Toxoplasma gondii microneme proteins 1 and 4 bind to Toll-like receptors 2 and 4 N-glycans triggering innate immune response

Aline Sardinha-Silva[1], Flávia C. Mendonça-Natividade[1], Camila F. Pinzan[1], Carla D. Lopes[1], Diego L. Costa[2], Damien Jacot[3], Fabricio F. Fernandes[1], André L. V. Zorzetto-Fernandes[1], Nicholas J. Gay[4], Alan Sher[2], Dragana Jankovic[2], Dominique Soldati-Favre[3], Michael E. Grigg[5], Maria Cristina Roque-Barreira[1*]

[1] Department of Cell and Molecular Biology and Pathogenic Bioagents, Ribeirão Preto Medical School, University of São Paulo- USP (FMRP/USP), Ribeirão Preto, São Paulo, 14049-900, Brazil

[2] Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[3] Department of Microbiology and Molecular Medicine, CMU, University of Geneva, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

[4] Department of Biochemistry, Cambridge University, 80 Tennis Court Road Cambridge CB2 1GA, United Kingdom

[5] Molecular Parasitology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

*Corresponding author:
Maria Cristina Roque-Barreira, Phone: +55-16-3315-3062, e-mail: mcrbarre@fmrp.usp.br

ABSTRACT

The infection of the host cell with *Toxoplasma gondii* involves the regulated secretion of microneme proteins (TgMICs). The complex formed by TgMIC1/4/6 on the *T. gondii* surface participates in the adhesion and invasion processes. Here, we show that TgMIC1- and TgMIC4-stimulated dendritic cells and macrophages produce proinflammatory cytokines through TLR2 and TLR4 signalling. This process depends on sugar recognition, since it was shown to be inhibited by point mutations introduced in the TgMIC1 and TgMIC4 carbohydrate-recognition domains. HEK cells transfected with TLR2 glycomutants were selectively unresponsive to TgMICs. Following parasite infection, phagocytes lacking TLR2 and TLR4 failed to generate an early IL-12 response in contrast to wild type cells. Moreover, TgMIC1-KO and TgMIC1/TgMIC4-DKO parasites stimulated a lower IL-12 response than wild type parasites. Together, our data reveal that TgMIC1 and TgMIC4 interact physically with TLR2 and TLR4 N-glycans to trigger an early IL-12 response to *T. gondii*, which may contribute to acute control of infection.

AUTHOR SUMMARY

Toxoplasmosis is caused by the protozoan *Toxoplasma gondii*, of the Apicomplexa phylum. It comprises important parasites able to infect a broad diversity of animals, including humans. A particularity of *T. gondii* is the ability to invade all nucleated cells of its hosts through an active process, which depends on the expression of surface adhesion proteins. These proteins are secreted by specialized organelles localized in the parasite apical region, such as micronemes. We show, in this study, that two of the microneme proteins from *T. gondii* play a function that goes beyond the adhesion activity. These microneme proteins denoted TgMIC1 and TgMIC4 have the

ability to recognize sugars. Through this property, they bind to sugars contained in receptors on mammalian cells surface. This binding starts the process of activation of innate immune cells and secretion of cytokines, which may act by making more efficient the host defence mechanisms against the parasite, especially in the beginning of the infection. It results in an appropriate response because it helps to control the parasite replication during the acute infection and favours host healthy.

INTRODUCTION

Toxoplasma gondii is a coccidian belonging to the phylum Apicomplexa and the causative agent of toxoplasmosis. The protozoan infects a variety of vertebrate hosts, including humans, about one-third of the world population being infected with it [1]. Toxoplasmosis can be fatal in immunocompromised individuals or when contracted congenitally [1], and is considered the second leading cause of death from foodborne illnesses in the United States [2].

T. gondii invades host cells through an active process that relies on the parasite's actomyosin system, concomitantly with the release of microneme proteins (TgMICs) and rhoptry proteins (ROPs and RONs) from specialized organelles in the apical region of the organism [3]. These proteins are secreted by tachyzoites [4, 5] and form complexes of soluble and transmembrane proteins. Some of the TgMICs act as adhesins, interacting tightly with host cell-membrane glycoproteins and receptors, and are involved in the formation of the moving junction [6]. This sequence of events ensures tachyzoite gliding motility, migration through host cells, and invasion [4, 7]. Among the released proteins, TgMIC1, TgMIC4, and TgMIC6 form a complex that plays a role in the adhesion and invasion of host cells [8, 9], contributing to the virulence of the parasite [10, 11].

Several studies have shown that host-cell invasion by apicomplexan parasites such as *T. gondii* involves carbohydrate recognition [12-15]. Interestingly, TgMIC1 and TgMIC4 have lectin domains [11, 16-18] that recognize oligosaccharides with sialic acid and D-galactose in the terminal position, respectively. Importantly, the parasite's Lac^+ subcomplex, consisting of TgMIC1 and TgMIC4, induces adherent spleen cells to release high levels of IL-12 [17], a cytokine critical for the host's protective response to *T. gondii* infection [19]. In addition, immunization with this native subcomplex, or with recombinant TgMIC1 and TgMIC4, protects mice against experimental toxoplasmosis [20, 21]. The induction of IL-12 is frequently due to the detection of the pathogen by innate immunity receptors, including members of the Toll-like receptor (TLR) family, whose stimulation most frequently involves MyD88 activation and leads to the priming of Th1 response, which protects the host against *T. gondii* [19, 22].

Interestingly, the extracellular leucine-rich repeat domains of TLR2 and TLR4 contain four and nine N-glycans, respectively [23]. Therefore, we hypothesized that TgMIC1 and TgMIC4 could target TLR2 and TLR4 N-glycans on antigen-presenting cells (APCs) and, through such interaction, trigger cell activation and IL-12 production. To investigate this possibility, we assayed the ability of recombinant TgMIC1 and TgMIC4 to bind and activate TLR2 and TLR4. Using several strategies, we demonstrated that TLR2 and TLR4 are indeed critical targets for both TgMIC1 and TgMIC4. These parasite and host cell structures establish lectin-carbohydrate interactions that contribute to the induction of IL-12 production during the early phase of *T. gondii* infection.

RESULTS

Lectin properties of recombinant TgMIC1 and TgMIC4 are consistent with those of the native Lac$^+$ subcomplex

Because the native TgMIC1/4 subcomplex purified from soluble *T. gondii* antigens has lectin properties, we investigated whether their recombinant counterparts retained the sugar-binding specificity. The glycoarray analysis revealed the interactions of: i) the Lac$^+$ subcomplex with glycans containing terminal α(2-3)-sialyl and β(1-4)- or β(1-3)-galactose; ii) TgMIC1 with α(2-3)-sialyl residues linked to β-galactosides; and iii) of TgMIC4 with oligosaccharides with terminal β(1-4)- or β(1-3)-galactose (Fig 1A). The combined specificities of the individual recombinant proteins correspond to the dual sugar specificity of the Lac$^+$ fraction, demonstrating that the sugar-recognition properties of the recombinant proteins are consistent with those of the native ones.

Based on the sugar recognition selectivity of TgMIC1 and TgMIC4, we tested two oligosaccharides (α(2-3)-sialyllactose and lacto-N-biose) for their ability to inhibit the interaction of the microneme proteins with the glycoproteins fetuin and asialofetuin [24]. Sialyllactose inhibited the binding of TgMIC1 to fetuin, and lacto-N-biose inhibited the binding of TgMIC4 to asialofetuin (Fig 1B). To ratify the carbohydrate recognition activity of TgMIC1 and TgMIC4, we generated point mutations into the carbohydrate recognition domains (CRDs) of the TgMICs to abolish their lectin properties [11, 18, 25]. These mutated forms, i.e. TgMIC1-T126A/T220A and TgMIC4-K469M, lost the capacity to bind to fetuin and asialofetuin, respectively (Fig 1B), having absorbance as low as that in the presence of the specific sugars. Thus, our results indicate that recombinant TgMIC1 and TgMIC4 maintained their lectin properties, and that the CRD function can be blocked either by competition with specific sugars or by targeted mutations.

TgMIC1 and TgMIC4 trigger the activation of DCs and macrophages

We have previously demonstrated that the native Lac^+ subcomplex stimulates murine adherent spleen cells to produce proinflammatory cytokines [20]. We evaluated whether recombinant TgMIC1 and TgMIC4 retained this property and exerted it on BMDCs and BMDMs. BMDCs (Figs 2A-D) and BMDMs (Figs 2E-H) produced high levels of proinflammatory cytokines as IL-12 (Figs 2A and 2E), TNF-α (Figs 2B and 2F), IL-6 (Figs 2C and 2G). Although conventional $CD4^+$ Th1 cells are known to be the major producers of IL-10 during murine *T. gondii* infection [26], we found that TgMIC1 and TgMIC4 are able to induce the production of this cytokine by BMDCs (Fig 2D) and BMDMs (Fig 2H). We verified that the two recombinant proteins induced the production of similar levels of IL-12, TNF-α, and IL-6 by both BMDCs (Figs 2A-C) and BMDMs (Figs 2E-G). Both microneme proteins induced the production of similar levels of IL-10 in BMDCs (Fig 2D); however, BMDMs produced significantly higher levels of IL-10 when stimulated with TgMIC1 than when stimulated with TgMIC4 (Fig 2H). These cytokine levels were similar to those induced by the TLR4 agonist LPS. Thus, recombinant TgMIC1 and TgMIC4 induce a proinflammatory response in innate immune cells, which is consistent with the results obtained for the native Lac^+ subcomplex [20]. LPS contamination of samples was avoided as described in the Material and Methods section.

The activation of macrophages by TgMIC1 and TgMIC4 depends on TLR2 and TLR4

To investigate the mechanisms through which TgMIC1 and TgMIC4 stimulate innate immune cells to produce cytokines, we assessed whether these microneme proteins can activate specific TLRs. To this end, BMDMs from WT, $MyD88^{-/-}$, $TRIF^{-/-}$, $TLR2^{-/-}$, $TLR4^{-/-}$, or $TLR2^{-/-}/TLR4^{-/-}$ (DKO) mice, as well as HEK293T cells transfected with TLR2 or TLR4, were cultured in the presence or absence of recombinant TgMIC1

and TgMIC4 for 48 hours. The production of IL-12 by BMDMs (Figs 3A-I) and IL-8 by HEK cells (Figs 3J and 3K) were used as an indicator of cell activation. IL-12 production by BMDMs from MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice was lower than that of BMDMs from WT mice (Figs 3A-D); no IL-12 was detected in cultures of DKO mice cells stimulated with either TgMIC1 or TgMIC4 (Fig 3E). These results show that TLR2 and TLR4 are both relevant for the activation of macrophages induced by TgMIC1 and TgMIC4. The residual cytokine production observed in macrophages from TLR2$^{-/-}$ or MyD88$^{-/-}$ mice may result from the activation of TLR4 (Figs 3A and 3C), and vice versa; e.g., the residual IL-12 levels produced by macrophages from TLR4$^{-/-}$ mice may result from TLR2 activation. The finding that microneme proteins fail to induce IL-12 production in DKO mice BMDMs suggests that cell activation triggered by TgMIC1 or TgMIC4 does not require the participation of other innate immunity receptors beyond TLR2 and TLR4. Nevertheless, since TLR9, TLR11, and TLR12 account for the production of IL-12 by macrophages stimulated with parasite components such as DNA or profilin [19, 22, 27], we investigated the involvement of these receptors, as well as TLR3 and TLR5, in the response to TgMIC1 or TgMIC4. BMDMs from TLR3$^{-/-}$, TLR5$^{-/-}$, TLR9$^{-/-}$, and TLR11$^{-/-}$/TLR12$^{-/-}$ mice stimulated with TgMIC1 or TgMIC4 produced similar levels of IL-12 as cells from WT (Figs 3F-I), indicating that the activation triggered by TgMIC1 or TgMIC4 does not depend on these receptors. Additionally, stimulation of HEK cells transfected with human TLR2 (Fig 3J) or TLR4 (Fig 3K) with optimal concentrations of TgMIC1 (S1A and S1C Figs) and TgMIC4 (S1B and S1D Figs) induced IL-8 production at levels that were higher than those detected in the absence of stimuli (medium), and similar to those induced by the positive controls. Finally, by means of a pull-down experiment, we demonstrated the

occurrence of physical interaction of TgMIC1 with TLR2 or TLR4 and TgMIC4 with TLR2 or TLR4 (Fig 3L).

