

1 **The lectin-specific activity of *Toxoplasma gondii* microneme proteins 1**  
2 **and 4 binds Toll-like receptor 2 and 4 N-glycans to regulate innate**  
3 **immune priming**

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## 24 ABSTRACT

25 Infection of host cells by *Toxoplasma gondii* is an active process, which is  
26 regulated by secretion of microneme (MICs) and rhoptry proteins (ROPs and RONs) from  
27 specialized organelles in the apical pole of the parasite. MIC1, MIC4 and MIC6 assemble  
28 into an adhesin complex, secreted on the parasite surface and function to promote  
29 infection competency. MIC1 and MIC4 are known to bind terminal sialic acid residues  
30 and galactose residues, respectively and to induce IL-12 production from splenocytes.  
31 Here we show that rMIC1- and rMIC4-stimulated dendritic cells and macrophages to  
32 produce proinflammatory cytokines, and they do so by engaging TLR2 and TLR4. This  
33 process depends on sugar recognition, since point mutations in the carbohydrate-  
34 recognition domains (CRD) of rMIC1 and rMIC4 inhibit innate immune cells activation.  
35 HEK cells transfected with TLR2 glycomutants were selectively unresponsive to MICs.  
36 Following *in vitro* infection, parasites lacking MIC1 or MIC4, as well as expressing MIC  
37 proteins with point mutations in their CRD, failed to induce wild-type (WT) levels of IL-  
38 12 secretion by innate immune cells. However, only MIC1 was shown to impact systemic  
39 levels of IL-12 and IFN- $\gamma$  *in vivo*. Together, our data show that MIC1 and MIC4 interact  
40 physically with TLR2 and TLR4 N-glycans to trigger IL-12 responses, and MIC1 is  
41 playing a significant role *in vivo* by altering *T. gondii* infection competency and murine  
42 pathogenesis.

43

## 44 AUTHOR SUMMARY

45 Toxoplasmosis is caused by the protozoan *Toxoplasma gondii*, belonging to the  
46 Apicomplexa phylum. This phylum comprises important parasites able to infect a broad  
47 diversity of animals, including humans. A particularity of *T. gondii* is its ability to  
48 invade virtually any nucleated cell of all warm-blooded animals through an active  
49 process, which depends on the secretion of adhesin proteins. These proteins are  
50 discharged by specialized organelles localized in the parasite apical region, and termed  
51 micronemes and rhoptries. We show in this study that two microneme proteins from *T.*  
52 *gondii* utilize their adhesion activity to stimulate innate immunity. These microneme  
53 proteins, denoted MIC1 and MIC4, recognize specific sugars on receptors expressed on  
54 the surface of mammalian immune cells. This binding activates these innate immune  
55 cells to secrete cytokines, which promotes efficient host defense mechanisms against the

56 parasite and regulate their pathogenesis. This activity promotes a chronic infection by  
57 controlling parasite replication during acute infection.

58

## 59 INTRODUCTION

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

66 *T. gondii* invades host cells through an active process that relies on the parasite  
67 actinomyosin system, concomitantly with the release of microneme proteins (MICs) and  
68 rhoptry neck proteins (RONs) from specialized organelles in the apical pole of the  
69 parasite [3]. These proteins are secreted by tachyzoites [4, 5] and form complexes  
70 composed of soluble and transmembrane proteins. Some of the MICs act as adhesins,  
71 interacting tightly with host cell-membrane glycoproteins and receptors, and are  
72 involved in the formation of the moving junction [6]. This sequence of events ensures  
73 tachyzoite gliding motility, migration through host cells, invasion and egress from  
74 infected cells [4, 7]. Among the released proteins, MIC1, MIC4, and MIC6 form a  
75 complex that, together with other *T. gondii* proteins, plays a role in the adhesion and  
76 invasion of host cells [8, 9], contributing to the virulence of the parasite [10, 11].

77 Several studies have shown that host-cell invasion by apicomplexan parasites  
78 such as *T. gondii* involves carbohydrate recognition [12-15]. Interestingly, MIC1 and  
79 MIC4 have lectin domains [11, 16-18] that recognize oligosaccharides with sialic acid  
80 and D-galactose in the terminal position, respectively. Importantly, the parasite's Lac<sup>+</sup>  
81 subcomplex, consisting of MIC1 and MIC4, induces adherent spleen cells to release IL-  
82 12 [17], a cytokine critical for the protective response of the host to *T. gondii* infection  
83 [19]. In addition, immunization with this native subcomplex, or with recombinant MIC1  
84 (rMIC1) and MIC4 (rMIC4), protects mice against experimental toxoplasmosis [20, 21].  
85 The induction of IL-12 is typically due to detection of the pathogen by innate immunity  
86 receptors, including members of the Toll-like receptor (TLR) family, whose stimulation  
87 involves MyD88 activation and priming of Th1 responses, which protects the host  
88 against *T. gondii* [19, 22]. It is also known that dysregulated expression of IL-12 and

89 IFN- $\gamma$  during acute toxoplasmosis can drive a lethal immune response, in which mice  
90 succumb to infection by severe immunopathology, the result of insufficient levels of IL-  
91 10 and/or a collapse in the regulatory CD4+Foxp3+ T cell population [23, 24].

92 Interestingly, regarding the innate immune receptors associated with IL-12  
93 response during several infections, the extracellular leucine-rich repeat domains of  
94 TLR2 and TLR4 contain four and nine N-glycans, respectively [25]. Therefore, we  
95 hypothesized that MIC1 and MIC4 bind TLR2 and TLR4 N-glycans on antigen-  
96 presenting cells (APCs) and, through this interaction, trigger immune cell activation and  
97 IL-12 production. To investigate this possibility, we assayed the ability of rMIC1 and  
98 rMIC4 to bind and activate TLR2 and TLR4. Using several strategies, we demonstrated  
99 that TLR2 and TLR4 are indeed critical targets for both MIC1 and MIC4. These  
100 parasite and host cell structures establish lectin-carbohydrate interactions that contribute  
101 to the induction of IL-12 production by innate immune cells, and we show here that the  
102 MIC1 lectin promotes *T. gondii* infection competency and regulates parasite virulence  
103 during *in vivo* infection.

104

## 105 **RESULTS**

### 106 **Lectin properties of recombinant MIC1 and MIC4 are consistent with those of the** 107 **native Lac<sup>+</sup> subcomplex**

108 The native MIC1/4 subcomplex purified from soluble *T. gondii* antigens has  
109 lectin properties, so we investigated whether their recombinant counterparts retained the  
110 sugar-binding specificity. The glycoarray analysis revealed the interactions of: i) the  
111 Lac<sup>+</sup> subcomplex with glycans containing terminal  $\alpha(2-3)$ -sialyl and  $\beta(1-4)$ - or  $\beta(1-3)$ -  
112 galactose; ii), rMIC1 with  $\alpha(2-3)$ -sialyl residues linked to  $\beta$ -galactosides; and iii) of  
113 rMIC4 with oligosaccharides with terminal  $\beta(1-4)$ - or  $\beta(1-3)$ -galactose (Fig 1A). The  
114 combined specificities of the individual recombinant proteins correspond to the dual  
115 sugar specificity of the Lac<sup>+</sup> fraction, demonstrating that the sugar-recognition  
116 properties of the recombinant proteins are consistent with those of the native ones.

117 Based on the sugar recognition selectivity of rMIC1 and rMIC4, we tested two  
118 oligosaccharides ( $\alpha(2-3)$ -sialyllactose and lacto-N-biose) for their ability to inhibit the  
119 interaction of the MICs with the glycoproteins fetuin and asialofetuin [26]. Sialyllactose  
120 inhibited the binding of rMIC1 to fetuin, and lacto-N-biose inhibited the binding of  
121 rMIC4 to asialofetuin (Fig 1B). To ratify the carbohydrate recognition activity of rMIC1

122 and rMIC4, we generated point mutations into the carbohydrate recognition domains  
123 (CRDs) of the rMICs to abolish their lectin properties [11, 18, 27]. These mutated  
124 forms, i.e. rMIC1-T126A/T220A and rMIC4-K469M, lost their capacity to bind to  
125 fetuin and asialofetuin, respectively (Fig 1B), having absorbance as low as that in the  
126 presence of the specific sugars. Thus, our results indicate that rMIC1 and rMIC4  
127 maintained their lectin properties, and that the CRD function can be blocked either by  
128 competition with specific sugars or by targeted mutations.

### 129 **rMIC1 and rMIC4 trigger the activation of DCs and macrophages**

130 We have previously demonstrated that the native Lac<sup>+</sup> subcomplex stimulates  
131 murine adherent spleen cells to produce proinflammatory cytokines [20]. We evaluated  
132 whether recombinant MIC1 and MIC4 retained this property and exerted it on BMDCs  
133 and BMDMs. BMDCs (Fig 2A-2D) and BMDMs (Fig 2E-2H) produced high levels of  
134 the proinflammatory cytokines IL-12 (Fig 2A and 2E), TNF- $\alpha$  (Fig 2B and 2F), and IL-  
135 6 (Fig 2C and 2G). This was not attributable to residual LPS contamination as the  
136 recombinant protein assays were done in the presence of polymyxin B, and LPS levels  
137 were less than 0.5ng/ml [see Materials and Methods section]. Although conventional  
138 CD4<sup>+</sup> Th1 cells are known to be the major producers of IL-10 during murine *T. gondii*  
139 infection [28], we also found that rMIC1 and rMIC4 induced the production of this  
140 cytokine by BMDCs (Fig 2D) and BMDMs (Fig 2H). We verified that the two  
141 recombinant proteins induced the production of similar levels of IL-12, TNF- $\alpha$ , and IL-  
142 6 by both BMDCs (Fig 2A-2C) and BMDMs (Fig 2E-2G). Both MICs induced the  
143 production of similar levels of IL-10 in BMDCs (Fig 2D); however, BMDMs produced  
144 significantly higher levels of IL-10 when stimulated with rMIC1 than when stimulated  
145 with rMIC4 (Fig 2H). These cytokine levels were similar to those induced by the TLR4  
146 agonist LPS. Thus, recombinant MIC1 and MIC4 induce a proinflammatory response in  
147 innate immune cells, which is consistent with the results obtained for the native Lac<sup>+</sup>  
148 subcomplex [20].

