 (data not shown), CD86 (data not-shown) and TLR-4 (Fig. 6B, 0 hpi).

Upon *T. cruzi* infection, the percentage of all markers was increased in both mMGL-/- and WT-M ϕ from two hours until 24 hpi. Similar expression levels of CD80 and CD86 were observed, but significantly less expression of MHC-II and TLR-4 was observed on mMGL-/- M ϕ at 2, 6, 12 and 24 hpi compared with WT M ϕ (Fig. 6 A and B, respectively).

Next, we asked whether on the altered activation of mMGL-/- M ϕ could reflect their ability to control *T. cruzi* infection. To address this question, *T. cruzi* epimastigotes were labeled with CFSE and used to infect M ϕ from healthy mMGL-/- and WT mice *in vitro*, then M ϕ were recovered and analyzed by flow cytometry. This allowed the comparison of parasite uptake between mMGL-/- and WT M ϕ . Interestingly mMGL-/- M ϕ harbored significantly more parasites after 2, 6, 12 and 24 hours post-infection (hpi) (Fig. 7 A) than their WT counterparts, which suggested that the presence of mMGL is important in delaying the entry of parasites into the host cell.

Figure 5. Mφ Phenotype from WT and mMGL-/- 21 days post-T. cruzi infection. Naive or infected Mφ from WT or mMGL-/- mice were recovered 21 days after *T. cruzi* infection. Mφ were left untreated or treated for 24 hours with LPS (5 μg/ml), POLY:IC (25 μg/ml) or TcAg (25 μg/ml), as indicated. The cells were recovered for RNA expression analysis A) of Arginase I B) Ym1 C), TNF-α D), iNOS E), IL-1β F), TLR-4 G), TLR-3 H) and TLR-2 I) by RT-PCR. Results are shown as the mean of triplicate samples (+/- S.E.M.) and are representative of three independent experiments. *P<0.05 by Student's t test (GraphPad Prism 6).