Cell activation induced by TgMIC1 and TgMIC4 results from the interaction of their CRDs with TLR2 and TLR4 N-glycans

We hypothesized that in order to trigger cell activation, TgMIC1 and TgMIC4 CRDs target oligosaccharides of the ectodomains of TLR2 (four N-linked glycans) [23] and TLR4 (nine N-linked glycans) [28]. This hypothesis was tested by stimulating BMDCs (Fig 4A) and BMDMs (Fig 4B) from WT mice with intact TgMIC1 and TgMIC4 or with the mutated forms of these microneme proteins, namely TgMIC1-T126A/T220A and TgMIC4-K469M, which lack carbohydrate binding activity [11, 18, 25]. IL-12 levels in culture supernatants were lower upon stimulation with TgMIC1-T126A/T220A or TgMIC4-K469M, showing that induction of cell activation requires intact TgMIC1 and TgMIC4 CRDs. The same microneme proteins were used to stimulate TLR2-transfected HEK293T cells (Fig 4C), and, similarly, lower IL-8 production was obtained in response to mutated TgMIC1 or TgMIC4 compared to that seen in response to intact proteins. These observations demonstrated that TgMIC1 and TgMIC4 CRDs are also necessary for inducing HEK cell activation.

We used an additional strategy to examine the ability of TgMIC1 and TgMIC4 to bind to TLR2 N-glycans. In this approach, HEK cells transfected with the fully N-glycosylated TLR2 ectodomain or with the TLR2 glycomutants [23] were stimulated with a control agonist (FSL-1) or with TgMIC1 or TgMIC4. HEK cells transfected with any TLR2 form, except those expressing totally unglycosylated TLR2 (mutant Δ-1,2,3,4), were able to respond to FSL-1 (Fig 4D), a finding that is consistent with the previous report that the Δ-1,2,3,4 mutant is not secreted by HEK293T cells [23]. Cells transfected with TLR2 lacking only the first N-glycan (mutant Δ-1) responded to all

stimuli. The response to the TgMIC1 stimulus was significantly reduced in cells transfected with five different TLR2 mutants, lacking the second, third, and fourth N-glycans (Fig 4D). Moreover, TgMIC4 stimulated IL-8 production was significantly reduced in cells transfected with the mutants lacking only the third N-glycan (Fig 4D).

These results indicate that TgMIC1 and TgMIC4 use their CRDs to induce TLR2- and TLR4-mediated cell activation. Among the TLR2 N-glycans, the TgMIC1 CRD likely targets the second, third, and the fourth glycan, while the TgMIC4 CRD is targets only the third.

The early activation of *T. gondii*-infected DCs depends on TLR2- and TLR4-mediated pathways

We investigated the biological relevance of the TLR2- and TLR4-mediated activation of innate immune cells during a *T. gondii* infection of DCs. Twenty-four hours after infection with WT tachyzoites of the RH strain, BMDCs from WT mice produced IL-12 levels that were as high as those induced by LPS or STAg, which were used as positive controls. Consistent with previous studies, BMDCs from MyD88$^{-/-}$ mice produced nearly 80% less IL-12 (Fig 5A). We then performed additional experiments in which BMDCs derived from WT or TLR2$^{-/-}$/TLR4$^{-/-}$ DKO mice were infected with *T. gondii*, and the kinetics of IL-12 production was evaluated. Lower levels of IL-12 were detected in the supernatants of DKO BMDCs at 6–24 h after infection than in those of WT cells. At 48 h after the infection, IL-12 levels were similar in both DKO and WT cells (Fig 5B). Our findings therefore indicated that TLR2 and TLR4 activation is required for the early induction of IL-12 production by DCs following *T. gondii* infection.

The early activation of *T. gondii*-infected innate immune cells is primarily triggered by TgMIC1

Because early IL-12 production by innate immune cells is induced by both *T. gondii* infection and stimulation with TgMICs, we investigated whether such production would be impaired in cells infected with *T. gondii* lacking TgMIC1 and/or TgMIC4. The assays were conducted by using TgMIC1 or TgMIC4 single-knockout parasites [8] and TgMIC1/TgMIC4 double knockout parasites. We generated the DKO strain from TgMIC1-KO parasites, which had the *TgMIC4* gene disrupted by the insertion of a chloramphenicol (cat) cassette with flanking regions of the endogenous *TgMIC4* locus (Fig 6A). After transfection and clone selection, we confirmed that the *TgMIC4* gene was absent in TgMIC1/TgMIC4-DKO parasites (Fig 6B) and there was no TgMIC4 protein expression (Fig 6C).

For the infection, we compared the kinetics of IL-12 production following the infection of BMDMs and BMDCs with WT, TgMIC1-KO, TgMIC4-KO, or TgMIC1/TgMIC4 DKO parasites, all derived from the RH strain. We found that the infection with the WT strain led to early IL-12 production (within 6 h) in BMDCs, but not in BMDMs. The levels of IL-12 produced by BMDCs (Figs 6D-F) were sustained when cells were infected with TgMIC4-KO parasites, but decreased significantly when infected with TgMIC1-KO or DKO parasites. Notably, during the first 12 h after infection, the levels of IL-12 were significantly lower and comparable to those in the negative controls (non-infected cells). At 24 and 48 h post-infection, the IL-12 levels were restored by 61.3–78.25%, but were still lower than those induced by the infection with WT parasites. A significant increase in IL-12 production by BMDMs after infection with WT parasites (Figs 6G-I), compared to non-infected cells, was detected only 24 h post infection. However, similar to that observed in BMDCs, infection with TgMIC1-KO and TgMIC1/TgMIC4 DKO parasites resulted in lower IL-12 production compared to the infection with WT parasites. The kinetics IL-12 production by BMDC

255 in response to infection with WT or with transformed *T. gondii* suggests that TgMIC1 is
256 crucial for stimulating the response of DCs in the early phases of *T. gondii* infection. In
257 later time points, other *T. gondii* components would act as IL-12 inducers, such as
258 profilin, which is a TLR11 and TLR12 agonist [27, 29], or GRA7 [30], GRA15 [31],
259 and GRA24 [32], which directly triggers intracellular signalling pathways in a TLR-
260 independent manner.
261

262 **DISCUSSION**

263 In this study, we report a new function for TgMIC1 and TgMIC4, two *T. gondii*
264 microneme proteins involved in the host-parasite relationship. We show that TgMIC1
265 and TgMIC4, by interacting with N-glycans of TLR2 and TLR4, trigger the activation
266 of innate immunity cells. This results in IL-12 secretion, known to lead to the
267 production of IFN-γ, a cytokine responsible for parasite clearance and development of a
268 protective T cell response by the host [19, 22]. This set of events explains, at least in
269 part, the resistance conferred by TgMIC1 and TgMIC4 administration against
270 experimental toxoplasmosis [20, 21].

271 *T. gondii* tachyzoites express microneme proteins either on their surface, as
272 transmembrane proteins, or secrete them in their soluble form. These proteins may form
273 complexes, such as those of TgMIC1, TgMIC4, and TgMIC6 (TgMIC1/4/6), in which
274 TgMIC6 is a transmembrane protein that anchors the two soluble molecules TgMIC1
275 and TgMIC4 [8]. Genetic disruption of any of the three encoding genes does not
276 interfere with parasite survival [8] nor its interaction with and attachment to the host
277 cells [10]; however, TgMIC1 has been demonstrated to play a role in invasion and
278 contribute to virulence in mice [10]. We previously isolated soluble TgMIC1/4, a
279 lactose-binding complex from soluble *T. gondii* antigens (STAg) [17], and its lectin

activity was confirmed by the fact that TgMIC1 binds to sialic acid [9] and TgMIC4 to β-galactose [18]. We also reported that TgMIC1/4 stimulates adherent splenic murine cells to produce IL-12 at levels as high as those induced by STAg [20]. The hypothesis that this result from interactions of microneme protein(s) with defined glycosylated receptor(s) expressed on the host cell surface was confirmed in the present study.

To make this analysis possible, we generated recombinant forms of TgMIC1 and TgMIC4, which retain their specific sialic acid- and β-galactose-binding properties conserved, as indicated by the results of their binding to fetuin and asialofetuin as well as the glycoarray assay. Both recombinant TgMIC1 and TgMIC4 triggered the production of proinflammatory and anti-inflammatory cytokines in DCs and macrophages via the recognition of TLR2 and TLR4 N-glycans, as well as by signalling through MyD88 and, partially, TRIF. Importantly, our results demonstrate that the binding of TgMIC1 and TgMIC4 to selected N-glycans in the structures of TLR2 and TLR4 induces cell activation. We further proposed that cell activation occurred due to TgMIC1 and TgMIC4 targeting N-glycans of both TLR2 and TLR4. The ligands for TgMIC1 and TgMIC4, α2-3-sialyllactosamine and β1-3- or β1-4-galactosamine, respectively, are very often terminal residues of mammalian N-glycans attached to numerous glycoconjugates on the surface of cells. Such interactions account mostly for the adhesion and invasion of host cells by the parasite, thus making these proteins important candidate *T. gondii* virulence factors [10]. However, as already mentioned, TgMIC1 and TgMIC4 target N-glycans on the ectodomains of TLR2 and TLR4, the established interactions culminate in an immunostimulatory effect, which limits parasite survival. In this sense, TgMIC1 and TgMIC4 may work as double-edged swords during *T. gondii* infection.

Several pathogens are known to synthesize lectins, which are most frequently reported to interact with glycoconjugates on host cells to promote adherence, invasion, and colonization of tissues [33-36]. Nonetheless, there are currently only a few examples of lectins from pathogens that recognize sugar moieties present in TLRs and induce IL-12 production by innate immunity cells. Paracoccin, a GlcNAc-binding lectin from the human pathogen *Paracoccidioides brasiliensis*, induces macrophage polarization towards the M1 phenotype [37] and the production of inflammatory cytokines through the interaction with TLR2 N-glycans [38]. Furthermore, the galactose-adherence lectin from *Entamoeba histolytica* activates TLR2 and induces IL-12 production [39]. In addition, the mammalian soluble lectin SP-A, found in lung alveoli, interacts with the TLR2 ectodomain [40]. The occurrence of cell activation and IL-12 production as a consequence of the recognition of TLR N-glycans has also been demonstrated by using plant lectins with different sugar-binding specificities [41, 42].