### 149 **The activation of macrophages by rMIC1 and rMIC4 depends on TLR2 and TLR4**

150 To investigate the mechanisms through which *T. gondii* MIC1 and MIC4  
151 stimulate innate immune cells to produce cytokines, we assessed whether these MICs  
152 can activate specific TLRs. To this end, BMDMs from WT, MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, TLR2<sup>-/-</sup>,  
153 TLR4<sup>-/-</sup>, or TLR2/4 DKO mice, as well as HEK293T cells transfected with TLR2 or  
154 TLR4, were cultured in the presence or absence of rMIC1 and rMIC4 for 48 hours. The

155 production of IL-12 by BMDMs (Fig 3A-3I) and IL-8 by HEK cells (Fig 3J and 3K)  
156 were used as an indicator of cell activation. IL-12 production by BMDMs from MyD88-  
157 <sup>-/-</sup>, TRIF<sup>-/-</sup>, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> mice was lower than that of BMDMs from WT mice  
158 (Fig 3A-3D); no IL-12 was detected in cultures of TLR2/4 DKO mice cells stimulated  
159 with either rMIC1 or rMIC4 (Fig 3E). These results show that TLR2 and TLR4 are both  
160 relevant for the activation of macrophages induced by rMIC1 and rMIC4. The residual  
161 cytokine production observed in macrophages from TLR2<sup>-/-</sup> or MyD88<sup>-/-</sup> mice may be  
162 the result of activation of TLR4 (Fig 3A and 3C), and vice versa; e.g., the residual IL-12  
163 levels produced by macrophages from TLR4<sup>-/-</sup> mice may be the result of TLR2  
164 activation. The finding that MICs fail to induce IL-12 production in DKO mice  
165 BMDMs suggests that cell activation triggered by *T. gondii* MIC1 or MIC4 does not  
166 require the participation of other innate immunity receptors beyond TLR2 and TLR4.  
167 Nevertheless, because parasite components such as DNA or profilin engage TLR9,  
168 TLR11, and TLR12 to produce IL-12 in macrophages [19, 22, 29], we investigated the  
169 involvement of these receptors, as well as TLR3 and TLR5, in the response to rMIC1 or  
170 rMIC4. BMDMs from TLR3<sup>-/-</sup>, TLR5<sup>-/-</sup>, TLR9<sup>-/-</sup>, and TLR11/12 DKO mice stimulated  
171 with rMIC1 or rMIC4 produced similar levels of IL-12 as cells from WT (Fig 3F-3I),  
172 indicating that the activation triggered by rMIC1 or rMIC4 does not depend on these  
173 receptors. Additionally, stimulation of HEK cells transfected with human TLR2 (Fig 3J)  
174 or TLR4 (Fig 3K) with optimal concentrations of rMIC1 (Fig S1A and S1C) and rMIC4  
175 (Fig S1B and S1D) induced IL-8 production at levels that were higher than those  
176 detected in the absence of stimuli (medium), and similar to those induced by the  
177 positive controls. Finally, by means of a pull-down experiment, we demonstrated a  
178 physical interaction between rMIC1 and TLR2 or TLR4 and between rMIC4 and TLR2  
179 or TLR4 (Fig 3L).

### 180 **Cell activation induced by rMIC1 and rMIC4 results from the interaction of their** 181 **CRDs with TLR2 and TLR4 N-glycans**

182 We hypothesized that in order to trigger cell activation, rMIC1 and rMIC4 CRDs  
183 target oligosaccharides of the ectodomains of TLR2 (four N-linked glycans) [25] and  
184 TLR4 (nine N-linked glycans) [30]. This hypothesis was tested by stimulating BMDCs  
185 (Fig 4A) and BMDMs (Fig 4B) from WT mice with intact rMIC1 and rMIC4 or with  
186 the mutated forms of these microneme proteins, namely rMIC1-T126A/T220A and  
187 rMIC4-K469M, which lack carbohydrate binding activity [11, 18, 27]. IL-12 levels in

188 culture supernatants were lower upon stimulation with rMIC1-T126A/T220A or rMIC4-  
189 K469M, showing that WT induction of cell activation requires intact rMIC1 and rMIC4  
190 CRDs. The same microneme proteins were used to stimulate TLR2-transfected  
191 HEK293T cells (Fig 4C), and similarly, lower IL-8 production was obtained in response  
192 to mutated rMIC1 or rMIC4 compared to that seen in response to intact proteins. These  
193 observations demonstrated that rMIC1 and rMIC4 CRDs are also necessary for inducing  
194 HEK cell activation.

195 We used an additional strategy to examine the ability of rMIC1 and rMIC4 to  
196 bind to TLR2 N-glycans. In this approach, HEK cells transfected with the fully N-  
197 glycosylated TLR2 ectodomain or with the TLR2 glycomutants [25] were stimulated  
198 with a control agonist (FSL-1) or with rMIC1 or rMIC4. HEK cells transfected with any  
199 TLR2 form, except those expressing totally unglycosylated TLR2 (mutant  $\Delta 1,2,3,4$ ),  
200 were able to respond to FSL-1 (Fig 4D), a finding that is consistent with the previous  
201 report that the  $\Delta 1,2,3,4$  mutant is not secreted by HEK293T cells [25]. Cells transfected  
202 with TLR2 lacking only the first or the third N-glycan (mutant  $\Delta 1$ ;  $\Delta 3$ ) responded to all  
203 stimuli. The response to the rMIC1 stimulus was significantly reduced in cells  
204 transfected with five different TLR2 mutants, lacking some combination of the second,  
205 third, and fourth N-glycans (Fig 4D). Moreover, rMIC4 stimulated IL-8 production was  
206 significantly reduced in cells transfected with the mutants lacking some combination of  
207 the third and fourth N-glycans (Fig 4D).

208 These results indicate that *T. gondii* MIC1 and MIC4 use their CRDs to induce  
209 TLR2- and TLR4-mediated cell activation. Among the TLR2 N-glycans, the rMIC1  
210 CRD likely targets the second, third, and fourth glycan, whereas the rMIC4 CRD targets  
211 only the third and fourth. Additionally, our findings suggested that TLR2 and TLR4  
212 activation is required to enhance the production of IL-12 by APCs following rMIC  
213 stimulation.

#### 214 **The IL-12 production during *T. gondii* *in vitro* infection depends partially on MIC1** 215 **and MIC4 proteins and their ability to recognize carbohydrates on APCs surface.**

216 Because IL-12 production is induced by rMICs that engage TLR2 and TLR4 N-  
217 glycans expressed on innate immune cells, we investigated whether such production is  
218 impaired when APCs are infected with *T. gondii* lacking MIC1 and/or MIC4 proteins,  
219 as well as complemented strains expressing mutant versions of these proteins that fail to  
220 bind TLR2 or TLR4 carbohydrates. We generated  $\Delta mic1$  and  $\Delta mic4$  strains in an RH

221 strain expressing GFP and Luciferase using CRISPR/Cas9 to replace the endogenous  
222 MIC gene with the drug-selectable marker HPT (HXGPRT – hypoxanthine-xanthine-  
223 guanine phosphoribosyl transferase) (Fig 5A and 5B). We then complemented MIC  
224 deficient parasites with mutated versions expressing an HA-tag, thus generating the  
225  $\Delta mic1::MIC1-T126A/T220A^{HA}$  or  $\Delta mic4::MIC4-K469M^{HA}$  strains (Fig 5A) that  
226 expressed endogenous levels of MIC1 and MIC4 as confirmed by Western Blotting (Fig  
227 5C).

228 IL-12 secretion by BMDCs and BMDMs infected with WT,  $\Delta mic1$ ,  
229  $\Delta mic1::MIC1-T126A/T220A$ ,  $\Delta mic4$  and  $\Delta mic4::K469M$  parasites was assessed at 24  
230 hours post infection. All mutant strains ( $\Delta mic1$ ,  $\Delta mic1::MIC1-T126A/T220A$ ,  $\Delta mic4$   
231 and  $\Delta mic4::K469M$ ) induced lower IL-12 secretion by BMDCs (Fig 5D) and BMDMs  
232 (Fig 5E) compared to that induced by WT parasites, indicating that engagement of  
233 TLR2 and TLR4 cell surface receptors by the MIC lectin-specific activity led to an early  
234 release of IL-12.

235 Using flow cytometry, we confirmed that parasites deficient in MIC1 or MIC4,  
236 or mutated in their carbohydrate recognition domain resulted in lower intracellular IL-  
237 12 production than WT infected BMDCs (Fig 5F-5H). Interestingly, the Toxo<sup>+</sup> BMDCs  
238 presented the same level of intracellular IL-12, independent of the *T. gondii* strain  
239 infected (Fig 5F and 5H). Whereas the Toxo<sup>-</sup> BMDCs produced less IL-12 when they  
240 were infected with knockout or CRD-mutated *T. gondii* compared to WT-infected cells  
241 (Fig 5G and 5H). Taken altogether, these results indicate that MIC1 and MIC4 induce  
242 IL-12 production in innate immune cells during *in vitro T. gondii* infection. It is known  
243 that other parasite factors act as IL-12 inducers, such as profilin, which is a TLR11 and  
244 TLR12 agonist [29, 31], or GRA7 [32], GRA15 [33], and GRA24 [34], which directly  
245 trigger intracellular signalling pathways in a TLR-independent manner, and these likely  
246 account for the majority of IL-12 released after 24 hours of intracellular infection.

247

248 **MIC1, but not MIC4, contributes to the cytokine storm and acute death during *in***  
249 ***vivo* murine infection with *T. gondii*.**

250 Given the importance of MIC1 and MIC4 as lectins that engage TLR2 and TLR4  
251 N-glycans to induce increased levels of IL-12 release during *T. gondii in vitro* infection,  
252 we investigated the biological relevance of these proteins during *in vivo* infection. Mice  
253 were injected with 50 tachyzoites of RH WT,  $\Delta mic1$ ,  $\Delta mic1::MIC1-T126A/T220A$ ,



254  $\Delta mic4$  and  $\Delta mic4::MIC4$ -K469M strains into the peritoneum of CD-1 outbred mice, a  
255 lethal dose that causes acute mortality. The survival curve showed that parasites  
256 deficient in MIC1 ( $\Delta mic1$  group) or mutated to remove MIC1 lectin binding activity  
257 ( $\Delta mic1::MIC1$ -T126A/T220A group) were less virulent, resulting in a slight, but  
258 significant ( $p=0.0017$ ) increase in mouse survival (12 days post-infection) compared to  
259 WT infected mice that all succumbed to infection by day 10 (Fig 6A). This was not the  
260 result of a difference in parasite load, which was equivalent across all *T. gondii*-infected  
261 mice at Day 5 (Fig 6D and 6I). Whereas, the absence of the MIC4 gene or MIC4 lectin  
262 activity did not change the survival curve (Fig 6E) indicating that MIC4 is less relevant  
263 than MIC1 during *in vivo* infection.

264 Acute mortality in CD-1 mice infected with Type I *T. gondii* is related to the  
265 induction of a cytokine storm, mediated by high levels of IFN- $\gamma$  production. Thus, we  
266 measured systemic levels of IFN- $\gamma$  and IL-12 in mice infected with WT,  $\Delta mic1$ ,  
267  $\Delta mic1::MIC1$ -T126A/T220A,  $\Delta mic4$  and  $\Delta mic4::MIC4$ -K469M strains. According to  
268 Kugler et al. (2013), the peak of systemic IL-12p40 and IFN- $\gamma$  during ME49-*T. gondii*  
269 infection is between days 5-6 post-infection, therefore, we measured these cytokines in  
270 the serum of CD-1-infected mice at day 5. Mice infected with  $\Delta mic1$  or  $\Delta mic1::MIC1$ -  
271 T126A/T220A strains had 3-5 fold lower systemic levels of IL-12 (Fig 6B;  $p=0.016$ )  
272 and IFN- $\gamma$  (Fig 6C;  $p\leq 0.0002$ ) than WT infected mice. In contrast, mice infected with  
273 parasites lacking the MIC4 gene, or those expressing the mutant version of MIC4  
274 showed no difference in IL-12 (Fig 6F) or IFN- $\gamma$  (Fig 6G) compared to WT infected  
275 mice. Hence, only MIC1 altered systemic levels of key cytokines induced during *T.*  
276 *gondii in vivo* infection, and mice survived longer with lower systemic levels of  
277 cytokines typically associated with acute mortality.