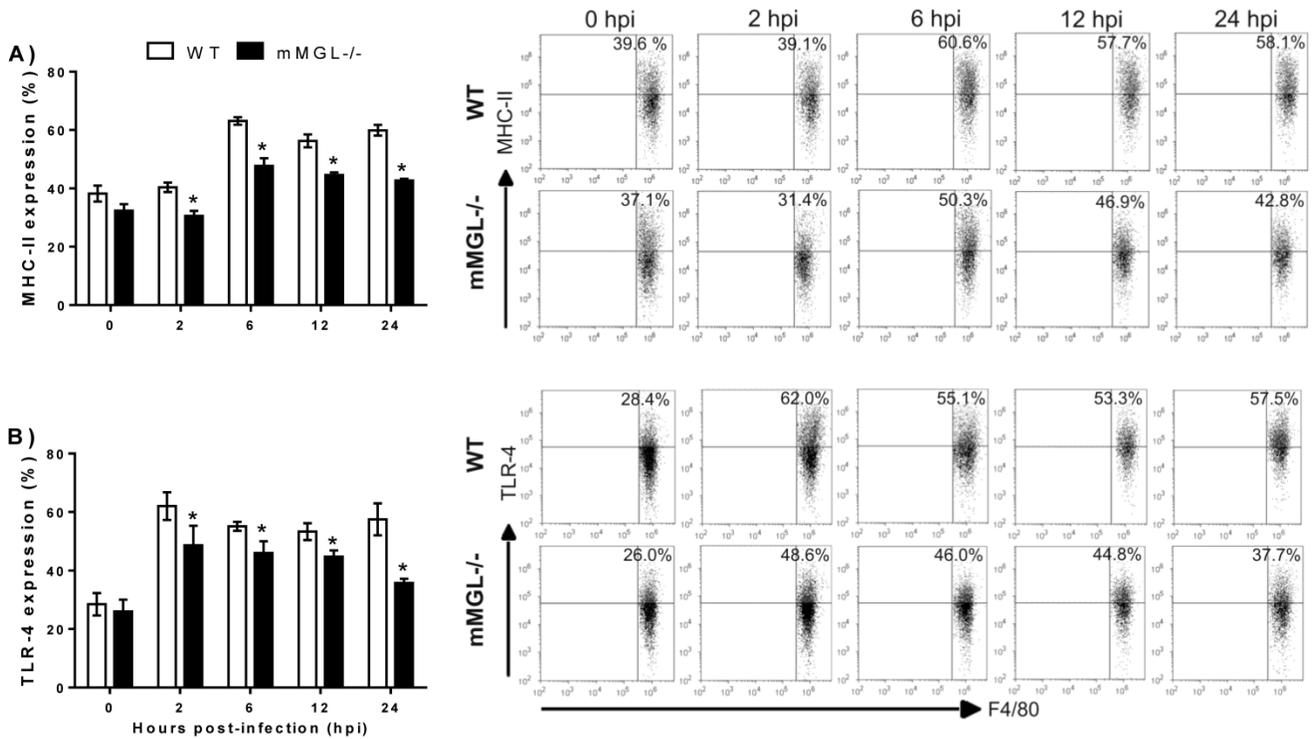


Figure 6. Effect of *T. cruzi* infection on the cell surface activation markers of WT and mMGL^{-/-} Mφ. Naive Mφ from WT or mMGL^{-/-} mice were infected *in vitro* with trypomastigotes of *T. cruzi* at 10:1 parasite to Mφ ratio. The cells were recovered and double-stained for F4/80 and MHC-II (A), and TLR-4 (B). Flow cytometry graphs show dot plots of F4/80-gated Mφ. Results are representative of three experiments. *P<0.05 by Student's t test (GraphPad Prism 6).