The binding of TgMIC1 and TgMIC4, as well as the lectins above, to TLR2 and TLR4 may be associated with the position of the specific sugar residue present on the receptor's N-glycan structure. Since the N-glycan structures of TLR2 and TLR4 are still unknown, we assume that the targeted TgMIC1 and TgMIC4 residues, e.g. sialic acid α2-3-linked to galactose β1-3- and β1-4-galactosamines, are appropriately placed in the receptors' oligosaccharides to allow the recognition phenomenon and trigger the activation of innate immune responses.

Several *T. gondii* components were shown to activate innate immune cells in a TLR-dependent manner, but independently of sugar recognition. This is the case for profilin (TgPRF), which is essential for the parasite's gliding motility based on actin polymerization; it is recognized by TLR11 [27] and TLR12 [29, 43]. In addition, *T. gondii*-derived glycosylphosphatidylinositol anchors activate TLR2 and TLR4 [44], and

329 parasite RNA and DNA were shown to be the ligands for TLR7 and TLR9, respectively
330 [19, 22, 43]. The stimulation of all of these TLRs culminates in MyD88 activation and
331 results in IL-12 production [19, 22]. Several other *T. gondii* components induce the
332 production of proinflammatory cytokines such as IL-12, independent of TLRs. For
333 example, the dense granule protein 7 (GRA7) induces MyD88-dependent NF-kB
334 activation, which is followed by IL-12, TNF-α, and IL-6 production [30]. In addition,
335 GRA15 activates NF-kB, promoting the release of IL-12 [31], and GRA24 triggers the
336 autophosphorylation of p38 MAP kinase, which culminates in proinflammatory
337 cytokine and chemokine secretion [32]. Furthermore, MIC3 was described to induce a
338 TNF-α secretory response and macrophage M1 polarization [45]. Thus, several *T.*
339 *gondii* components, including TgMIC1 and TgMIC4, can favour protective immunity to
340 the parasite by inducing the production of proinflammatory cytokines, which are known
341 to control the pathogen's replication during the acute phase of infection and favour the
342 development of a Th1 adaptive response [19].

343 Our results regarding soluble TgMIC1 and TgMIC4 confirmed our hypothesis
344 that they are associated with the induction of the innate immune response against *T.*
345 *gondii* through TLR2- and TLR4-dependent pathways. We demonstrated that, during
346 the first 24 h of infection, IL-12 production by DCs is dependent on TLR2 and TLR4
347 activation. This is consistent with previous studies that highlight the importance of TLR
348 signalling pathways, as well as the MyD88 adapter molecule, as essential for conferring
349 resistance to *T. gondii* infection [27, 44, 46, 47].

350 Finally, our results suggest that TgMIC1, in particular, contributes to the
351 secretion of IL-12 by macrophages and DCs during the first 48 h of infection. More
352 specifically, TgMIC1 seemed to be exclusively responsible for IL-12 production during
353 the first 12 h of infection, since DCs infected with TgMIC1-KO parasites did not

produce IL-12 during this period. The same activation pattern was generated by TgMIC1/TgMIC4 DKO tachyzoites. Although we did not detect active participation of TgMIC4 in the activation of infected innate immune cells, we still speculate that it contributes to the induction of IL-12 production during *T. gondii* infection. We have clearly demonstrated this property of TgMIC4 when performing *in vitro* assays with recombinant TgMIC4, which induced IL-12 production by APCs. We did not observe a decrease in IL-12 production when APCs were infected with TgMIC4-KO parasites probably because TgMIC1 is still expressed and properly targeted in the absence of TgMIC4. Therefore, TgMIC1 expression alone appears to be sufficient to induce IL-12 secretion by APCs. On the other hand, TgMIC4 and TgMIC6 rely on the expression of TgMIC1 to be transported through the secretory pathway, to the micronemes and, consequently, to the protozoan's cell surface [8, 18]. Thus, in TgMIC1-KO parasites, the TgMIC4 and TgMIC6 proteins remain inside intracellular compartments such as the endoplasmic reticulum and Golgi apparatus. The subsequent reintroduction of TgMIC1 expression in knockout parasites restored the transport of TgMIC4 and TgMIC6 to the cell surface[8]. Marchant and collaborators [18] have proposed a model that explains the expression and secretion of TgMIC1 and TgMIC4 in *T. gondii*: TgMIC1 forms a disulphide-linked trimer, and each TgMIC1 monomer engages a TgMIC4 monomer through its β-finger motif, creating a heterohexamer [15]. According to this model and previous studies on the issue, the interaction between TgMIC1 and TgMIC4 is required for TgMIC4 to correctly target micronemes [8, 48]. These data indicate that TgMIC1-KO parasites do not secrete TgMIC4, while TgMIC4-KO parasites do secrete TgMIC1. By analysing the infection of DCs and macrophages by TgMIC4-KO parasites, we observed that early IL-12 production is stimulated by TgMIC1 in compensation for the absence of TgMIC4. Infection with TgMIC1-KO tachyzoites yielded results that were

similar to those obtained using DKO parasites, because TgMIC1 and TgMIC4 are not expressed on the surface of *T. gondii* nor secreted by TgMIC1-KO or DKO strains.

Our findings demonstrate, for the first time, the importance of the recognition of the host's carbohydrates by *T. gondii* lectins, namely the interaction of TgMIC1 and TgMIC4 with carbohydrates of TLR2 and TLR4, which triggers protective innate immune cytokine in vitro. Studies are in progress to test the role of this novel recognition pathway in the control of acute infection in vivo. If confirmed, the parasite lectin-host TLR interactions described here could serve as potential targets for therapeutic intervention strategies against toxoplasmosis.

METHODS

Ethics statement

All experiments were conducted in accordance to the Brazilian Federal Law 11,794/2008 establishing procedures for the scientific use of animals, and State Law establishing the Animal Protection Code of the State of Sao Paulo. All efforts were made to minimize suffering, and the animal experiments were approved by the Ethics Committee on Animal Experimentation (*Comissão de Ética em Experimentação Animal* - CETEA) of the Ribeirao Preto Medical School, University of Sao Paulo (protocol number 065/2012), following the guidelines of the National Council for Control of Animal Experimentation (*Conselho Nacional de Controle de Experimentação Animal* - CONCEA).

Lac^+ fraction and recombinant TgMIC1 and TgMIC4

The lactose-eluted (Lac^+) fraction was obtained as previously reported [17, 21]. Briefly, the total soluble tachyzoite antigen (STAg) fraction was loaded into a lactose column (Sigma-Aldrich, St. Louis, MO) and equilibrated with PBS containing 0.5 M

NaCl. The material adsorbed to the resin was eluted with 0.1 M lactose in equilibrating buffer and dialyzed against ultrapure water. The obtained fraction was denoted as Lac^+ and confirmed to contain TgMIC1 and TgMIC4. For the recombinant proteins, TgMIC1 and TgMIC4 sequences were amplified from cDNA of the *T. gondii* strain ME49 with a 6-histidine tag added on the N-terminal, cloned into pDEST17 vector (Gateway Cloning, Thermo Fisher Scientific Inc., Grand Island, NY), and used to transform DH5α *E. coli* chemically competent cells for ampicillin expression selection, as described before [21]. The plasmids with TgMIC1-T126A/T220A and TgMIC4-K469M were synthesized by GenScript (China) using a pET28a vector, and the TgMIC sequences carrying the mutations were cloned between the *Nde*I and *Bam*H I sites. All plasmids extracted from DH5α *E. coli* were transformed in *E. coli* BL21-DE3 chemically competent cells to produce recombinant proteins that were then purified from inclusion bodies and refolded by gradient dialysis, as described previously [21]. Endotoxin concentrations were measured in all proteins samples using the Limulus Amebocyte Lysate Kit – QCL-1000 (Lonza, Basel, Switzerland). The TgMIC1, TgMIC1-T126A/T220A, TgMIC4 and TgMIC4-K469M contained 7.2, 3.2, 3.5 and 1.1 EU endotoxin/μg of protein, respectively. Endotoxin was removed by passing over two polymyxin-B columns (Affi-Prep® Polymyxin Resin; Bio-Rad, Hercules, CA). Additionally, prior to all *in vitro* cell-stimulation assays, the proteins samples were incubated with 50 μg/mL of polymyxin B sulphate salt (Sigma-Aldrich, St. Louis, MO) for 30 min at 37 °C to remove possible residual LPS.

Glycan array

The carbohydrate-binding profile of microneme proteins was determined by Core H (Consortium for Functional Glycomics, Emory University, Atlanta, GA), using a printed glycan microarray, as described previously [49]. Briefly, TgMIC1-Fc,

TgMIC4-Fc, and Lac^{+}-Fc in binding buffer (1% BSA, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% (w/v) Tween 20, and 20 mM Tris-HCl, pH 7.4) were applied onto a covalently printed glycan array and incubated for 1 h at 25 °C, followed by incubation with Alexa Fluor 488-conjugate (Invitrogen, Thermo Fisher Scientific Inc., Grand Island, NY). Slides were scanned, and the average signal intensity was calculated. The common features of glycans with stronger binding are depicted in Fig. 1a. The average signal intensity detected for all of the glycans was calculated and set as the baseline.

Sugar-inhibition assay

Ninety-six-well microplates were coated with 1 μg/well of fetuin or asialofetuin, glycoproteins diluted in 50 μL of carbonate buffer (pH 9.6) per well, followed by overnight incubation at 4 °C. TgMIC1 or TgMIC4 proteins (both wild type (WT) and mutated forms), previously incubated or not with their corresponding sugars, i.e. α(2-3)-sialyllactose for TgMIC1 and lacto-N-biose for TgMIC4 (V-lab, Dextra, LA, UK), were added into coated wells and incubated for 2 h at 25 °C. After washing with PBS, *T. gondii*-infected mouse serum (1:50) was used as the source of the primary antibody. The assay was then developed with anti-mouse peroxidase-conjugated secondary antibody, and the absorbance was measured at 450 nm in a microplate-scanning spectrophotometer (Power Wave-X; BioTek Instruments, Inc., Winooski, VT).

Mice and parasites

Female C57BL/6 (WT), $MyD88^{-/-}$, $TRIF^{-/-}$, $TLR2^{-/-}$, $TLR3^{-/-}$, $TLR4^{-/-}$, double knockout (DKO) $TLR2^{-/-}$/$TLR4^{-/-}$, $TLR5^{-/-}$, and $TLR9^{-/-}$ mice (all from the C57BL/6 background), 8 to 12 weeks of age, were acquired from the University of São Paulo - Ribeirão Preto campus animal facility, Ribeirão Preto, São Paulo, Brazil, and housed in the animal facility of the Department of Cell and Molecular Biology - Ribeirão Preto Medical School, under specific pathogen-free conditions. The $TLR11^{-/-}$/$TLR12^{-/-}$ DKO

mice were maintained at American Association of Laboratory Animal Care-accredited animal facilities at NIAID/NIH.