### 278 **MIC1 wild type complemented strain restores the cytokine storm and acute** 279 **mortality kinetics during *in vivo* infection with *T. gondii*.**

280 To formally show that MIC1 alters systemic levels of pro-inflammatory  
281 cytokines associated with acute mortality, we complemented  $\Delta mic1$  parasites at the  
282 endogenous locus with a Type I allele of MIC1 expressing an HA tag (MIC1<sup>HA</sup>).  
283 Western blotting for either MIC1 or HA expression showed WT levels of MIC1  
284 expression in the complemented parasites  $\Delta mic1::MIC1$ <sup>HA</sup> (Fig 7A). The complemented  
285 strain restored WT virulence kinetics during *in vivo* infection and all mice died acutely,  
286 in contrast to  $\Delta mic1$  or  $\Delta mic1::MIC1$ -T126A/T220A parasites, that had a slight, but

287 significant delay in their acute mortality kinetics (Fig 7B;  $p=0.0082$ ). Systemic levels of  
288 IFN- $\gamma$  (Fig 7C) and parasite load (Fig 7D and 7E) from mice infected with the  
289 complemented strain were indistinguishable from WT. To better resolve the apparent  
290 difference in acute mortality, parasites were injected into the right footpad to monitor  
291 mouse weight loss and survival kinetics [35]. Mice infected locally in the footpad with  
292  $\Delta mic1$  survived significantly longer, or did not die (Fig 7G;  $p=0.0031$ ), and lost less  
293 weight during acute infection (Fig 7F) than those infected with WT or  $\Delta mic1::MIC1$   
294 complemented parasites. Further, mice infected with  $\Delta mic1::MIC1$ -T126A/T220A  
295 parasites that fail to bind TLR2 and TLR4 N-glycans *in vivo* also lost less weight and  
296 survived significantly longer than WT or  $\Delta mic1::MIC1$  complemented parasites (Fig 7F  
297 and G). In conclusion, our results suggest that MIC1 operates in two distinct ways; as an  
298 adhesin protein that promotes parasite infection competency, and as a lectin that  
299 engages TLR N-glycans to induce a stronger proinflammatory immune response, one  
300 that is unregulated and results in acute mortality upon RH infection of CD-1 mice.

301

## 302 **DISCUSSION**

303 In this study, we report a new function for MIC1 and MIC4, two *T. gondii*  
304 microneme proteins involved in the host-parasite relationship. We show that rMIC1 and  
305 rMIC4, by interacting directly with N-glycans of TLR2 and TLR4, trigger a  
306 noncanonical carbohydrate recognition-dependent activation of innate immune cells.  
307 This results in IL-12 secretion and the production of IFN- $\gamma$ , a pivotal cytokine that  
308 mediates parasite clearance and the development of a protective T cell response [19,  
309 22], but in some cases, as seen during RH infection of CD-1 mice, promotes a  
310 dysregulated cytokine storm and acute mortality, as seen during RH infection of CD-1  
311 mice [36]. This MIC-TLR activation event explains, at least in part, the resistance  
312 conferred by rMIC1 and rMIC4 administration against experimental toxoplasmosis [20,  
313 21].

314 *T. gondii* tachyzoites express microneme proteins either on their surface or  
315 secrete them in their soluble form. These proteins may form complexes, such as those of  
316 MIC1, MIC4, and MIC6 (MIC1/4/6), in which MIC6 is a transmembrane protein that  
317 anchors the two soluble molecules MIC1 and MIC4 [8]. Genetic disruption of each one  
318 of these three genes does not interfere with parasite survival [8] nor its interaction with,  
319 and attachment to, host cells [10]; however, MIC1 has been shown to play a role in

320 invasion and contributes to virulence in mice [10]. We previously isolated soluble  
321 MIC1/4, a lactose-binding complex from soluble *T. gondii* antigens (STAg) [17], and its  
322 lectin activity was confirmed by the ability of MIC1 to bind sialic acid [9] and MIC4 to  
323  $\beta$ -galactose [18]. We also reported that MIC1/4 stimulates adherent splenic murine cells  
324 to produce IL-12 at levels as high as those induced by STAg [20]. Recently, it was also  
325 demonstrated that MIC1, MIC4 and MIC6 are capable of inducing IFN- $\gamma$  production  
326 from memory T cells in mice chronically infected with *T. gondii* [37]. Our data herein  
327 shows that MIC1/4 binds to and activates TLRs via a novel lectin-carbohydrate  
328 interaction, rather than by its cognate receptor-ligand binding groove, establishing  
329 precisely how the interactions of microneme protein(s) with defined glycosylated  
330 receptor(s) expressed on the host cell surface are capable of altering innate priming of  
331 the immune system.

332 To formally demonstrate the MIC1/MIC4 binding to glycosylated TLR cell  
333 surface receptors we generated recombinant forms of MIC1 and MIC4, which retained  
334 their specific sialic acid- and  $\beta$ -galactose-binding properties as indicated by the results  
335 of their binding to fetuin and asialofetuin as well as the glycoarray assay. Both  
336 recombinant MIC1 and MIC4 triggered the production of proinflammatory and anti-  
337 inflammatory cytokines in DCs and macrophages via their specific recognition of TLR2  
338 and TLR4 N-glycans, as well as by signaling through MyD88 and, partially, TRIF.  
339 Importantly, our results establish how binding of rMIC1 and rMIC4 to specific N-  
340 glycans present on TLR2 and TLR4 induces cell activation through this novel lectin-  
341 carbohydrate interaction. The ligands for MIC1 and MIC4,  $\alpha$ 2-3-sialyllactosamine and  
342  $\beta$ 1-3- or  $\beta$ 1-4-galactosamine, respectively, are terminal N-glycan residues found on a  
343 wide-spectrum of mammalian cell surface-associated glycoconjugates. Thus, it is  
344 possible that additional lectin-carbohydrate interactions may exist between MIC1/4 and  
345 other cell surface receptors beyond TLR2 and TLR4. Such interactions likely evolved to  
346 facilitate adhesion and promote the infection competency of a wide-variety of host cells  
347 infected by *T. gondii*, further underscoring how these proteins exist as important  
348 virulence factors [10] beyond immune priming. However, it is the immunostimulatory  
349 capacity of rMIC1 and rMIC4 to target N-glycans on the ectodomains of TLR2 and  
350 TLR4 that likely rationalizes how these microneme proteins function as a double-edged  
351 sword during *T. gondii* infection. Mice infected by Type I strains die acutely due to a  
352 failure to regulate the cytokine storm induced by high levels of IL-12 and IFN- $\gamma$  [38,

353 39]. In this study, *T. gondii* Type I strains engineered to be deficient in MIC1 or  
354 defective in binding TLR2/4 N-glycans lost less weight, survived significantly longer,  
355 and produced less IL-12 and IFN- $\gamma$ . Future studies that test whether the  
356 immunostimulatory effect of MIC1/4 alters the pathogenesis and cyst burden of Type II  
357 strains of *T. gondii* should be pursued to formally demonstrate that Type II parasites  
358 rely on MIC1/4 induction of Th1-biased cytokines in order to limit tachyzoite  
359 proliferation and induce a life-long persistent bradyzoite infection.

360 Several pathogens are known to synthesize lectins, which are most frequently  
361 reported to interact with glycoconjugates on host cells to promote adherence, invasion,  
362 and colonization of tissues [40-43]. Nonetheless, there are currently only a few  
363 examples of lectins from pathogens that recognize sugar moieties present in TLRs and  
364 induce IL-12 production by innate immune cells. Paracoccin, a GlcNAc-binding lectin  
365 from the human pathogen *Paracoccidioides brasiliensis*, induces macrophage  
366 polarization towards the M1 phenotype [44] and the production of inflammatory  
367 cytokines through its interaction with TLR2 N-glycans [45]. Furthermore, the galactose-  
368 adherence lectin from *Entamoeba histolytica* activates TLR2 and induces IL-12  
369 production [46]. In addition, the mammalian soluble lectin SP-A, found in lung alveoli,  
370 interacts with the TLR2 ectodomain [47]. The occurrence of cell activation and IL-12  
371 production as a consequence of the recognition of TLR N-glycans has also been  
372 demonstrated using plant lectins with different sugar-binding specificities [48, 49].

373 The binding of MIC1 and MIC4, as well as the lectins above, to TLR2 and  
374 TLR4 may be associated with the position of the specific sugar residue present on the  
375 receptor's N-glycan structure. Since the N-glycan structures of TLR2 and TLR4 are still  
376 unknown, we assume that the targeted MIC1 and MIC4 residues, e.g. sialic acid  $\alpha$ 2-3-  
377 linked to galactose  $\beta$ 1-3- and  $\beta$ 1-4-galactosamines, are appropriately placed in the  
378 receptors' oligosaccharides to allow the recognition phenomenon and trigger the  
379 activation of innate immune responses.

380 Several *T. gondii* proteins have previously been shown to activate innate  
381 immune cells in a TLR-dependent manner, but independent of sugar recognition. This is  
382 the case for profilin (TgPRF), which is essential for the parasite's gliding motility based  
383 on actin polymerization; it is recognized by TLR11 [29] and TLR12 [31, 50]. In  
384 addition, *T. gondii*-derived glycosylphosphatidylinositol anchors activate TLR2 and  
385 TLR4 [51], and parasite RNA and DNA are ligands for TLR7 and TLR9, respectively

386 [19, 22, 50]. The stimulation of all of these TLRs culminate in MyD88 activation which  
387 results in IL-12 production [19, 22]. Several other *T. gondii* secreted effector proteins  
388 regulate the production of proinflammatory cytokines such as IL-12, independent of  
389 TLRs. For example, the dense granule protein 7 (GRA7) induces MyD88-dependent  
390 NF- $\kappa$ B activation, which facilitates IL-12, TNF- $\alpha$ , and IL-6 production [32]. MIC3 is  
391 reported to induce TNF- $\alpha$  secretion and macrophage M1 polarization [52], whereas  
392 GRA15 expressed by Type II strains activates NF- $\kappa$ B, promoting the release of IL-12  
393 [33], and GRA24 triggers the autophosphorylation of p38 MAP kinase and  
394 proinflammatory cytokine and chemokine secretion [34]. In contrast, TgIST interferes  
395 with IFN- $\gamma$  induction by actively inhibiting STAT1-dependent proinflammatory gene  
396 expression indicating that the parasite is capable of both activating as well as inhibiting  
397 effector arms of the host immune response to impact its pathogenesis *in vivo* [53]. Thus,  
398 multiple secretory effector proteins of *T. gondii*, including MIC1 and MIC4, appear to  
399 work in tandem to ultimately promote protective immunity by either inducing or  
400 dampening the production of proinflammatory cytokines, the timing of which is central  
401 to controlling both the parasite's proliferation during the acute phase of infection and  
402 the induction of an effective immune response capable of establishing a chronic  
403 infection [19].

404 Our results regarding soluble MIC1 and MIC4 confirmed our hypothesis that  
405 these two effector proteins induce the innate immune response against *T. gondii* through  
406 TLR2- and TLR4-dependent pathways. This is consistent with previous studies that  
407 highlight the importance of TLR signaling, as well as the MyD88 adapter molecule, as  
408 essential for conferring resistance to *T. gondii* infection [29, 51, 54, 55]. In addition, we  
409 show that both MIC1 and MIC4 on the parasite surface contribute to the secretion of IL-  
410 12 by macrophages and DCs during *in vitro* infection, but only MIC1 plays a significant  
411 role during *in vivo* infection, demonstrated by its ability to promote a dysregulated  
412 induction of systemic levels of IFN- $\gamma$  and a proinflammatory cytokine storm that leads  
413 to acute mortality during murine infection.

414

## 415 **METHODS**

### 416 **Ethics statement**

417 All experiments were conducted in accordance to the Brazilian Federal Law  
418 11,794/2008 establishing procedures for the scientific use of animals, and State Law

419 establishing the Animal Protection Code of the State of Sao Paulo. All efforts were  
420 made to minimize suffering, and the animal experiments were approved by the Ethics  
421 Committee on Animal Experimentation (*Comissão de Ética em Experimentação Animal*  
422 - CETEA) of the Ribeirao Preto Medical School, University of Sao Paulo (protocol  
423 number 065/2012), following the guidelines of the National Council for Control of  
424 Animal Experimentation (*Conselho Nacional de Controle de Experimentação Animal* -  
425 CONCEA).