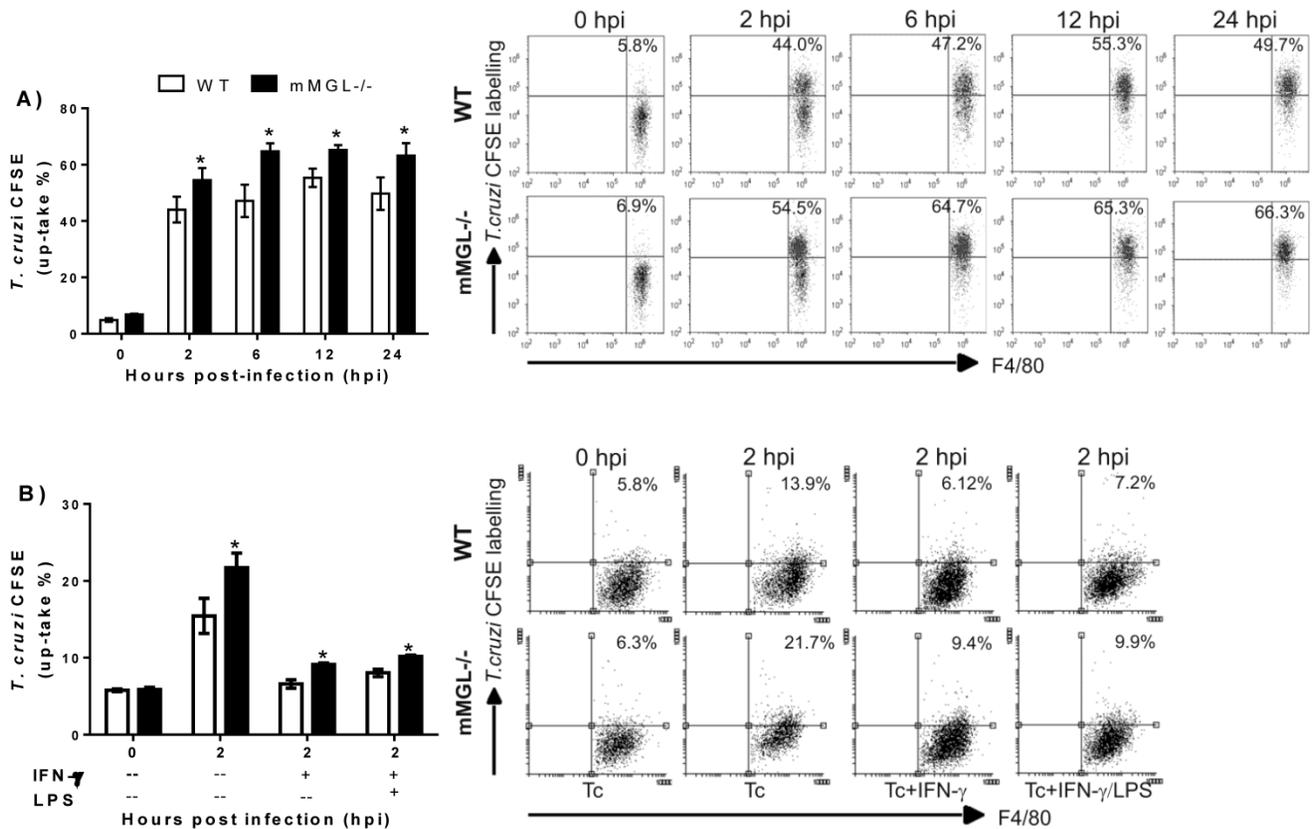


Figure 7. Untreated, IFN-γ and LPS/IFN-γ primed mMGL^{-/-} Mφ take up most *Trypanosoma cruzi* parasites. Peritoneal derived WT or mMGL^{-/-} Mφ were untreated (A) or treated 24 h with IFN-γ or LPS/IFN-γ (B), followed by infection with *T. cruzi* at 10:1 parasite to Mφ ratio. *T. cruzi*-CFSE-labelling that were not taken up were washed off 2 hours post infection, and parasite uptake determined by flow cytometry at 0, 2, 6 and 12 hours post infection (A), or 0 and 2 hours post infection (B). Flow cytometry graphs show dot plots of F4/80-gated Mφ. Results are representative of three experiments to A) and two experiments to B). *P<0.05 by Student's t test (GraphPad Prism 6).

To investigate whether the Th1 activating signals, like IFN- γ or IFN- γ /LPS could alter the mMGL-/- M ϕ activation. Thioglycollate-elicited M ϕ mMGL-/- and WT were left untreated or treated 24 h with IFN- γ or LPS/IFN- γ , infected with CFSE labeled epimastigotes of *T. cruzi* and analyzed by cytometry. As expected, both stimulus IFN- γ and LPS/IFN- γ act to reduce the parasite uptake on WT as mMGL-/- M ϕ . However, this effect was significantly lesser on mMGL-/- M ϕ , which showed higher number of parasites compared with WT M ϕ (Fig. 7 B). This result demonstrates that mMGL is important to IFN- γ or LPS/IFN- γ stimulus works to activate efficiently M ϕ .

Discussion

New evidence has recently emerged about the role of CLRs in modulating the activation and function of APC (18, 49, 50). Some of these studies suggest that MGL improves DC antigen presentation and triggers distinct signaling pathways that regulate APC functions through the expression of specific molecules and cytokines that modulate the innate and adaptive immune response (24, 51). This hypothesis has been driven by the knowledge that MGL works as a molecular target for Gal/GalNAc or Tn-carrying tumor-associated antigens on DCs (24, 25, 52) and is supported by subsequent data showing that mMGL recognizes Gal/GalNAc residues carried by glycoproteins and/or glycosphingolipids present on the helminth *Schistosoma mansoni* inducing phagocytosis, endocytosis, enhancing parasite glycopeptide presentation and promoting the polarization to Th2 responses (27, 53). Despite evidence pointing to a role for MGL in the immune response to these parasites, the function of MGL in the host defense against protozoa or other helminth parasites rich in Gal/GalNAc residues has not been determined. Therefore, in this study, we evaluated the *in vivo* role of mMGL in *T. cruzi* infection using MGL-/- and WT mice.

First, we demonstrated by Jacalin-Peroxidase lectin blotting that a pathogenic *T. cruzi* strain (Queretaro) has Gal residues on its membrane antigens that can be recognized by mMGL receptors on APCs. This observation is in line with previous reports from other *T. cruzi* strains which demonstrated that this parasite displays highly glycosylated membrane proteins related to the invasion of host cells through recognition by CLRs (31, 54), mainly DEC205 and SIGN (18, 55). However, the role of CLRs *in vivo* has not been determined. Here, we used mMGL-/- mice to demonstrate for the first time that the CLR mMGL plays a critical role in the recognition of and resistance to a protozoan infection. We showed that mMGL-/- mice are highly susceptible to experimental *T. cruzi* infection, harboring prolonged and higher

parasitemia levels, severe cardiac immunopathology and increased mortality. These findings indicate that mMGL plays a major role in the host defense against acute *T. cruzi* infection.