A clonal isolate of the RH-Δhxgprt strain of *T. gondii* was used as the recipient strain to generate TgMIC1-KO and TgMIC4-KO strains, as described previously [8]. The TgMIC4-KO was produced from the TgMIC1-KO recipient strain to generate the TgMIC1/TgMIC4-DKO strain. Briefly, the previously described [8] TgMIC4-KO vector containing a chloramphenicol-resistant cassette was transfected in the TgMIC1-KO strain. Double homologous recombination was performed using the CRISPR/Cas9-based genome editing to facilitate the targeting of the *TgMIC4* locus. The specific gRNA/Cas9 were generated using the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA), with pSAG1::CAS9-GFP-U6::sgUPRT as the template [50], using the primers ATGCAGTTGTACTCATCTCCGTTTTAGAGCTAGAAATAGC and AACTTGACATCCCCATTTAC. Twenty micrograms of gRNA was transfected along with 40 μg of the linearized KO vector. Parasites were transfected and selected as previously described [51, 52].

Strains were maintained in human foreskin fibroblast (HFF) cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 0.25 mM gentamicin, 10 U/mL penicillin, and 10 μg/mL streptomycin (Gibco, Thermo Fisher Scientific Inc., Grand Island, NY).

Bone marrow-derived dendritic cells and macrophages

Bone marrows of WT, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$, TLR3$^{-/-}$, TLR4$^{-/-}$, DKO TLR2$^{-/-}$/TLR4$^{-/-}$, TLR5$^{-/-}$, TLR9$^{-/-}$, and DKO TLR11$^{-/-}$/TLR12$^{-/-}$ mice were harvested from femurs and hind leg bones. Cells were washed with RPMI medium and resuspended in RPMI medium with 10% FBS, 10 U/mL penicillin, and 10 μg/mL

streptomycin (Gibco). For dendritic cell (DC) differentiation, we added 10 ng/mL of recombinant murine GM-CSF (Prepotech, Rocky Hill, NJ), and 10 ng/mL murine recombinant IL-4 (eBioscience, San Diego, CA); for macrophage differentiation, 30% of L929 conditioned medium was added to RPMI medium with 10% FBS. The cells were cultured in 100 × 20 mm dish plates (Costar; Corning Inc., Corning, NY), supplemented with respective conditioned media at days 3 and 6 for DCs, and at day 4 for macrophages. DCs were incubated for 8–9 days and macrophages for 7 days; the cells were then harvested and plated into 24-well plates at 5×10^5 cells/well for protein stimulations or *T. gondii* infections, followed by ELISA.

HEK293T cells transfection

Human embryonic kidney 293T (HEK293T) cells, originally acquired from American Tissue Culture Collection (ATCC, Rockville, MD), were used as an expression tool [53] for TLR2 and TLR4 [38, 54]. The cells grown in DMEM supplemented with 10% FBS (Gibco), and were seeded at 3.5×10^5 cells/mL in 96-well plates (3.5×10^4 cells/well) 24 h before transfection. Then, HEK293T cells were transiently transfected (70-80% confluence) with human TLR2 plasmids as described previously [23] or with CD14, CD36, MD-2 and TLR4 [55] using Lipofectamine 2000 (Invitrogen) with 60 ng of NF-κB Luc, an NF-κB reporter plasmid, and 0.5 ng of *Renilla* luciferase plasmid, together with 60 ng of each gene of single and multiple glycosylation mutants and of TLR2 WT genes [23]. After 24 h of transfection, the cells were stimulated overnight with positive controls: P3C (Pam3CSK4; EMC Microcollections, Tübingen, Germany), fibroblast stimulating ligand-1 (FSL-1; EMC Microcollections), or LPS Ultrapure (standard LPS, *E. coli* 0111:B4; Sigma-Aldrich); or with the negative control for cell stimulation (the medium). Cells transfected with empty vectors, incubated either with the medium or with agonists (FSL-1 or P3C), were

503 also assayed; negative results were required for each system included in the study. IL-8
504 was detected in the culture supernatants. The absence of Mycoplasma contamination in
505 the cell culture was certified by indirect fluorescence staining as described previously
506 [56].

507 **Cytokine measurement**

508 The quantification of human IL-8 and mouse IL-12p40, IL-6, TNF-α, and IL-10
509 in the supernatant of the cultures was performed by ELISA, following the
510 manufacturer's instructions (OptEIA set; BD Biosciences, San Jose, CA). Human and
511 murine recombinant cytokines were used to generate standard curves and determine
512 cytokine concentrations. The absorbance was read at 450 nm using the Power Wave-X
513 spectrophotometer (BioTek Instruments).

514 **TLR2-FLAG and TLR4-FLAG plasmids**

515 The pcDNA4/TO-FLAG plasmid was kindly provided by Dr. Dario Simões
516 Zamboni. The pcDNA4-FLAG-TLR2 and pcDNA4-FLAG-TLR4 plasmids were
517 constructed as follows. RNA from a P388D1 cell line (ATCC, Rockville, MD) was
518 extracted and converted to cDNA with Maxima H Minus Reverse Transcriptase
519 (Thermo-Fisher Scientific, Waltham, MA USA) and oligo(dT). TLR2 and TLR4 were
520 amplified from total cDNA from murine macrophages by using Phusion High-Fidelity
521 DNA Polymerase and the phosphorylated primers TLR2_F:
522 ATGCTACGAGCTCTTTGGCTCTTCTGG, TLR2_R:
523 CTAGGACTTTATTGCAGTTCTCAGATTTACCCAAAAC, TLR4_F:
524 TGCTTAGGATCCATGATGCCTCCCTGGCTCCTG and TLR4_R:
525 TGCTTAGCGGCCGCTCAGGTCCAAGTTGCCGTTTCTTG. The fragments were
526 isolated from 1% agarose/Tris-acetate-ethylenediaminetetraacetic acid gel, purified with
527 GeneJET Gel Extraction Kit (Thermo-Fisher Scientific), and inserted into the

pcDNA4/TO-FLAG vector by using the restriction enzymes sites for NotI and XbaI (Thermo-Fisher Scientific) for TLR2, and BamHI and NotI (Thermo-Fisher Scientific) for TLR4. Ligation reactions were performed by using a 3:1 insert/vector ratio with T4 DNA Ligase (Thermo-Fisher Scientific) and transformed into chemically competent *Escherichia coli* DH5α cells. Proper transformants were isolated from LB agar medium plates under ampicillin selection (100 μg/mL) and analyzed by PCR, restriction fragment analysis, and DNA sequencing. All reactions were performed according to the manufacturer's instructions.

Pull-down assay

We used the lysate of HEK293T cells transfected (70-80% confluence) with plasmids containing TLR2-FLAG or TLR4-FLAG. After 24 h of transfection, the HEK cells were lysed with a non-denaturing lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, and 2 mM EDTA) supplemented with a protease inhibitor (Roche, Basel, Switzerland). After 10 min of incubation on ice, the lysate was subjected to centrifugation (16,000 *g*, at 4 °C for 5 min). The protein content in the supernatant was quantified by the BCA method, aliquoted, and stored at -80 °C. For the pull-down assay, 100 μg of the lysate from TLR2-FLAG- or TLR4-FLAG-transfected HEK cells were incubated with 10 μg of TgMIC1 or TgMIC4 overnight at 4 °C. Since these proteins had a histidine tag, the samples were purified on nickel-affinity resin (Ni Sepharose High Performance; GE Healthcare, Little Chalfont, UK) after incubation for 30 min at 25 °C and centrifugation of the fraction bound to nickel to pull down the TgMIC-His that physically interacted with TLR-FLAG (16,000 *g*, 4 °C, 5 min). After washing with PBS, the samples were resuspended in 100 μL of SDS loading dye with 5 μL of 2-mercaptoethanol, heated for 5 min at 95 ºC, and 25 μL of total volume was run on 10% SDS-PAGE. After transferring to a nitrocellulose membrane (Millipore, Billerica, MA), immunoblotting

553 was performed by following the manufacturer's protocol. First, the membrane was
554 incubated with anti-FLAG monoclonal antibodies (1:2,000) (Clone G10, ab45766,
555 Sigma-Aldrich) to detect the presence of TLR2 or TLR4. The same membrane was then
556 subjected to secondary probing and was developed with anti-TgMIC1 or anti-TgMIC4
557 polyclonal antibodies (IgY; 1:20,000) and followed by incubation with secondary
558 polyclonal anti-chicken IgY-HRP (1:4,000) (A9046, Sigma-Aldrich) to confirm the
559 presence of TgMIC1 and TgMIC4.

560 ***Toxoplasma gondii* infection**

561 Bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived
562 macrophages (BMDMs) were infected with WT RH-Δhxgprt, TgMIC1-KO, TgMIC4-
563 KO, or TgMIC1/TgMIC4-DKO strains recovered from T25 flasks with HFF cell
564 cultures. The T25 flasks were washed with RPMI medium to completely remove
565 parasites, and the collected material was centrifuged for 5 min at 50 *g* to remove HFF
566 cell debris. The resulting pellet was discarded, and the supernatant containing the
567 parasites was centrifuged for 10 min at 1,000 *g* and resuspended in RPMI medium for
568 counting and concentration adjustments. BMDCs and BMDMs were dispensed in 24-
569 well plates at 5×10^5 cells/well (in RPMI medium supplemented with 10% FBS),
570 followed by infection with 3 parasites per cell (multiplicity of infection, MOI 3). Then,
571 the plate was centrifuged for 3 min at 200 *g* to synchronize the contact between cells
572 and parasites and incubated at 37 °C. The supernatants were collected at 6, 12, 24, and
573 48 h after infection for quantification of IL-12p40.

574 **Statistical analysis**

575 The data were plotted and analysed using GraphPad Prism 6.0 software
576 (GraphPad, La Jolla, CA). Statistical significance of the obtained results was calculated

using analysis of variance (One-way ANOVA) followed by Bonferroni's multiple comparisons. Differences were considered significant when the *P* value was <0.05.

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REFERENCES

1. Dubey JP. Toxoplasmosis of animals and humans. 2nd ed. Boca Raton, FL, USA: CRC Press; 2009. 336 p.

2. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States--major pathogens. Emerging infectious diseases. 2011;17(1):7-15. Epub 2011/01/05. doi: 10.3201/eid1701.P1110110.3201/eid1701.091101p1. PubMed PMID: 21192848; PubMed Central PMCID: PMC3375761.

600 3. Carruthers VB, Sibley LD. Sequential protein secretion from three distinct
601 organelles of Toxoplasma gondii accompanies invasion of human fibroblasts. Eur J Cell
602 Biol. 1997;73(2):114-23. Epub 1997/06/01. PubMed PMID: 9208224.
603 4. Carruthers VB, Giddings OK, Sibley LD. Secretion of micronemal proteins is
604 associated with toxoplasma invasion of host cells. Cell Microbiol. 1999;1(3):225-35.
605 Epub 2001/02/24. PubMed PMID: 11207555.
606 5. Lovett JL, Sibley LD. Intracellular calcium stores in Toxoplasma gondii govern
607 invasion of host cells. J Cell Sci. 2003;116(Pt 14):3009-16. Epub 2003/06/05. doi:
608 10.1242/jcs.00596. PubMed PMID: 12783987.
609 6. Tomley FM, Soldati DS. Mix and match modules: structure and function of
610 microneme proteins in apicomplexan parasites. Trends in parasitology. 2001;17(2):81-8.
611 Epub 2001/03/03. PubMed PMID: 11228014.
612 7. Carruthers VB, Tomley FM. Microneme proteins in apicomplexans. Subcell
613 Biochem. 2008;47:33-45. Epub 2008/06/03. PubMed PMID: 18512339; PubMed
614 Central PMCID: PMC2847500.
615 8. Reiss M, Viebig N, Brecht S, Fourmaux MN, Soete M, Di Cristina M, et al.
616 Identification and characterization of an escorter for two secretory adhesins in
617 Toxoplasma gondii. J Cell Biol. 2001;152(3):563-78. Epub 2001/02/07. PubMed PMID:
618 11157983; PubMed Central PMCID: PMC2196004.
619 9. Friedrich N, Santos JM, Liu Y, Palma AS, Leon E, Saouros S, et al. Members of
620 a novel protein family containing microneme adhesive repeat domains act as sialic acid-
621 binding lectins during host cell invasion by apicomplexan parasites. J Biol Chem.
622 2010;285(3):2064-76. Epub 2009/11/11. doi: 10.1074/jbc.M109.060988. PubMed
623 PMID: 19901027; PubMed Central PMCID: PMC2804363.