#### 426 **Lac<sup>+</sup> fraction and recombinant MIC1 and MIC4**

427 The lactose-eluted (Lac<sup>+</sup>) fraction was obtained as previously reported [17, 21].  
428 Briefly, the total soluble tachyzoite antigen (STAg) fraction was loaded into a lactose  
429 column (Sigma-Aldrich, St. Louis, MO) and equilibrated with PBS containing 0.5 M  
430 NaCl. The material adsorbed to the resin was eluted with 0.1 M lactose in equilibrating  
431 buffer and dialyzed against ultrapure water. The obtained fraction was denoted as Lac<sup>+</sup>  
432 and confirmed to contain MIC1 and MIC4. For the recombinant proteins, rMIC1 and  
433 rMIC4 sequences were amplified from cDNA of the *T. gondii* strain ME49 with a 6-  
434 histidine tag added on the N-terminal, cloned into pDEST17 vector (Gateway Cloning,  
435 Thermo Fisher Scientific Inc., Grand Island, NY), and used to transform DH5α *E. coli*  
436 chemically competent cells for ampicillin expression selection, as described before [21].  
437 The plasmids with rMIC1-T126A/T220A and rMIC4-K469M were synthesized by  
438 GenScript (New Jersey, US) using a pET28a vector, and the MIC sequences carrying  
439 the mutations were cloned between the *Nde*I and *Bam*H I sites. All plasmids extracted  
440 from DH5α *E. coli* were transformed in *E. coli* BL21-DE3 chemically competent cells  
441 to produce recombinant proteins that were then purified from inclusion bodies and  
442 refolded by gradient dialysis, as described previously for rMIC1 and rMIC4 wild type  
443 forms [21]. Endotoxin concentrations were measured in all protein samples using the  
444 Limulus Amebocyte Lysate Kit – QCL-1000 (Lonza, Basel, Switzerland). The rMIC1,  
445 rMIC1-T126A/T220A, rMIC4 and rMIC4-K469M contained 7.2, 3.2, 3.5 and 1.1 EU  
446 endotoxin/μg of protein, respectively. Endotoxin was removed by passing over two  
447 polymyxin-B columns (Affi-Prep Polymyxin Resin; Bio-Rad, Hercules, CA).  
448 Additionally, prior to all *in vitro* cell-stimulation assays, the proteins samples were  
449 incubated with 50 μg/mL of polymyxin B sulphate salt (Sigma-Aldrich, St. Louis, MO)  
450 for 30 min at 37 °C to remove possible residual LPS.

451

## 452 **Glycan array**

453 The carbohydrate-binding profile of microneme proteins was determined by  
454 Core H (Consortium for Functional Glycomics, Emory University, Atlanta, GA), using  
455 a printed glycan microarray, as described previously [56]. Briefly, rMIC1-Fc, rMIC4-  
456 Fc, and Lac<sup>+</sup>-Fc in binding buffer (1% BSA, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>,  
457 0.05% (w/v) Tween 20, and 20 mM Tris-HCl, pH 7.4) were applied onto a covalently  
458 printed glycan array and incubated for 1 hour at 25 °C, followed by incubation with  
459 Alexa Fluor 488-conjugate (Invitrogen, Thermo Fisher Scientific Inc., Grand Island,  
460 NY). Slides were scanned, and the average signal intensity was calculated. The common  
461 features of glycans with stronger binding are depicted in Fig. 1a. The average signal  
462 intensity detected for all of the glycans was calculated and set as the baseline.

## 463 **Sugar-inhibition assay**

464 Ninety-six-well microplates were coated with 1 µg/well of fetuin or asialofetuin,  
465 glycoproteins diluted in 50 µL of carbonate buffer (pH 9.6) per well, followed by  
466 overnight incubation at 4 °C. Recombinant MIC1 or MIC4 proteins (both wild type  
467 (WT) and mutated forms), previously incubated or not with their corresponding sugars,  
468 i.e. α(2-3)-sialyllactose for MIC1 and lacto-N-biose for MIC4 (V-lab, Dextra, LA, UK),  
469 were added into coated wells and incubated for 2 h at 25 °C. After washing with PBS,  
470 *T. gondii*-infected mouse serum (1:50) was used as the source of the primary antibody.  
471 The assay was then developed with anti-mouse peroxidase-conjugated secondary  
472 antibody, and the absorbance was measured at 450 nm in a microplate-scanning  
473 spectrophotometer (Power Wave-X; BioTek Instruments, Inc., Winooski, VT).

## 474 **Mice and parasites**

475 Female C57BL/6 (WT), MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, TLR2<sup>-/-</sup>, TLR3<sup>-/-</sup>, TLR4<sup>-/-</sup>, double  
476 knockout (DKO) TLR2<sup>-/-</sup>/TLR4<sup>-/-</sup>, TLR5<sup>-/-</sup>, and TLR9<sup>-/-</sup> mice (all from the C57BL/6  
477 background), 8 to 12 weeks of age, were acquired from the University of São Paulo -  
478 Ribeirão Preto campus animal facility, Ribeirão Preto, São Paulo, Brazil, and housed in  
479 the animal facility of the Department of Cell and Molecular Biology - Ribeirão Preto  
480 Medical School, under specific pathogen-free conditions. The TLR11<sup>-/-</sup>/TLR12<sup>-/-</sup> DKO  
481 mice were maintained at American Association of Laboratory Animal Care-accredited  
482 animal facilities at NIAID/NIH. For the *in vivo* infections, female CD-1 outbred mice, 6  
483 weeks of age were acquired from Charles River Laboratories, Germantown, MD, USA.

484 A clonal isolate of the *T. gondii* RH- $\Delta ku80/\Delta hpt$  strain was used to generate the  
485 GFP/Luciferase strain, which was the recipient strain to generate the single-knockout  
486 parasites. The GFP/Luc sequence was inserted into the UPRT locus of *Toxoplasma* by  
487 double crossover homologous recombination using CRISPR/Cas-based genome editing  
488 and selected for FUDR resistance to facilitate the targeted GFP/Luc gene cassette  
489 knock-in. The MIC1 and MIC4 genes were replaced by the drug-selectable marker *hpt*  
490 (*hxgprt* - hypoxanthine-xanthine-guanine phosphoribosyl transferase) flanked by LoxP  
491 sites. For all gene deletions, 30  $\mu$ g of guide RNA was transfected along with 15  $\mu$ g of a  
492 repair oligo. Parasites were transfected and selected as previously described [57, 58].  
493 For the MIC gene complementation, the sequence was amplified from RH genomic  
494 DNA with the addition of one copy of HA-tag sequence  
495 (TACCCATACGATGTTCCAGATTACGCT) before the stop codon, and cloned into  
496 pCR2.1-TOPO vector, followed by site-directed mutagenesis using the Q-5 kit (New  
497 England Biolabs) in order to generate point mutations into MIC1 (MIC1-  
498 T126A/T220A) and MIC4 (MIC4-K469M) sequences. For transfections, 30  $\mu$ g of guide  
499 RNA was transfected along with 20  $\mu$ g of linearized pTOPO vector containing the MIC  
500 mutated sequences.

501 Strains were maintained in human foreskin fibroblast (HFF) cells grown in  
502 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-  
503 inactivated foetal bovine serum (FBS), 0.25 mM gentamicin, 10 U/mL penicillin, and  
504 10  $\mu$ g/mL streptomycin (Gibco, Thermo Fisher Scientific Inc., Grand Island, NY).

#### 505 **Bone marrow-derived dendritic cells and macrophages**

506 Bone marrows of WT, MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, TLR2<sup>-/-</sup>, TLR3<sup>-/-</sup>, TLR4<sup>-/-</sup>, DKO  
507 TLR2<sup>-/-</sup>/TLR4<sup>-/-</sup>, TLR5<sup>-/-</sup>, TLR9<sup>-/-</sup>, and DKO TLR11<sup>-/-</sup>/TLR12<sup>-/-</sup> mice were harvested  
508 from femurs and hind leg bones. Cells were washed with RPMI medium and  
509 resuspended in RPMI medium with 10% FBS, 10 U/mL penicillin, and 10  $\mu$ g/mL  
510 streptomycin (Gibco). For dendritic cell (DC) differentiation, we added 10 ng/mL of  
511 recombinant murine GM-CSF (Preprotech, Rocky Hill, NJ), and 10 ng/mL murine  
512 recombinant IL-4 (eBioscience, San Diego, CA); for macrophage differentiation, 30%  
513 of L929 conditioned medium was added to RPMI medium with 10% FBS. The cells  
514 were cultured in 100  $\times$  20 mm dish plates (Costar; Corning Inc., Corning, NY),  
515 supplemented with respective conditioned media at days 3 and 6 for DCs, and at day 4  
516 for macrophages. DCs were incubated for 8–9 days and macrophages for 7 days; the



517 cells were then harvested and plated into 24-well plates at  $5 \times 10^5$  cells/well for protein  
518 stimulations or *T. gondii* infections, followed by ELISA. Cell purity was analyzed by  
519 flow cytometry. Eighty-five percent of differentiated dendritic cells were  
520 CD11b<sup>+</sup>/CD11c<sup>+</sup>, while 94% of differentiated macrophages were CD11b<sup>+</sup>.

#### 521 **HEK293T cells transfection**

522 Human embryonic kidney 293T (HEK293T) cells, originally acquired from  
523 American Tissue Culture Collection (ATCC, Rockville, MD), were used as an  
524 expression tool [59] for TLR2 and TLR4 [45, 60]. The cells grown in DMEM  
525 supplemented with 10% FBS (Gibco), and were seeded at  $3.5 \times 10^5$  cells/mL in 96-well  
526 plates ( $3.5 \times 10^4$  cells/well) 24 h before transfection. Then, HEK293T cells were  
527 transiently transfected (70-80% confluence) with human TLR2 plasmids as described  
528 previously [25] or with CD14, CD36, MD-2 and TLR4 [61] using Lipofectamine 2000  
529 (Invitrogen) with 60 ng of NF- $\kappa$ B Luc, an NF- $\kappa$ B reporter plasmid, and 0.5 ng of  
530 *Renilla* luciferase plasmid, together with 60 ng of each gene of single and multiple  
531 glycosylation mutants and of TLR2 WT genes [25]. After 24 h of transfection, the cells  
532 were stimulated overnight with positive controls: P3C (Pam3CSK4; EMC  
533 Microcollections, Tübingen, Germany), fibroblast stimulating ligand-1 (FSL-1; EMC  
534 Microcollections), or LPS Ultrapure (standard LPS, *E. coli* 0111:B4; Sigma-Aldrich); or  
535 with the negative control for cell stimulation (the medium). Cells transfected with  
536 empty vectors, incubated either with the medium or with agonists (FSL-1 or P3C), were  
537 also assayed; negative results were required for each system included in the study. IL-8  
538 was detected in the culture supernatants. The absence of Mycoplasma contamination in  
539 the cell culture was certified by indirect fluorescence staining as described previously  
540 [62].