M ϕ , specifically CAM ϕ , mediate microbial destruction and play an important role in controlling parasite replication during the acute phase of *T. cruzi* infection by enhancing their microbicidal activity by increasing NO and the proinflammatory cytokines IL-12, TNF- α and MIF (56, 57). We showed that the peritoneal M ϕ population generated at 21 days after *T. cruzi* infection in mMGL-/- mice produced significantly less NO, TNF- α , IL-12 and IL-10. Moreover, this M ϕ population did not respond as expected to *ex vivo* proinflammatory stimuli (LPS) or to TcAg, but they did respond efficiently to POLY:IC. The lack of proinflammatory cytokines and NO production in early infection in response to *ex vivo* stimuli with LPS or TcAg in mMGL-/- M ϕ could be explained by the following: 1) The development of different M ϕ phenotypes in mMGL-/- compared with WT infected *T. cruzi* mice. However, this possibility was discarded because we did not observe a phenotype switch in mMGL-/- M ϕ . 2) Inadequate M ϕ activation that could be dependent on a surface receptor because with POLY:IC stimuli, a ligand to intracellular TLR-3, the mMGL-/- M ϕ produced similar levels of cytokines compared with WT M ϕ . Thus, mMGL may be required for the efficient production of NO, TNF- α , IL-12 and IL-10 in innate immunity against *T. cruzi* infection.

These observations suggest there was no clear phenotype switch in mMGL-/- M ϕ toward an AAM profile. However, our data may reflect a less mature activation state in mMGL-/- M ϕ , independently of whether the M ϕ come from *T. cruzi* infected mice, which suggests that mMGL-/- M ϕ may require signaling through a pathogen recognition receptor such as mMGL before becoming responsive to LPS or TcAg.

The current model states that ligand binding to CLRs elicits signaling cascades that modulate immune responses. Some CLRs, such as Dectin-1 and CLEC9A/DNGR-1, clearly promote immunity through ITAM-like motifs within their cytoplasmic tails, leading to the production of several inflammatory cytokines (49). Strikingly, DC-SIGN and DCIR do not seem to act individually, as their signaling pathways require co-triggering of a TLR molecule for their effects to become apparent. Also, MGL induces IL-10 secretion after antibody crosslinking stimulation (58) and has been reported to trigger the phosphorylation of extracellular signal-regulated kinase 1, 2 (ERK1, 2) and nuclear factor- κ B activation. However, this finding has not been tested with "natural ligands" but

with strong activation such as direct antibody stimulation.

On the other hand, MGL engagement improved DC performance as antigen-presenting cells, promoting the up-regulation of maturation markers (HLAII-DR, CD83, CD86, CD40), enhancing motility, and increasing antigen-specific CD8⁺ activation (25). Recently, the capacity of MGL to modulate TLR-2 signaling has been reported (51). In accordance with this, we showed that upon *in vitro* *T. cruzi* infection, mMGL^{-/-} Mφ displayed significantly less expression of MHC-II and TLR-4. This is consistent with previous studies showing that MCH-II and TLR-4 expression is essential to develop “activated” Mφ with the ability to kill *T. cruzi* parasites (10). Because TLR4-mediated responses to *T. cruzi* have been reported as one of the main pathways for inducing early cytokine production (12), the low expression levels of TLR-4 on mMGL^{-/-} Mφ may explain the low proinflammatory cytokine production by these cells. It is possible that mMGL engagement may couple to TLR-4 signal transduction for increased TNF-α and IL-1β secretion by Mφ during *T. cruzi* infection. Consistent with this, mMGL^{-/-} Mφ were infected more than WT Mφ. This defect on mMGL^{-/-} Mφ was persistent even when mMGL^{-/-} Mφ were previously primed with IFN-γ or LPS/IFN-γ. The ability of WT Mφ under IFN-γ or IFN-γ/LPS stimulation are able to control parasite number more effectively than mMGL^{-/-} Mφ may be explained by signals that WT Mφ encounter with *T. cruzi* or IFN-γ/LPS causing the upregulation of TLR4. Interestingly, no changes were observed in CD80, CD86, TLR-3 and TLR-2 expression, which suggests that the role of mMGL in upregulating costimulatory molecules may depend on the type of triggering ligand and the time of exposure.

These results, together with the finding that mMGL^{-/-} mice were more susceptible to *T. cruzi* infection, suggest that mMGL is required for optimal Mφ activation and may synergize with TLR-4-induced pathways to produce TNF-α, IL-1β and NO during the early phase of *T. cruzi* infection. These observations contribute to the understanding of the inflammatory properties of the mMGL molecule, pointing to its potential role as an important modulator of the immune response during *T. cruzi* infection, and perhaps in other parasitic diseases. Moreover, mMGL may act as a CLR that plays a critical role in determining the quality of the adaptive immune response to this parasite. A better characterization of the effects of mMGL on APCs involved in innate and adaptive immunity in response to parasitic diseases is therefore of great interest.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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