624 10. Cerede O, Dubremetz JF, Soete M, Deslee D, Vial H, Bout D, et al. Synergistic
625 role of micronemal proteins in Toxoplasma gondii virulence. J Exp Med.
626 2005;201(3):453-63. Epub 2005/02/03. doi: 10.1084/jem.20041672. PubMed PMID:
627 15684324; PubMed Central PMCID: PMC2213027.
628 11. Blumenschein TM, Friedrich N, Childs RA, Saouros S, Carpenter EP,
629 Campanero-Rhodes MA, et al. Atomic resolution insight into host cell recognition by
630 Toxoplasma gondii. EMBO J. 2007;26(11):2808-20. Epub 2007/05/12. doi:
631 10.1038/sj.emboj.7601704. PubMed PMID: 17491595; PubMed Central PMCID:
632 PMC1888667.
633 12. Monteiro VG, Soares CP, de Souza W. Host cell surface sialic acid residues are
634 involved on the process of penetration of Toxoplasma gondii into mammalian cells.
635 FEMS Microbiol Lett. 1998;164(2):323-7. Epub 1998/07/31. PubMed PMID: 9682481.
636 13. Ortega-Barria E, Boothroyd JC. A Toxoplasma lectin-like activity specific for
637 sulfated polysaccharides is involved in host cell infection. J Biol Chem.
638 1999;274(3):1267-76. Epub 1999/01/09. PubMed PMID: 9880495.
639 14. Carruthers VB, Hakansson S, Giddings OK, Sibley LD. Toxoplasma gondii uses
640 sulfated proteoglycans for substrate and host cell attachment. Infect Immun.
641 2000;68(7):4005-11. Epub 2000/06/17. PubMed PMID: 10858215; PubMed Central
642 PMCID: PMC101681.
643 15. Paing MM, Tolia NH. Multimeric assembly of host-pathogen adhesion
644 complexes involved in apicomplexan invasion. PLoS Pathog. 2014;10(6):e1004120.
645 Epub 2014/06/20. doi: 10.1371/journal.ppat.1004120. PubMed PMID: 24945143;
646 PubMed Central PMCID: PMC4055764.
647 16. Brecht S, Carruthers VB, Ferguson DJ, Giddings OK, Wang G, Jakle U, et al.
648 The toxoplasma micronemal protein MIC4 is an adhesin composed of six conserved

649 apple domains. J Biol Chem. 2001;276(6):4119-27. Epub 2000/10/29. doi:
650 10.1074/jbc.M008294200. PubMed PMID: 11053441.
651 17. Lourenco EV, Pereira SR, Faca VM, Coelho-Castelo AA, Mineo JR, Roque-
652 Barreira MC, et al. Toxoplasma gondii micronemal protein MIC1 is a lactose-binding
653 lectin. Glycobiology. 2001;11(7):541-7. Epub 2001/07/12. PubMed PMID: 11447133.
654 18. Marchant J, Cowper B, Liu Y, Lai L, Pinzan C, Marq JB, et al. Galactose
655 recognition by the apicomplexan parasite Toxoplasma gondii. J Biol Chem.
656 2012;287(20):16720-33. Epub 2012/03/09. doi: 10.1074/jbc.M111.325928. PubMed
657 PMID: 22399295; PubMed Central PMCID: PMC3351351.
658 19. Yarovinsky F. Toll-like receptors and their role in host resistance to Toxoplasma
659 gondii. Immunol Lett. 2008;119(1-2):17-21. Epub 2008/07/12. doi:
660 10.1016/j.imlet.2008.05.007. PubMed PMID: 18617274.
661 20. Lourenco EV, Bernardes ES, Silva NM, Mineo JR, Panunto-Castelo A, Roque-
662 Barreira MC. Immunization with MIC1 and MIC4 induces protective immunity against
663 Toxoplasma gondii. Microbes Infect. 2006;8(5):1244-51. Epub 2006/04/18. doi:
664 10.1016/j.micinf.2005.11.013. PubMed PMID: 16616574.
665 21. Pinzan CF, Sardinha-Silva A, Almeida F, Lai L, Lopes CD, Lourenco EV, et al.
666 Vaccination with Recombinant Microneme Proteins Confers Protection against
667 Experimental Toxoplasmosis in Mice. PLoS One. 2015;10(11):e0143087. Epub
668 2015/11/18. doi: 10.1371/journal.pone.0143087. PubMed PMID: 26575028; PubMed
669 Central PMCID: PMC4648487.
670 22. Yarovinsky F. Innate immunity to Toxoplasma gondii infection. Nat Rev
671 Immunol. 2014;14(2):109-21. Epub 2014/01/25. doi: 10.1038/nri3598. PubMed PMID:
672 24457485.

673 23. Weber AN, Morse MA, Gay NJ. Four N-linked glycosylation sites in human
674 toll-like receptor 2 cooperate to direct efficient biosynthesis and secretion. J Biol Chem.
675 2004;279(33):34589-94. Epub 2004/06/03. doi: 10.1074/jbc.M403830200. PubMed
676 PMID: 15173186.
677 24. Santos A, Carvalho FC, Roque-Barreira MC, Zorzetto-Fernandes AL, Gimenez-
678 Romero D, Monzo I, et al. Evidence for Conformational Mechanism on the Binding of
679 TgMIC4 with beta-Galactose-Containing Carbohydrate Ligand. Langmuir.
680 2015;31(44):12111-9. Epub 2015/10/22. doi: 10.1021/acs.langmuir.5b03141. PubMed
681 PMID: 26488670.
682 25. Hager KM, Carruthers VB. MARveling at parasite invasion. Trends in
683 parasitology. 2008;24(2):51-4. Epub 2008/01/22. doi: 10.1016/j.pt.2007.10.008.
684 PubMed PMID: 18203663; PubMed Central PMCID: PMC2662992.
685 26. Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, et
686 al. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective
687 regulatory IL-10 during intracellular protozoan infection. J Exp Med. 2007;204(2):273-
688 83. Epub 2007/02/07. doi: 10.1084/jem.20062175. PubMed PMID: 17283209; PubMed
689 Central PMCID: PMC2118735.
690 27. Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS,
691 et al. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science.
692 2005;308(5728):1626-9. Epub 2005/04/30. doi: 10.1126/science.1109893. PubMed
693 PMID: 15860593.
694 28. da Silva Correia J, Ulevitch RJ. MD-2 and TLR4 N-linked glycosylations are
695 important for a functional lipopolysaccharide receptor. J Biol Chem. 2002;277(3):1845-
696 54. Epub 2001/11/14. doi: 10.1074/jbc.M109910200. PubMed PMID: 11706042.

697 29. Koblansky AA, Jankovic D, Oh H, Hieny S, Sungnak W, Mathur R, et al.
698 Recognition of profilin by Toll-like receptor 12 is critical for host resistance to
699 Toxoplasma gondii. Immunity. 2013;38(1):119-30. Epub 2012/12/19. doi:
700 10.1016/j.immuni.2012.09.016. PubMed PMID: 23246311; PubMed Central PMCID:
701 PMC3601573.
702 30. Yang CS, Yuk JM, Lee YH, Jo EK. Toxoplasma gondii GRA7-Induced TRAF6
703 Activation Contributes to Host Protective Immunity. Infect Immun. 2015;84(1):339-50.
704 Epub 2015/11/11. doi: 10.1128/IAI.00734-15. PubMed PMID: 26553469; PubMed
705 Central PMCID: PMC4693986.
706 31. Rosowski EE, Lu D, Julien L, Rodda L, Gaiser RA, Jensen KD, et al. Strain-
707 specific activation of the NF-kappaB pathway by GRA15, a novel Toxoplasma gondii
708 dense granule protein. J Exp Med. 2011;208(1):195-212. Epub 2011/01/05. doi:
709 10.1084/jem.20100717. PubMed PMID: 21199955; PubMed Central PMCID:
710 PMC3023140.
711 32. Braun L, Brenier-Pinchart MP, Yogavel M, Curt-Varesano A, Curt-Bertini RL,
712 Hussain T, et al. A Toxoplasma dense granule protein, GRA24, modulates the early
713 immune response to infection by promoting a direct and sustained host p38 MAPK
714 activation. The Journal of experimental medicine. 2013;210(10):2071-86. Epub
715 2013/09/18. doi: 10.1084/jem.20130103. PubMed PMID: 24043761; PubMed Central
716 PMCID: PMC3782045.
717 33. Takabatake N, Okamura M, Yokoyama N, Ikehara Y, Akimitsu N, Arimitsu N,
718 et al. Glycophorin A-knockout mice, which lost sialoglycoproteins from the red blood
719 cell membrane, are resistant to lethal infection of Babesia rodhaini. Vet Parasitol.
720 2007;148(2):93-101. Epub 2007/07/27. doi: 10.1016/j.vetpar.2007.06.011. PubMed
721 PMID: 17651898.

722 34. Persson KE, McCallum FJ, Reiling L, Lister NA, Stubbs J, Cowman AF, et al.
723 Variation in use of erythrocyte invasion pathways by Plasmodium falciparum mediates
724 evasion of human inhibitory antibodies. J Clin Invest. 2008;118(1):342-51. Epub
725 2007/12/08. doi: 10.1172/JCI32138. PubMed PMID: 18064303; PubMed Central
726 PMCID: PMC2117763.
727 35. Favila MA, Geraci NS, Jayakumar A, Hickerson S, Mostrom J, Turco SJ, et al.
728 Differential Impact of LPG-and PG-Deficient Leishmania major Mutants on the
729 Immune Response of Human Dendritic Cells. PLoS Negl Trop Dis.
730 2015;9(12):e0004238. Epub 2015/12/03. doi: 10.1371/journal.pntd.0004238. PubMed
731 PMID: 26630499; PubMed Central PMCID: PMC4667916.
732 36. Nogueira PM, Assis RR, Torrecilhas AC, Saraiva EM, Pessoa NL, Campos MA,
733 et al. Lipophosphoglycans from Leishmania amazonensis Strains Display
734 Immunomodulatory Properties via TLR4 and Do Not Affect Sand Fly Infection. PLoS
735 Negl Trop Dis. 2016;10(8):e0004848. Epub 2016/08/11. doi:
736 10.1371/journal.pntd.0004848. PubMed PMID: 27508930; PubMed Central PMCID:
737 PMC4980043.
738 37. Freitas MS, Oliveira AF, da Silva TA, Fernandes FF, Goncales RA, Almeida F,
739 et al. Paracoccin Induces M1 Polarization of Macrophages via Interaction with TLR4.
740 Front Microbiol. 2016;7:1003. Epub 2016/07/28. doi: 10.3389/fmicb.2016.01003.
741 PubMed PMID: 27458431; PubMed Central PMCID: PMC4932198.
742 38. Alegre-Maller AC, Mendonca FC, da Silva TA, Oliveira AF, Freitas MS, Hanna
743 ES, et al. Therapeutic administration of recombinant Paracoccin confers protection
744 against paracoccidioides brasiliensis infection: involvement of TLRs. PLoS Negl Trop
745 Dis. 2014;8(12):e3317. doi: 10.1371/journal.pntd.0003317. PubMed PMID: 25474158;
746 PubMed Central PMCID: PMC4256291.