#### 541 **Cytokine measurement**

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

#### 548 **TLR2-FLAG and TLR4-FLAG plasmids**

549 The pcDNA4/TO-FLAG plasmid was kindly provided by Dr. Dario Simões  
550 Zamboni. The pcDNA4-FLAG-TLR2 and pcDNA4-FLAG-TLR4 plasmids were  
551 constructed as follows. RNA from a P388D1 cell line (ATCC, Rockville, MD) was  
552 extracted and converted to cDNA with Maxima H Minus Reverse Transcriptase  
553 (Thermo-Fisher Scientific, Waltham, MA USA) and oligo(dT). TLR2 and TLR4 were  
554 amplified from total cDNA from murine macrophages by using Phusion High-Fidelity  
555 DNA Polymerase and the phosphorylated primers TLR2\_F:  
556 ATGCTACGAGCTCTTTGGCTCTTCTGG, TLR2\_R:  
557 CTAGGACTTTATTGCAGTTCTCAGATTTACCCAAAAC, TLR4\_F:  
558 TGCTTAGGATCCATGATGCCTCCCTGGCTCCTG and TLR4\_R:  
559 TGCTTAGCGGCCGCTCAGGTCCAAGTTGCCGTTTCTTG. The fragments were  
560 isolated from 1% agarose/Tris-acetate-ethylenediaminetetraacetic acid gel, purified  
561 with GeneJET Gel Extraction Kit (Thermo-Fisher Scientific), and inserted into the  
562 pcDNA4/TO-FLAG vector by using the restriction enzymes sites for NotI and XbaI  
563 (Thermo-Fisher Scientific) for TLR2, and BamHI and NotI (Thermo-Fisher Scientific)  
564 for TLR4. Ligation reactions were performed by using a 3:1 insert/vector ratio with T4  
565 DNA Ligase (Thermo-Fisher Scientific) and transformed into chemically competent  
566 *Escherichia coli* DH5 $\alpha$  cells. Proper transformants were isolated from LB agar  
567 medium plates under ampicillin selection (100  $\mu$ g/mL) and analyzed by PCR,  
568 restriction fragment analysis, and DNA sequencing. All reactions were performed  
569 according to the manufacturer's instructions.

#### 570 **Pull-down assay and Western Blot**

571 We used the lysate of HEK293T cells transfected (70-80% confluence) with  
572 plasmids containing TLR2-FLAG or TLR4-FLAG. After 24 h of transfection, the HEK  
573 cells were lysed with a non-denaturing lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl,  
574 and 2 mM EDTA) supplemented with a protease inhibitor (Roche, Basel, Switzerland).  
575 After 10 min of incubation on ice, the lysate was subjected to centrifugation (16,000 g,  
576 at 4 °C for 5 min). The protein content in the supernatant was quantified by the BCA  
577 method, aliquoted, and stored at -80 °C. For the pull-down assay, 100  $\mu$ g of the lysate  
578 from TLR2-FLAG- or TLR4-FLAG-transfected HEK cells were incubated with 10  $\mu$ g  
579 of TgMIC1 or TgMIC4 overnight at 4 °C. Since these proteins had a histidine tag, the  
580 samples were purified on nickel-affinity resin (Ni Sepharose High Performance; GE

581 Healthcare, Little Chalfont, UK) after incubation for 30 min at 25 °C and centrifugation  
582 of the fraction bound to nickel to pull down the TgMIC-His that physically interacted  
583 with TLR-FLAG (16,000 g, 4 °C, 5 min). After washing with PBS, the samples were  
584 resuspended in 100 µL of SDS loading dye with 5 µL of 2-mercaptoethanol, heated for  
585 5 min at 95 °C, and 25 µL of total volume was run on 10% SDS-PAGE. After  
586 transferring to a nitrocellulose membrane (Millipore, Billerica, MA), immunoblotting  
587 was performed by following the manufacturer's protocol. First, the membrane was  
588 incubated with anti-FLAG monoclonal antibodies (1:2,000) (Clone G10, ab45766,  
589 Sigma-Aldrich) to detect the presence of TLR2 or TLR4. The same membrane was then  
590 subjected to secondary probing and was developed with anti-TgMIC1 (IgY; 1:20,000)  
591 or anti-TgMIC4 (IgY; 1:8,000) polyclonal antibodies and followed by incubation with  
592 secondary polyclonal anti-chicken IgY-HRP (1:4,000) (A9046, Sigma-Aldrich) to  
593 confirm the presence of TgMIC1 and TgMIC4.

#### 594 ***In vitro* infections**

595 Bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived  
596 macrophages (BMDMs) were infected with WT ( $\Delta ku80/\Delta hpt$ ),  $\Delta mic1$ ,  $\Delta mic1::MIC1$ -  
597 T126A/T220A,  $\Delta mic4$  or  $\Delta mic4::MIC4$ -K469M (Type I, RH background) strains  
598 recovered from T25 flasks with HFF cell cultures. The T25 flasks were washed with  
599 RPMI medium to completely remove parasites, and the collected material was  
600 centrifuged for 5 min at 50 g to remove HFF cell debris. The resulting pellet was  
601 discarded, and the supernatant containing the parasites was centrifuged for 10 min at  
602 1,000 g and resuspended in RPMI medium for counting and concentration adjustments.  
603 BMDCs and BMDMs were dispensed in 24-well plates at  $5 \times 10^5$  cells/well (in RPMI  
604 medium supplemented with 10% FBS), followed by infection with 3 parasites per cell  
605 (multiplicity of infection, MOI 3). Then, the plate was centrifuged for 3 min at 200 g to  
606 synchronize the contact between cells and parasites and incubated at 37 °C. The  
607 supernatants were collected at 6, 12, 24, and 48 h after infection for quantification of  
608 IL-12p40.

#### 609 ***In vivo* infections and Luciferase assay**

610 Six-week-old female CD-1 outbred mice were infected by intraperitoneal  
611 injection with 50 tachyzoites of RH engineered strains diluted in 500 µl of phosphate-  
612 buffered saline. The mice were weighed daily and survival was evaluated

613 Bioluminescent detection of firefly luciferase activity was performed at day 5  
614 post-infection using an IVIS BLI system from Xenogen to monitor parasite burden.  
615 Mice were injected with 3 milligrams (200 µl) of D-luciferin (PerkinElmer) substrate,  
616 and after 5 minutes the mice were imaged for 300 seconds to detect the photons emitted.

617

### 618 **Statistical analysis**

619 The data were plotted and analysed using GraphPad Prism 7.0 software  
620 (GraphPad, La Jolla, CA). Statistical significance of the obtained results was calculated  
621 using analysis of variance (One-way ANOVA) followed by Bonferroni's multiple  
622 comparisons test. Differences were considered significant when the *P* value was <0.05.

623

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637

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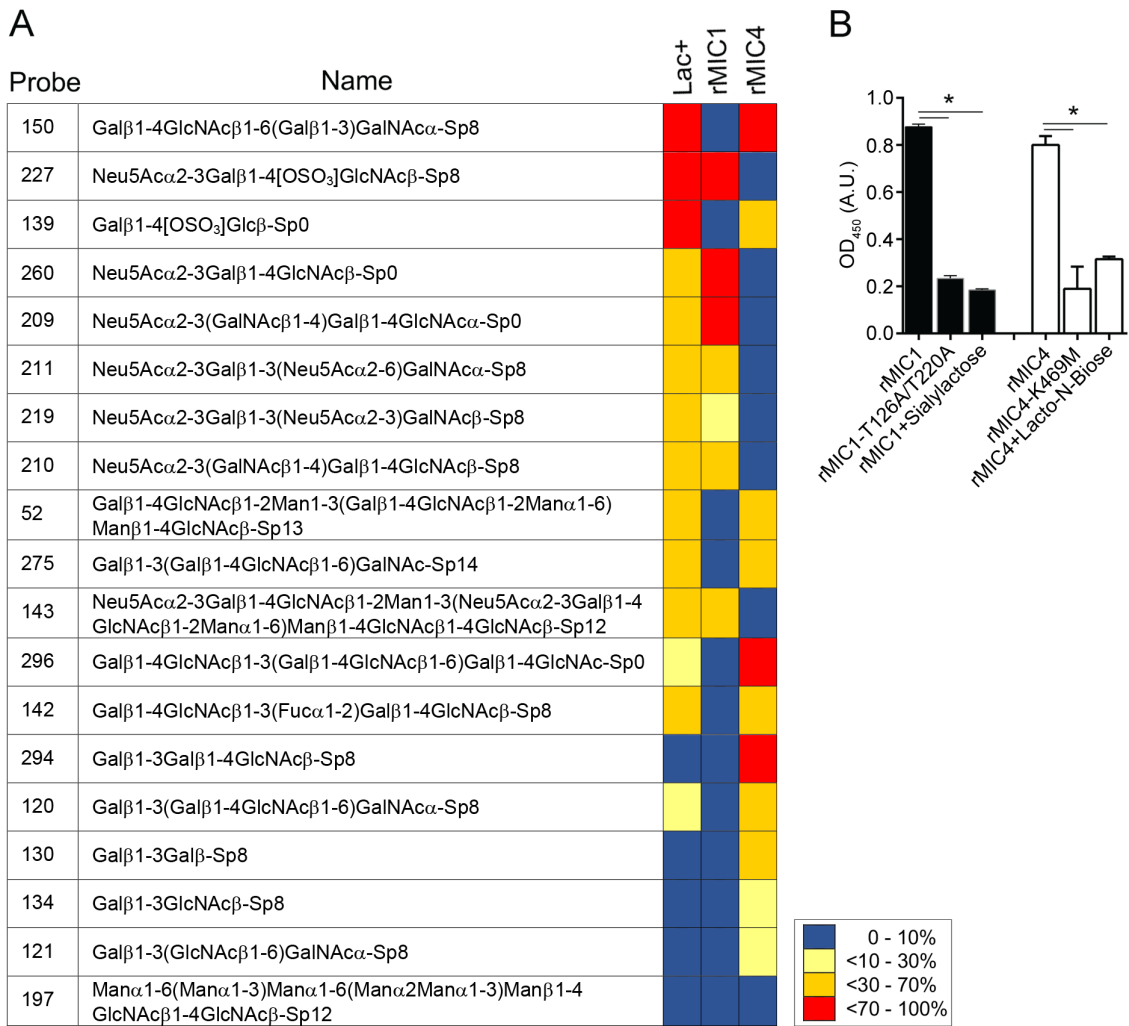
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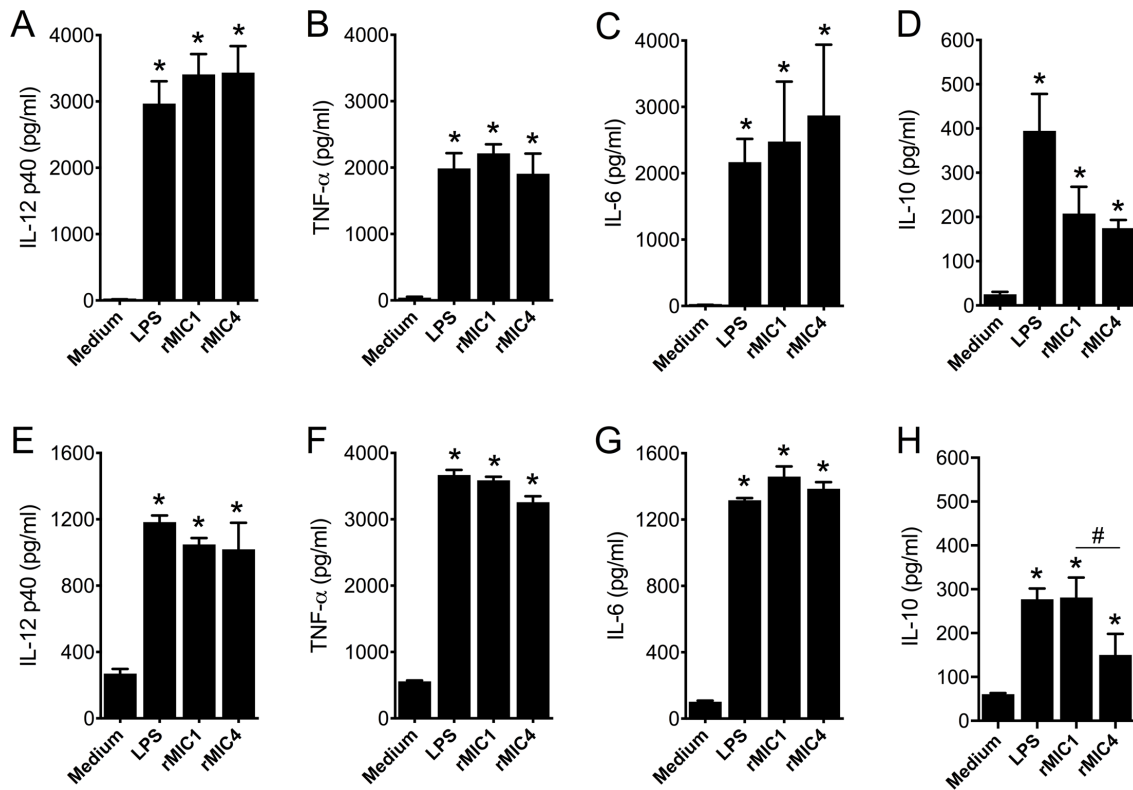
904 **FIGURES**



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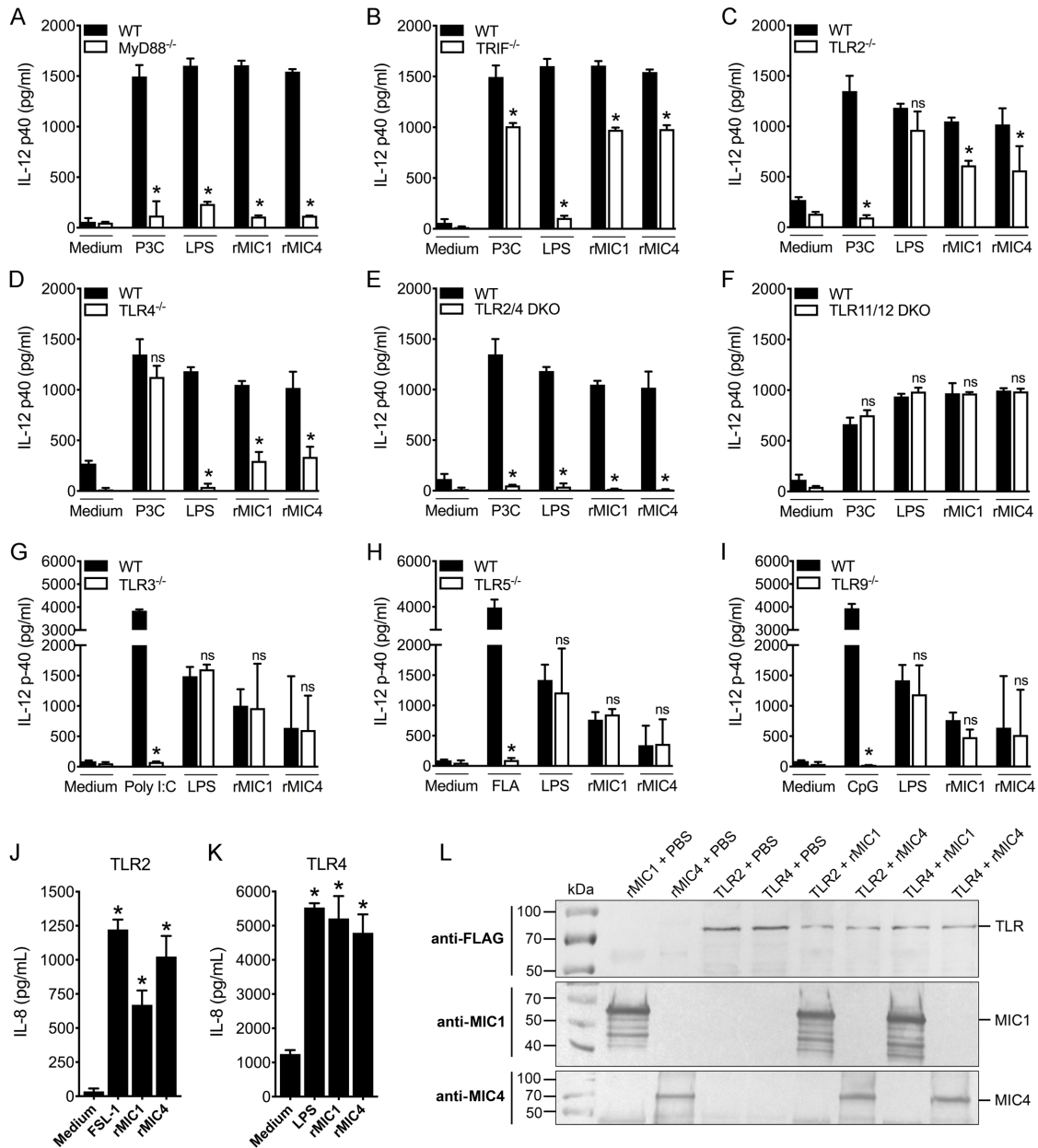
906 **Fig 1. Lectin activity of rMIC1 and rMIC4.** (A) Glycoarray of the native MIC1/MIC4  
 907 subcomplex (Lac+) and of the recombinant forms of MIC1 and MIC4. In total, 320  
 908 oligosaccharide probes were analysed by reading their fluorescence intensities, and the 20  
 909 best recognized glycans are shown. The results were represented as previously reported  
 910 [18]. (B) The activity and inhibition assays were performed in microplates coated with  
 911 glycoproteins with or without sialic acid, fetuin (black bars), or asialofetuin (white bars),  
 912 separately. After coating, wild type or mutated rMIC1 and rMIC4, pre-incubated with  
 913 PBS or their corresponding sugars, were added to the wells. Later, bound proteins were  
 914 detected through the addition of serum from *T. gondii*-infected mice. Data in (B) are  
 915 expressed as mean ±S.D. of triplicate wells and significance was calculated with  
 916 ANOVA followed by Bonferroni's multiple comparisons test. \*p<0.05. Data are  
 917 representative of two (B) independent experiments. Gal: galactose; GalNAc: N-  
 918 acetylgalactosamine; Glc: glucose; Man: mannose; Fuc: fucose; Neu5Ac: N-

919 acetylneuraminic acid; wt: wild type protein; mut: protein with a mutation in the  
920 carbohydrate-recognition domain (CRD); ns: not significant.  
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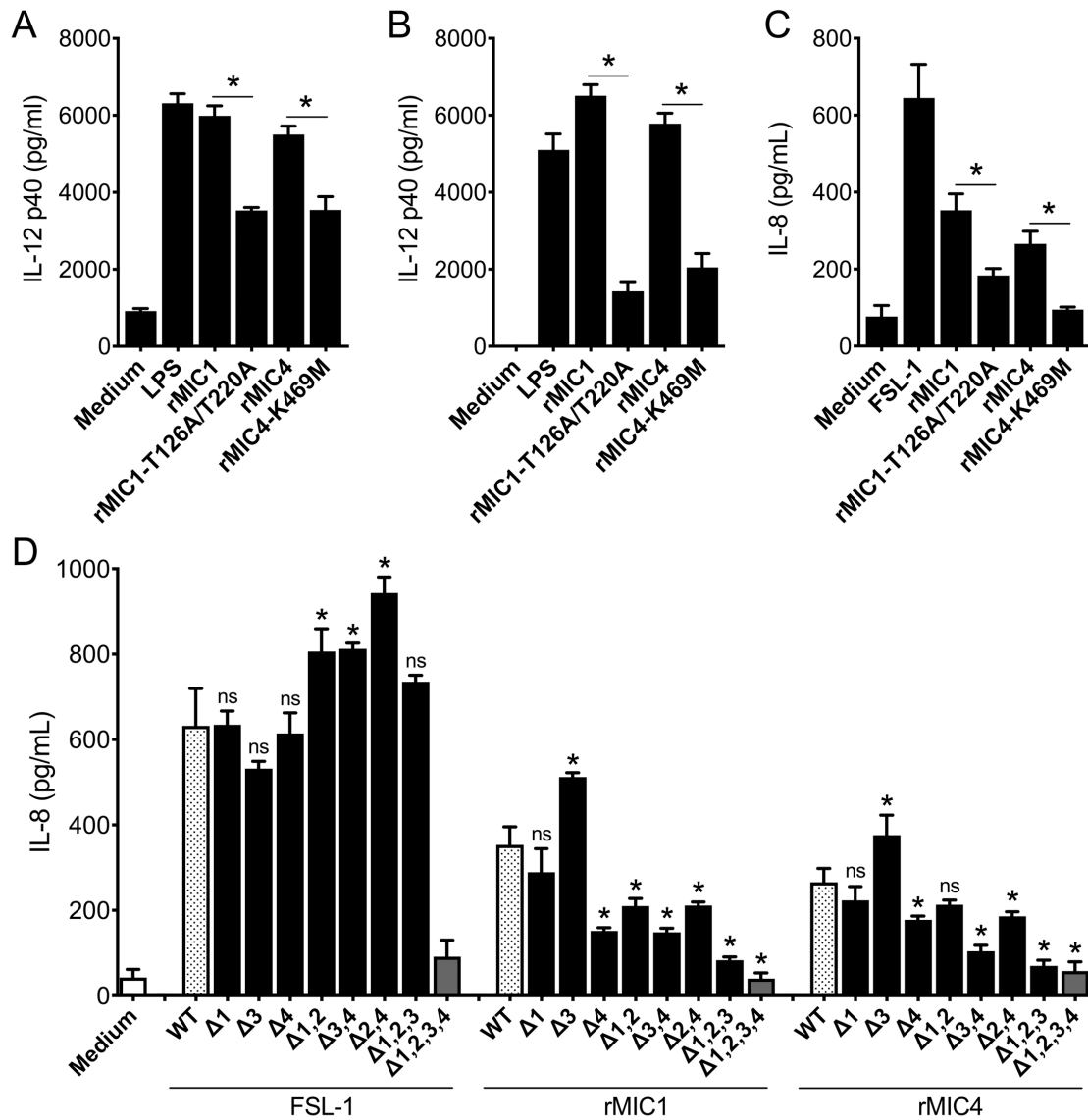
923 **Fig 2. Microneme proteins stimulate cytokine production by dendritic cells and**  
924 **macrophages. (A-D)** Bone marrow-derived dendritic cells (BMDCs) and **(E-H)** bone  
925 marrow-derived macrophages (BMDMs) from C57BL/6 mice were stimulated with  
926 rMIC1 (5 µg/mL) and rMIC4 (5 µg/mL) for 48 h. LPS (100 ng/mL) was used as  
927 positive control. The levels of IL-12p40, TNF-α, and IL-6 were measured by ELISA.  
928 For this assay, rMIC1 and rMIC4 were passed through polymyxin B column, followed  
929 by incubation with polymyxin B sulphate salt media preparation that was added to the  
930 culture (see Material and Methods). Data are expressed as mean ±S.D. of triplicate wells  
931 and significance was calculated with ANOVA followed by Bonferroni's multiple  
932 comparisons test. \*p<0.05. Data are representative of three independent experiments.  
933