747 39. Campbell D, Mann BJ, Chadee K. A subunit vaccine candidate region of the
748 Entamoeba histolytica galactose-adherence lectin promotes interleukin-12 gene
749 transcription and protein production in human macrophages. Eur J Immunol.
750 2000;30(2):423-30. Epub 2000/02/12. doi: 10.1002/1521-4141(200002)30:2<423::AID-
751 IMMU423>3.0.CO;2-0. PubMed PMID: 10671197.
752 40. Murakami S, Iwaki D, Mitsuzawa H, Sano H, Takahashi H, Voelker DR, et al.
753 Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-alpha
754 secretion in U937 cells and alveolar macrophages by direct interaction with toll-like
755 receptor 2. J Biol Chem. 2002;277(9):6830-7. Epub 2001/11/29. doi:
756 10.1074/jbc.M106671200. PubMed PMID: 11724772.
757 41. Unitt J, Hornigold D. Plant lectins are novel Toll-like receptor agonists.
758 Biochem Pharmacol. 2011;81(11):1324-8. Epub 2011/03/23. doi:
759 10.1016/j.bcp.2011.03.010. PubMed PMID: 21420389.
760 42. Souza MA, Carvalho FC, Ruas LP, Ricci-Azevedo R, Roque-Barreira MC. The
761 immunomodulatory effect of plant lectins: a review with emphasis on ArtinM
762 properties. Glycoconj J. 2013;30(7):641-57. Epub 2013/01/10. doi: 10.1007/s10719-
763 012-9464-4. PubMed PMID: 23299509; PubMed Central PMCID: PMC3769584.
764 43. Andrade WA, Souza Mdo C, Ramos-Martinez E, Nagpal K, Dutra MS, Melo
765 MB, et al. Combined action of nucleic acid-sensing Toll-like receptors and
766 TLR11/TLR12 heterodimers imparts resistance to Toxoplasma gondii in mice. Cell
767 Host Microbe. 2013;13(1):42-53. Epub 2013/01/08. doi: 10.1016/j.chom.2012.12.003.
768 PubMed PMID: 23290966; PubMed Central PMCID: PMC3552114.
769 44. Debierre-Grockiego F, Campos MA, Azzouz N, Schmidt J, Bieker U, Resende
770 MG, et al. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived

771 from Toxoplasma gondii. J Immunol. 2007;179(2):1129-37. Epub 2007/07/10. PubMed
772 PMID: 17617606.
773 45. Qiu J, Wang L, Zhang R, Ge K, Guo H, Liu X, et al. Identification of a TNF-
774 alpha inducer MIC3 originating from the microneme of non-cystogenic, virulent
775 Toxoplasma gondii. Scientific reports. 2016;6:39407. Epub 2016/12/22. doi:
776 10.1038/srep39407. PubMed PMID: 28000706; PubMed Central PMCID:
777 PMC5175157.
778 46. Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, et al.
779 Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and
780 regulates parasite-induced IL-12 production by dendritic cells. J Immunol.
781 2002;168(12):5997-6001. Epub 2002/06/11. PubMed PMID: 12055206.
782 47. Plattner F, Yarovinsky F, Romero S, Didry D, Carlier MF, Sher A, et al.
783 Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction
784 of an interleukin-12 response. Cell Host Microbe. 2008;3(2):77-87. Epub 2008/03/04.
785 doi: 10.1016/j.chom.2008.01.001. PubMed PMID: 18312842.
786 48. Saouros S, Edwards-Jones B, Reiss M, Sawmynaden K, Cota E, Simpson P, et
787 al. A novel galectin-like domain from Toxoplasma gondii micronemal protein 1 assists
788 the folding, assembly, and transport of a cell adhesion complex. J Biol Chem.
789 2005;280(46):38583-91. Epub 2005/09/17. doi: 10.1074/jbc.C500365200. PubMed
790 PMID: 16166092.
791 49. von Gunten S, Smith DF, Cummings RD, Riedel S, Miescher S, Schaub A, et al.
792 Intravenous immunoglobulin contains a broad repertoire of anticarbohydrate antibodies
793 that is not restricted to the IgG2 subclass. J Allergy Clin Immunol. 2009;123(6):1268-
794 76 e15. Epub 2009/05/16. doi: 10.1016/j.jaci.2009.03.013. PubMed PMID: 19443021;
795 PubMed Central PMCID: PMC2777748.

796 50. Shen B, Brown KM, Lee TD, Sibley LD. Efficient gene disruption in diverse
797 strains of Toxoplasma gondii using CRISPR/CAS9. mBio. 2014;5(3):e01114-14. Epub
798 2014/05/16. doi: 10.1128/mBio.01114-14. PubMed PMID: 24825012; PubMed Central
799 PMCID: PMC4030483.
800 51. Soldati D, Boothroyd JC. Transient transfection and expression in the obligate
801 intracellular parasite Toxoplasma gondii. Science. 1993;260(5106):349-52. Epub
802 1993/04/16. PubMed PMID: 8469986.
803 52. Kim K, Soldati D, Boothroyd JC. Gene replacement in Toxoplasma gondii with
804 chloramphenicol acetyltransferase as selectable marker. Science. 1993;262(5135):911-
805 4. Epub 1993/11/05. PubMed PMID: 8235614.
806 53. Aricescu AR, Owens RJ. Expression of recombinant glycoproteins in
807 mammalian cells: towards an integrative approach to structural biology. Curr Opin
808 Struct Biol. 2013;23(3):345-56. doi: 10.1016/j.sbi.2013.04.003. PubMed PMID:
809 23623336; PubMed Central PMCID: PMC4757734.
810 54. Mariano VS, Zorzetto-Fernandes AL, da Silva TA, Ruas LP, Nohara LL,
811 Almeida IC, et al. Recognition of TLR2 N-glycans: critical role in ArtinM
812 immunomodulatory activity. PLoS One. 2014;9(6):e98512. doi:
813 10.1371/journal.pone.0098512. PubMed PMID: 24892697; PubMed Central PMCID:
814 PMC4043963.
815 55. Carneiro AB, Iaciura BM, Nohara LL, Lopes CD, Veas EM, Mariano VS, et al.
816 Lysophosphatidylcholine triggers TLR2- and TLR4-mediated signaling pathways but
817 counteracts LPS-induced NO synthesis in peritoneal macrophages by inhibiting NF-
818 kappaB translocation and MAPK/ERK phosphorylation. PLoS One. 2013;8(9):e76233.
819 doi: 10.1371/journal.pone.0076233. PubMed PMID: 24312681; PubMed Central
820 PMCID: PMC3848743.

821 56. Young L, Sung J, Stacey G, Masters JR. Detection of Mycoplasma in cell
822 cultures. Nat Protoc. 2010;5(5):929-34. doi: 10.1038/nprot.2010.43. PubMed PMID:
823 20431538.

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825 **AUTHOR INFORMATION**

826 **Aline Sardinha-Silva**

827 Present Address: Molecular Parasitology Section, Laboratory of Parasitic Diseases,
828 National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000
829 Rockville Pike, Bethesda, MD 20892, USA.

830

831 **AFFILIATIONS**

832 **Department of Cell and Molecular Biology and Pathogenic Bioagents, Ribeirão**
833 **Preto Medical School, University of São Paulo- USP (FMRP/USP), Ribeirão Preto,**
834 **São Paulo, 14049-900, Brazil**

835 Aline Sardinha-Silva, Flávia C. Mendonça-Natividade, Camila F. Pinzan, Carla D.
836 Lopes, Fabrício F. Fernandes, André L. V. Zorzetto-Fernandes & Maria Cristina Roque-
837 Barreira

838

839 **Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of**
840 **Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD**
841 **20892, USA**

842 Diego L. Costa, Alan Sher & Dragana Jankovic

843

844 **Department of Microbiology and Molecular Medicine, CMU, University of**
845 **Geneva, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland**

Damien Jacot & Dominique Soldati-Favre

Department of Biochemistry, Cambridge University, 80 Tennis Court Road Cambridge CB2 1GA, United Kingdom

Nicholas J. Gay

Molecular Parasitology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Michael E. Grigg

CONTRIBUTIONS

A.S.S., F.C.M.N. and M.C.R.B. designed the experiments; A.S.S., F.C.M.N., C.F.P., C.D.L., D.L.C., F.F.F., A.L.V.Z.F. and D.Jan. performed the experiments; A.S.S., F.C.M.N. and C.D.L. and processed and analysed the data; D.Jac. and D.S.F. generated the *Toxoplasma gondii* knockout strains; N.J.G., M.E.G and A.S. provided reagents/material and contributed for the final manuscript writing; A.S.S., F.C.M.N., and M.C.R.B. wrote the manuscript; and all authors commented on the manuscript.