934

935 **Fig 3. The IL-12 production induced by rMICs is dependent on binding to TLR2**  
 936 **and TLR4.** (A-I) Bone marrow-derived macrophages from WT, TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>,  
 937 double knockout TLR2<sup>-/-</sup>/TLR4<sup>-/-</sup>, TLR3<sup>-/-</sup>, TLR5<sup>-/-</sup>, TLR9<sup>-/-</sup>, and double knockout  
 938 TLR11<sup>-/-</sup>/TLR12<sup>-/-</sup> mice, all of the C57BL/6 background, were stimulated with rMIC1  
 939 or rMIC4 (5 µg/mL) for 48 h. Pam3CSK4 (P3C) (1 µg/mL), LPS (100 ng/mL), Poly I:C  
 940 (10 µg/mL), Flagellin (FLA) (1 µg/mL) and CpG (25 µg/mL) were used as positive  
 941 controls. IL-12p40 levels were measured by ELISA. (J and K) Transfected HEK293T  
 942 cells expressing TLR2 were stimulated with rMIC1 (750 nM) or rMIC4 (500 nM), and  
 943 rMIC1 (200 nM) or rMIC4 (160 nM) for HEK cells expressing TLR4, for 24 h. FSL-1  
 944 (100 ng/mL) and LPS (100 ng/mL) were used as positive controls. IL-8 levels were

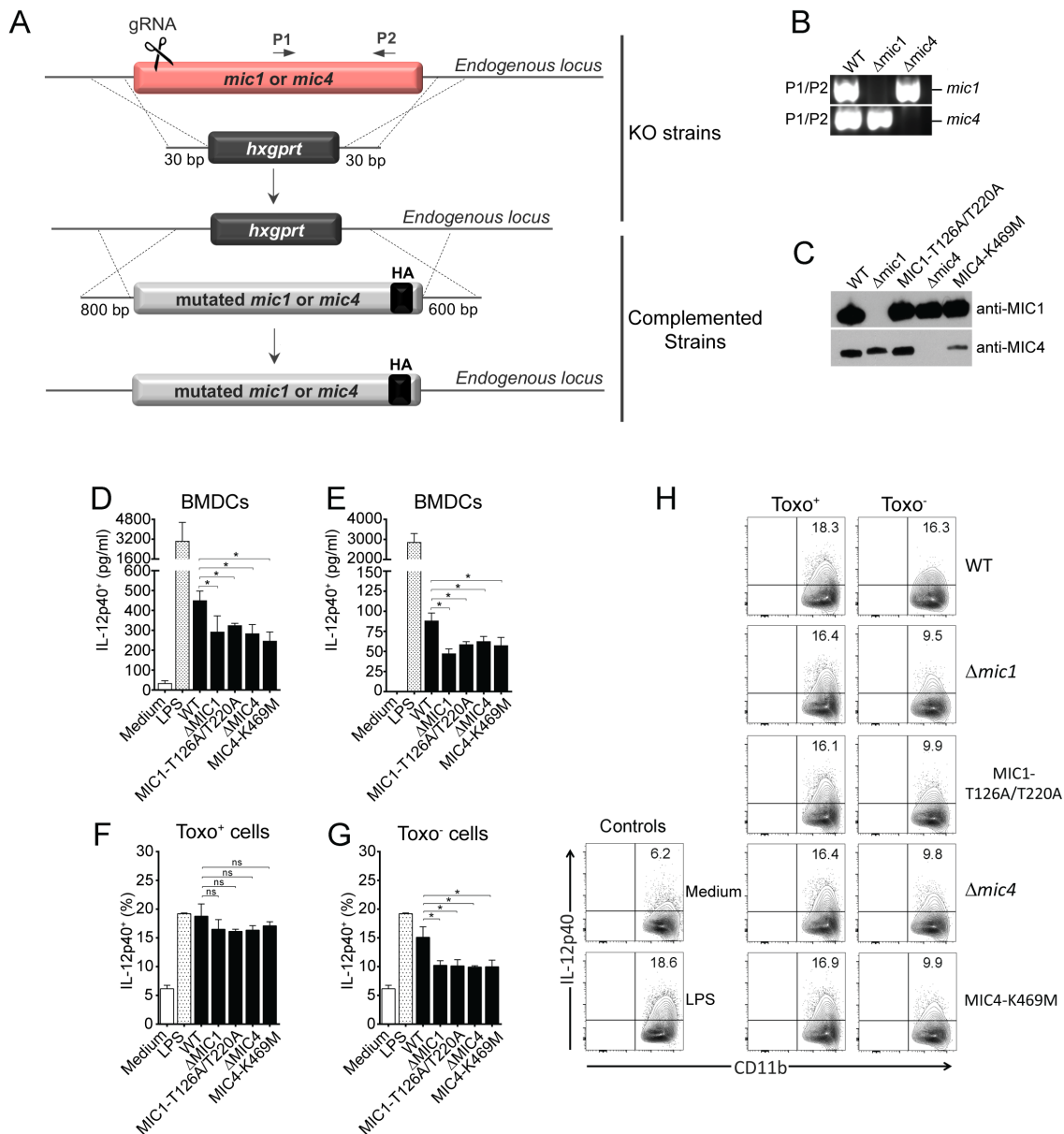
945 measured by ELISA. (L) The interaction between rMICs and TLRs was evaluated by  
 946 western blot. HEK293T cells transiently expressing TLR2-FLAG and TLR4-FLAG  
 947 were lysed and incubated with His-rMIC1 (rMIC1<sup>His</sup>) or His-rMIC4 (rMIC4<sup>His</sup>). His-  
 948 rMICs were subjected to Ni<sup>2+</sup>-affinity resin pull-down (lanes 6 to 9) and analysed for  
 949 TLR2 and TLR4 binding by protein blotting with antibodies specific for FLAG-tag and  
 950 then for rMIC (IgY, polyclonal). For these assays, rMIC1 and rMIC4 were passed  
 951 through polymyxin B column, followed by incubation with polymyxin B sulphate salt  
 952 media preparation that was added to the culture (see Material and Methods). Data in (A-  
 953 K) are expressed as mean  $\pm$ S.D. of triplicate wells and significance was calculated with  
 954 ANOVA followed by Bonferroni's multiple comparisons test. \* $p$ <0.05. Data are  
 955 representative of three (A-K) and two (L) independent experiments.  
 956



957



958 **Fig 4. The cellular activation induced by rMICs via TLRs depends on**  
959 **carbohydrate recognition. (A)** Bone marrow-derived macrophages and **(B)** bone  
960 marrow-derived dendritic cells from C57BL/6 mice and **(C)** transfected HEK293T cells  
961 expressing fully glycosylated TLR2 were stimulated with rMIC1 (WT) and rMIC4  
962 (WT) or with their mutated forms, rMIC1-T126A/T220A and rMIC4-K469M, 5 µg/mL  
963 of each, for 48 h. LPS (100 ng/mL) and FSL-1 (100 ng/mL) were used as positive  
964 controls. IL-12p40 and IL-8 levels were measured by ELISA. **(D)** HEK293T cells  
965 expressing fully glycosylated TLR2 (with 4 N-glycans, WT) or glycosylation mutants  
966 of TLR2 ( $\Delta$ -1;  $\Delta$ -4;  $\Delta$ -1,2;  $\Delta$ -3,4;  $\Delta$ -2,4;  $\Delta$ -1,2,3;  $\Delta$ -1,2,3,4) were stimulated with rMIC1  
967 or rMIC4. FSL-1 (100 ng/mL) was used as positive control. IL-8 levels were measured  
968 by ELISA. The statistical analysis compared fully glycosylated TLR2 (WT) and TLR2  
969 mutants for the N-glycosylation sites for the same stimuli. For these assays, rMIC1,  
970 rMIC1-T126A/T220A, rMIC4 and rMIC4-K469M were passed through polymyxin B  
971 column, followed by incubation with polymyxin B sulphate salt media preparation that  
972 was added to the culture (see Material and Methods). Data are expressed as mean  $\pm$ S.D.  
973 of triplicate wells and significance was calculated with ANOVA followed by  
974 Bonferroni's multiple comparisons test. \* $p$ <0.05. Data are representative of three  
975 independent experiments.  
976



977

978 **Fig 5. The IL-12 production during *T. gondii* in vitro infection partially depends on**

979 **MICs and their ability to recognize carbohydrates on APCs surface. (A)** Schematic

980 representation of knockout and complementation constructs for MIC1 and MIC4 loci.

981 The endogenous loci were disrupted using the hypoxanthine-xanthine-guanine

982 phosphoribosyl transferase (HPT)-selectable marker and CRISPR methodology. (B)

983 PCR analysis for MIC1 and MIC4 loci of gDNA from parental (WT RH- $\Delta ku80/\Delta hpt$ -

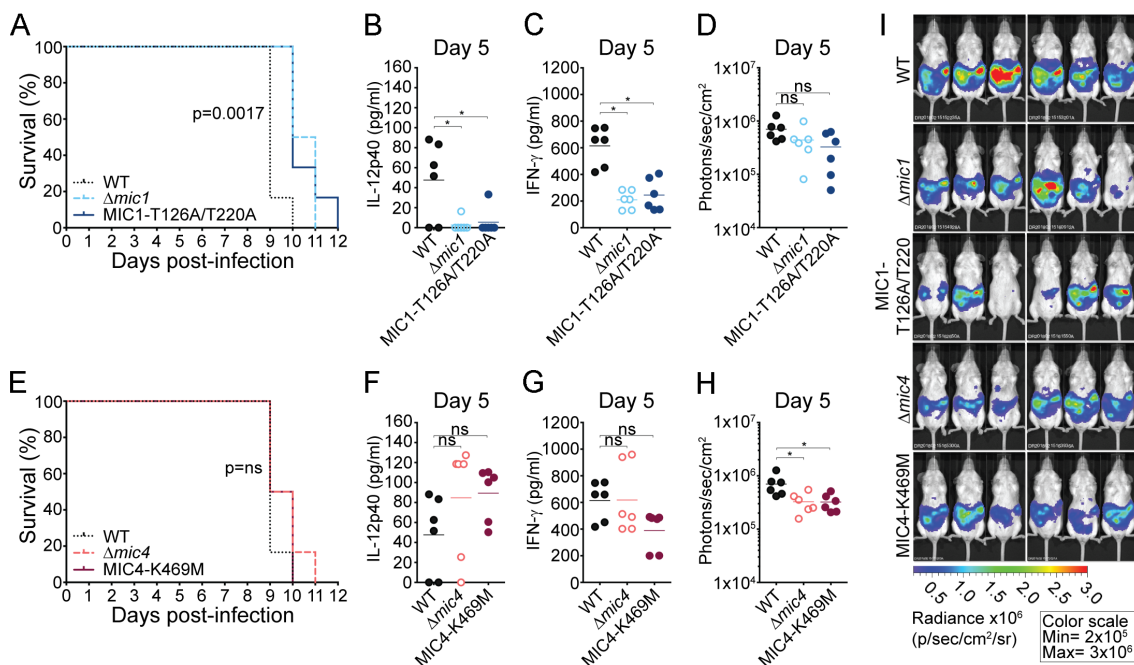
984 GFP/Luc) and knockout (RH- $\Delta ku80/\Delta mic1$ -GFP/Luc and RH- $\Delta ku80/\Delta mic4$ -GFP/Luc)

985 strains. (C) Western blot analysis of an equal loading of whole cell lysates

986 corresponding to  $3 \times 10^6$  tachyzoites ( $1 \times 10^8$ /mL) from WT,  $\Delta mic1$ ,  $\Delta mic1::MIC1$ -