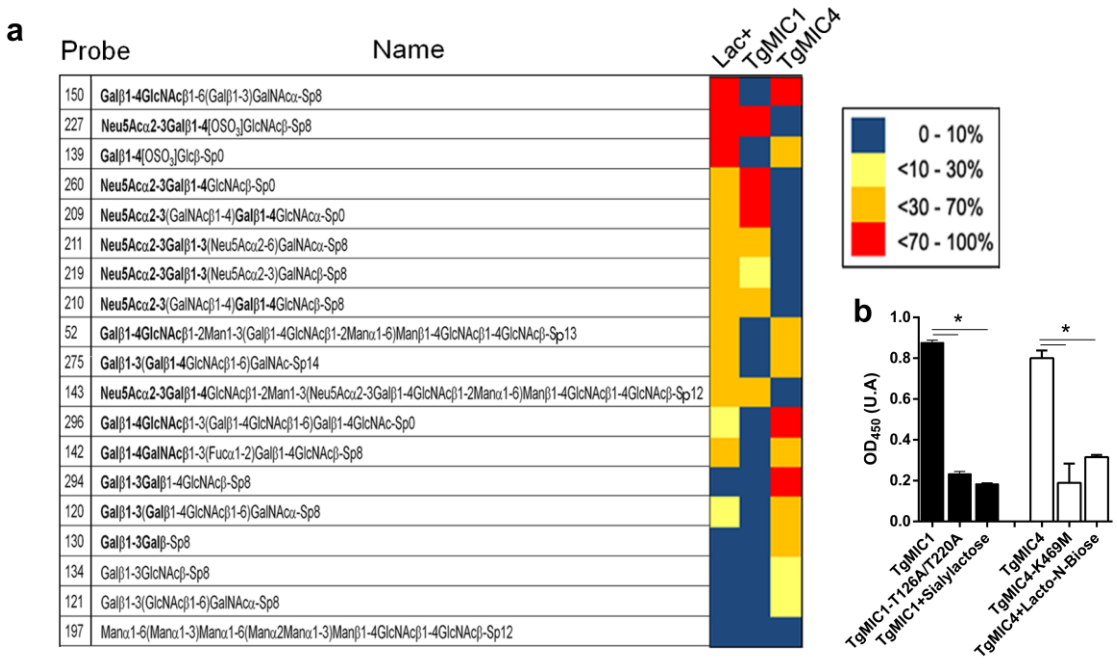
COMPETING INTERESTS

The authors declare no competing financial interests

CORRESPONDING AUTHOR

Maria Cristina Roque-Barreira, Phone: +55-16-3315-3062, e-mail: mcrbarre@fmrp.usp.br

871 **FIGURES and LEGENDS**



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873 **Fig 1. Lectin activity of TgMIC1 and TgMIC4. (A)** Glycoarray of the native
874 TgMIC1/TgMIC4 subcomplex (Lac+) and of the recombinant forms of TgMIC1 and
875 TgMIC4. In total, 320 oligosaccharide probes were analysed by reading their
876 fluorescence intensities, and the 20 best recognized glycans are shown. The results were
877 represented as previously reported [18]. **(B)** The activity and inhibition assays were
878 performed in microplates coated with glycoproteins with or without sialic acid, fetuin
879 (black bars), or asialofetuin (white bars), separately. After coating, wild type or mutated
880 TgMIC1 and TgMIC4, pre-incubated with PBS or their corresponding sugars, were
881 added to the wells. Later, bound proteins were detected through the addition of serum
882 from *T. gondii*-infected mice. Data in **(B)** are expressed as mean ±S.D. of triplicate wells
883 and significance was calculated with ANOVA. *P<0.05. Data are representative of two
884 **(B)** independent experiments. Gal: galactose; GalNAc: *N*-acetylgalactosamine; Glc:
885 glucose; Man: mannose; Fuc: fucose; Neu5Ac: *N*-acetylneuraminic acid; wt: wild type

886 protein; mut: protein with a mutation in the carbohydrate-recognition domain (CRD); ns:
887 not significant.
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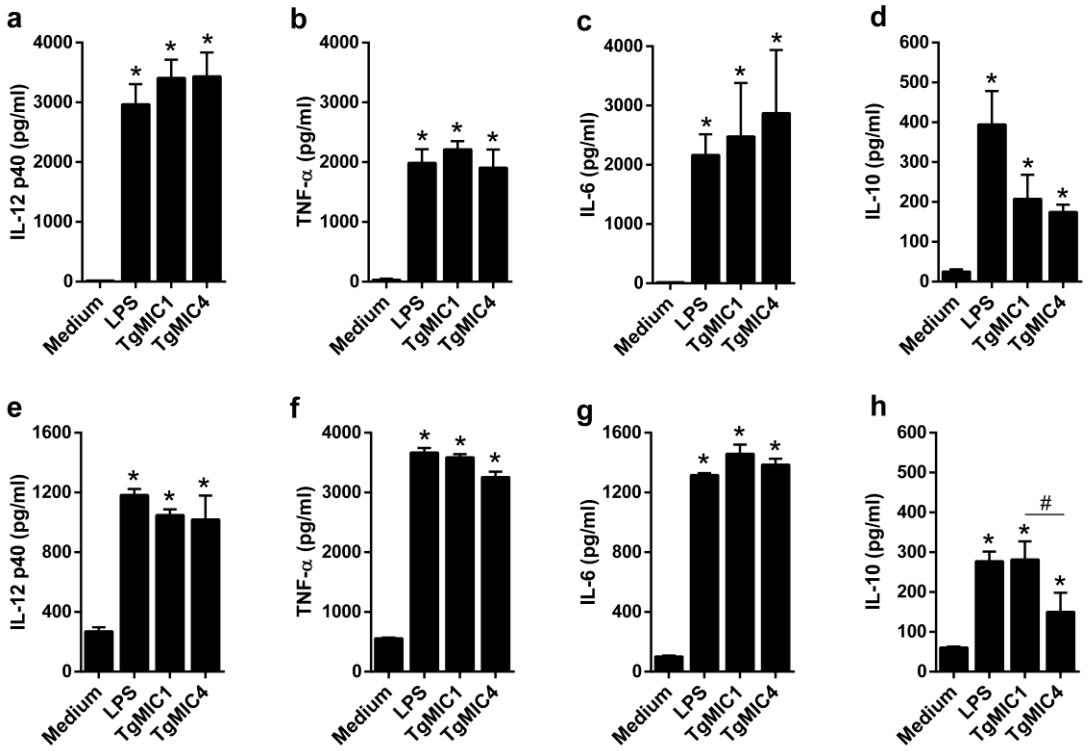
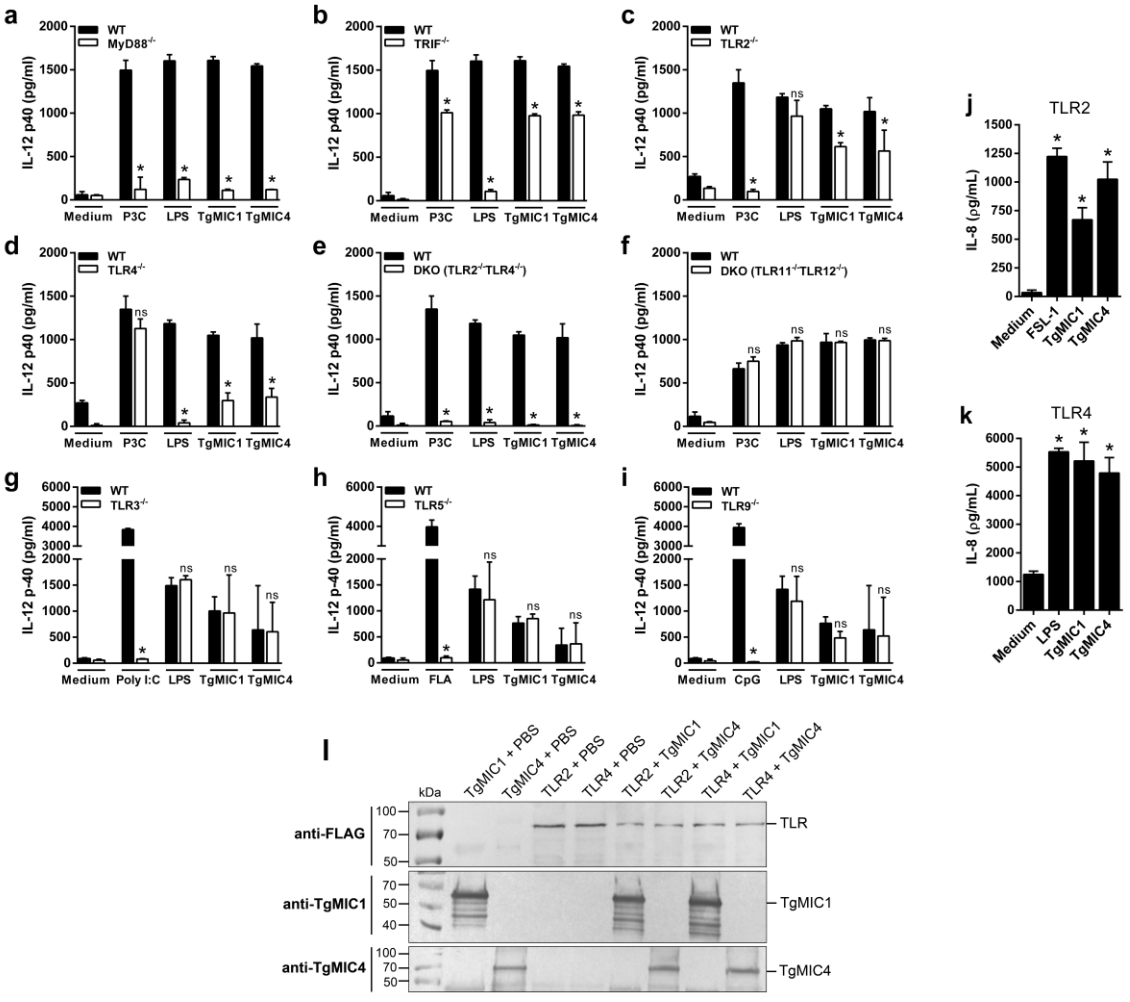


Fig 2. Microneme proteins stimulate proinflammatory cytokine production by dendritic cells and macrophages. (A-D) Bone marrow-derived dendritic cells and **(E-H)** bone marrow-derived macrophages from C57BL/6 mice were stimulated with TgMIC1 (5 μg/mL) and TgMIC4 (5 μg/mL) for 48 h. LPS (100 ng/mL) was used as positive control. The levels of IL-12p40, TNF-α, and IL-6 were measured by ELISA. Data are expressed as mean ±S.D. of triplicate wells and significance was calculated with ANOVA. *P<0.05. Data are representative of three independent experiments.



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912 **Fig 3. The IL-12 production induced by TgMICs is dependent on binding to TLR2**
913 **and TLR4. (A-I)** Bone marrow-derived macrophages from WT, TLR2$^{-/-}$, TLR4$^{-/-}$,
914 double knockout TLR2$^{-/-}$/TLR4$^{-/-}$, TLR3$^{-/-}$, TLR5$^{-/-}$, TLR9$^{-/-}$, and double knockout
915 TLR11$^{-/-}$/TLR12$^{-/-}$ mice, all of the C57BL/6 background, were stimulated with TgMIC1
916 or TgMIC4 (5 μg/mL) for 48 h. Pam3CSK4 (P3C) (1 μg/mL), LPS (100 ng/mL), Poly
917 I:C (10 μg/mL), Flagellin (FLA) (1 μg/mL) and CpG (25 μg/mL) were used as positive
918 controls. IL-12p40 levels were measured by ELISA. **(J and K)** Transfected HEK293T
919 cells expressing TLR2 were stimulated with TgMIC1 (750 nM) or TgMIC4 (500 nM),
920 and TgMIC1 (200 nM) or TgMIC4 (160 nM) for HEK cells expressing TLR4, for 24 h.
921 FSL-1 (100 ng/mL) and LPS (100 ng/mL) were used as positive controls. IL-8 levels
922 were measured by ELISA. **(L)** The interaction between TgMICs and TLRs was

evaluated by western blot. HEK293T cells transiently expressing TLR2-Flag and TLR4-Flag were lysed and incubated with His-TgMIC1 (WT) or His-TgMIC4 (WT). His-TgMICs were subjected to Ni-affinity resin pull-down and analysed for TLR2 and TLR4 binding by protein blotting with antibodies specific for Flag and then for TgMIC. Data in **(A-K)** are expressed as mean ±S.D. of triplicate wells and significance was calculated with ANOVA. *P<0.05. Data are representative of three **(A-K)** and two **(L)** independent experiments.