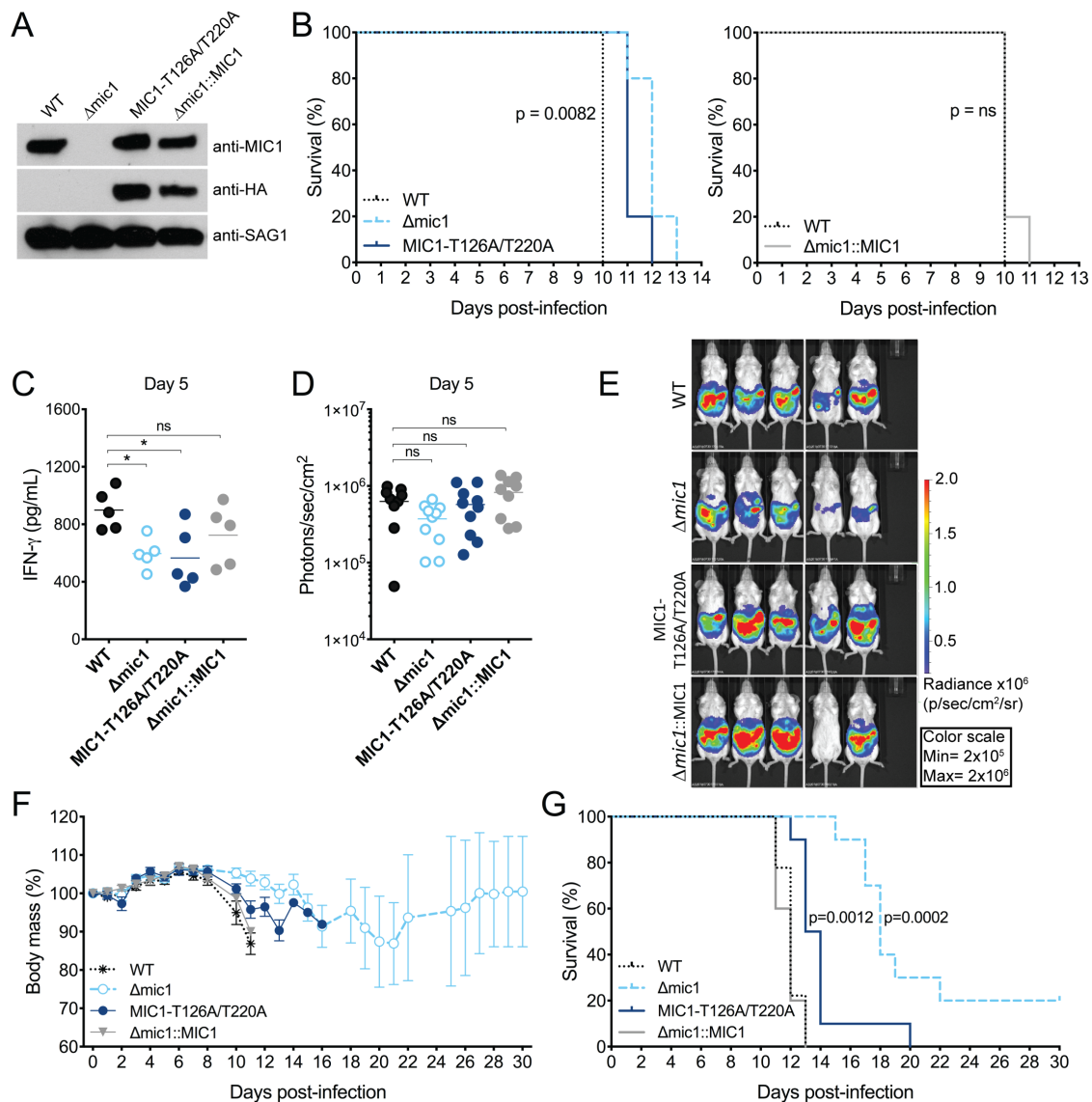
987 T126A/T220A,  $\Delta mic4$  and  $\Delta mic4::K469M$  parasites. The membrane was probed with

988 anti-MIC1 (IgY, 1:20,000) and anti-MIC4 (IgY, 1:8,000). **(D)** Bone marrow-derived  
 989 dendritic cells (BMDCs) and **(E)** Bone marrow-derived macrophages (BMDMs) from  
 990 C57BL/6 were infected with WT,  $\Delta mic1$ ,  $\Delta mic1::MIC1-T126A/T220A$ ,  $\Delta mic4$  and  
 991  $\Delta mic4::K469M$  strains (MOI 3). LPS (100 ng/mL) was used as positive control. Cell-  
 992 culture supernatants were collected 24 hours post-infection and the IL-12p40 production  
 993 was analyzed by ELISA. **(F and G)** Frequency of IL-12p40<sup>+</sup> BMDCs (CD11b<sup>+</sup>IL-  
 994 12p40<sup>+</sup>) after 20-24 hours of *in vitro* infection with WT,  $\Delta mic1$ ,  $\Delta mic1::MIC1$ -  
 995 T126A/T220A,  $\Delta mic4$  and  $\Delta mic4::K469M$  strains (MOI 1). Brefeldin A was added to  
 996 the culture for 8 hours. LPS (100 ng/mL) was used as positive control. **(H)**  
 997 Representative dot plots showing IL-12p40 staining in *T. gondii* infected or non-  
 998 infected (SAG1<sup>+</sup> or SAG1<sup>-</sup>) CD11b<sup>+</sup> cells after 20-24 hours of *in vitro* infection with  
 999 WT,  $\Delta mic1$ ,  $\Delta mic1::MIC1-T126A/T220A$ ,  $\Delta mic4$  and  $\Delta mic4::K469M$  strains (MOI 1).  
 1000 Brefeldin A was added to the culture for 8 hours. LPS (100 ng/mL) was used as positive  
 1001 control. Data are expressed as mean  $\pm$  S.D. of triplicate wells and significance was  
 1002 calculated with ANOVA followed by Bonferroni's multiple comparisons test. \* $p < 0.05$ .  
 1003 Data are representative of three **(D and E)** and two **(F-H)** independent experiments.  
 1004



1005  
 1006 **Fig 6. MIC1 lectin activity, but not MIC4, contributes to virulence in mice during**  
 1007 ***in vivo* infection with *T. gondii*.** CD-1 mice were infected intraperitoneally with RH  
 1008 engineered strains of *T. gondii* at an infectious dose of 50 tachyzoites/mouse (n=6).  
 1009 Mortality kinetics of mice infected with **(A)** WT,  $\Delta mic1$  and  $\Delta mic1::MIC1$ -

1010 T126A/T220A strains or (E) WT,  $\Delta mic4$  and  $\Delta mic4::MIC4-K469M$  parasites. At day 5  
 1011 post-infection the sera were collected for measuring systemic (B and F) IL-12p40 and  
 1012 (C and G) IFN- $\gamma$ . (D, H and I) Bioluminescent detection in photons/sec/cm<sup>2</sup> shows  
 1013 parasite burden 5 days post-infection. Data are expressed as mean  $\pm$  S.D. and  
 1014 significance was calculated with ANOVA followed by Bonferroni's multiple  
 1015 comparisons test. \* $p < 0.05$ . Data are representative of three independent experiments,  
 1016 with total  $n = 16$ .  
 1017

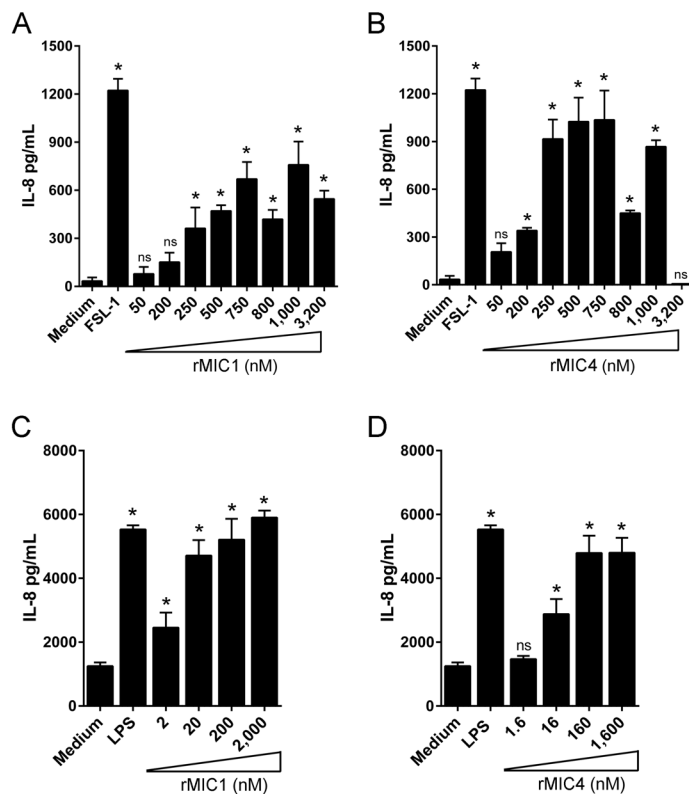


1018  
 1019 **Fig 7. MIC1 wild type complemented strain restores virulence in mice during *in***  
 1020 ***vivo* infection with *T. gondii*.** (A) Western blot analysis of an equal loading of whole  
 1021 cell lysates corresponding to  $3 \times 10^6$  tachyzoites ( $1 \times 10^8$ /mL) from WT,  $\Delta mic1$ ,  
 1022  $\Delta mic1::MIC1$ -T126A/T220A and  $\Delta mic1::MIC1$  parasites. The membrane was probed

1023 with anti-MIC1 (IgY, 1:20,000), anti-HA (rabbit, 1:5,000) and anti-SAG1 (rabbit,  
 1024 1:10,000). **(B)** Mortality kinetics of CD-1 mice infected intraperitoneally with WT,  
 1025  $\Delta mic1$ ,  $\Delta mic1::MIC1$ -T126A/T220A and  $\Delta mic1::MIC1$  at an infectious dose of 50  
 1026 tachyzoites/mouse (n=5). **(C)** At day 5 post-infection the sera were collected for  
 1027 measuring systemic IFN- $\gamma$ . **(D and E)** Bioluminescent detection in photons/sec/cm<sup>2</sup>  
 1028 shows parasite burden 5 days post-infection. **(F and G)** Body mass and mortality  
 1029 kinetics of CD-1 mice infected subcutaneously with WT,  $\Delta mic1$ ,  $\Delta mic1::MIC1$ -  
 1030 T126A/T220A and  $\Delta mic1::MIC1$  using an infectious dose of 10<sup>4</sup> tachyzoites/mouse.  
 1031 Data are expressed as mean  $\pm$ S.D. and significance were calculated with ANOVA  
 1032 followed by Bonferroni's multiple comparisons test. \*p<0.05. Data are representative of  
 1033 two independent experiments, total n=10.

1034

1035 **SUPPLEMENTARY INFORMATION**



1036

1037 **S1 Fig. Effect of different concentrations of rMIC1 and rMIC4 on the transfected**  
 1038 **HEK cells.** HEK293T cells expressing **(A and B)** TLR2 or **(C and D)** TLR4 were  
 1039 stimulated with increasing concentrations of **(A and C)** rMIC1 and **(B and D)** rMIC4  
 1040 for 24 h. FSL-1 (100 ng/mL) LPS (100 ng/mL) were used as positive controls. IL-8  
 1041 levels were measured by ELISA. Data are expressed as mean  $\pm$ S.D. of triplicate wells

1042 and significance was calculated with ANOVA. \* $p < 0.05$ . Data are representative of two  
1043 independent experiments.