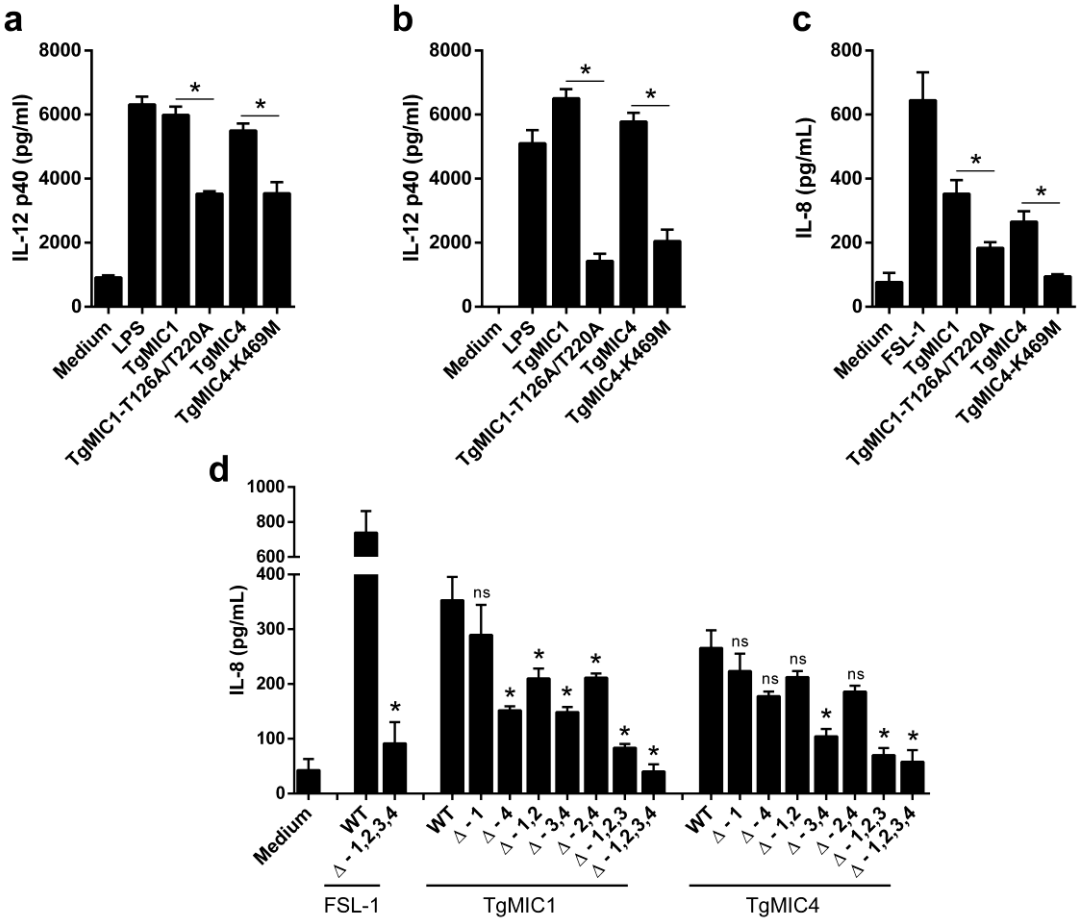
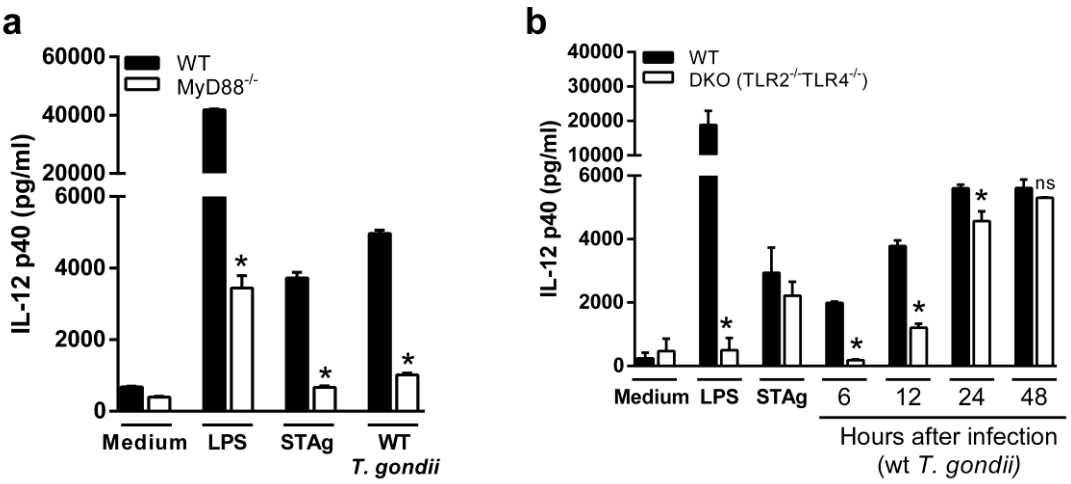


Fig 4. The cellular activation induced by TgMICs via TLRs depends on carbohydrate recognition. (A) Bone marrow-derived macrophages and **(B)** bone marrow-derived dendritic cells from C57BL/6 mice and **(C)** transfected HEK293T cells expressing fully glycosylated TLR2 were stimulated with TgMIC1 (WT) and TgMIC4 (WT) or with their mutated forms, TgMIC1-T126A/T220A and TgMIC4-K469M, 5 μg/mL of each, for 48 h. LPS (100 ng/mL) and FSL-1 (100 ng/mL) were used as positive controls. IL-12p40 and IL-8 levels were measured by ELISA. **(D)** HEK293T cells expressing fully glycosylated TLR2 (with 4 N-glycans, WT) or glycosylation mutants of TLR2 (Δ-1; Δ-4; Δ-1,2; Δ-3,4; Δ-2,4; Δ-1,2,3; Δ-1,2,3,4) were stimulated with TgMIC1 or TgMIC4. FSL-1 (100 ng/mL) was used as positive control. IL-8 levels were measured by ELISA. The statistical analysis compared fully glycosylated TLR2

(WT) and TLR2 mutants for the N-glycosylation sites for the same stimuli. Data are expressed as mean ±S.D. of triplicate wells and significance was calculated with ANOVA. *P<0.05. Data are representative of three independent experiments.



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971 **Fig 5. Initial production of IL-12 by dendritic cells during *T. gondii* infection**

972 **depends on TLR2 and TLR4.** Bone marrow-derived dendritic cells from WT, **(A)**

973 MyD88$^{-/-}$, and **(B)** DKO TLR2$^{-/-}$/TLR4$^{-/-}$ mice (C57BL/6 background) were infected

974 with WT *T. gondii* (RH strain, MOI 3). LPS (500 ng/mL) and STAg (10 μg/mL)

975 (soluble *T. gondii* antigen) were used as positive controls. Cell-culture supernatants

976 were collected after 6, 12, 24, and 48 h. IL-12p40 production was analysed by ELISA.

977 Data are expressed as mean ±S.D. of triplicate wells and significance was calculated

978 with ANOVA. *P<0.05. Data are representative of two **(A)** and three **(B)** independent

979 experiments.

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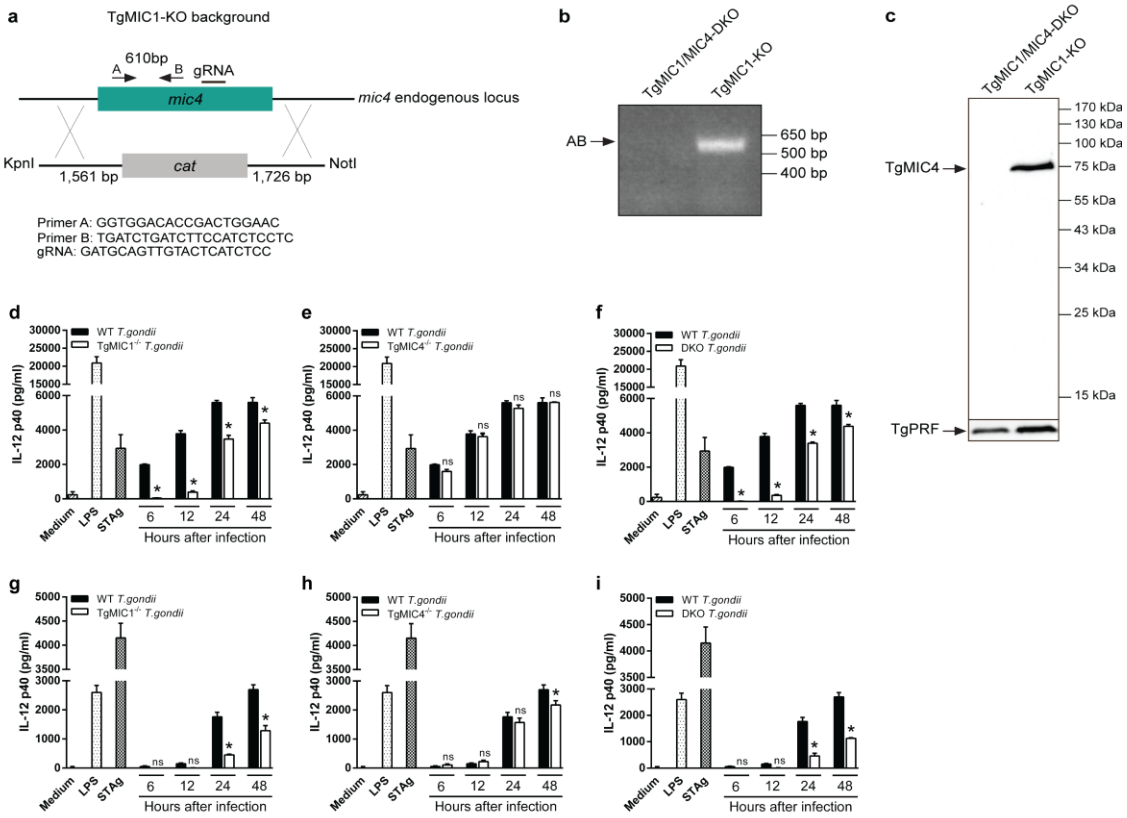

Fig 6. TgMIC1 is important for inducing the initial production of IL-12 during *in vitro* infection with *T. gondii*. (A) Schematic representation of chloramphenicol (cat) cassette with flanking TgMIC4 regions that was inserted into the endogenous *TgMIC4* locus in the recipient TgMIC1-KO strain. **(B)** Genomic DNA was isolated from both strains, and *TgMIC4* gene expression (610 bp from the upstream region) was analysed by PCR. **(C)** Western blot analyses of an equal loading of whole cell lysates corresponding to 5×10^6 tachyzoites from TgMIC1/TgMIC4-DKO and TgMIC1-KO parasites. The membrane was probed with the anti-MIC4 rabbit antibody. **(D-F)** Bone marrow-derived dendritic cells and **(G-I)** bone marrow-derived macrophages from C57BL/6 mice were infected with WT, TgMIC1-KO, TgMIC4-KO, or DKO TgMIC1/TgMIC4-dKO *T. gondii* (RH strain, MOI 3:1). LPS (500 ng/mL) and STAg (10 μg/mL) (soluble *T. gondii* antigen) were used as positive controls. Cell-culture supernatants were collected after 6, 12, 24, and 48 h. IL-12 production was analysed by

ELISA. Data are expressed as mean ±S.D. of triplicate wells and significance was calculated with ANOVA. *P<0.05. Data in **(D-I)** are representative of three independent experiments.