Role of CD4$^+$Foxp3$^+$ regulatory T cells in protection induced by a live, attenuated, replicating Type-I vaccine strain of *Toxoplasma gondii*

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Abstract

Vaccination with the live attenuated *Toxoplasma gondii* Mic1.3KO strain induced long-lasting immunity against challenge with *Toxoplasma gondii* Type I and Type II strains. The involvement of T regulatory (Treg) cells in the protection mechanism was investigated. Intraperitoneal injection of Mic1.3KO induced a weak and transient influx of $CD4^+Foxp3^+$ T regulatory cells followed by recruitment/expansion of $CD4^+Foxp3^-CD25^+$ effector cells and control of the parasite at the site of infection. The local and systemic cytokine responses associated with this recruitment of Treg were of TH1/Treg-like type. In contrast, injection of RH, the wild-type strain from which the vaccinal strain is derived, induced a low $CD4^+Foxp3^+$ cell influx and uncontrolled multiplication of the parasites at this local site, followed by death of the mice. The associated local and systemic cytokine responses were of TH1/TH17-like type.

In addition, *in vivo* Treg induction in the RH-infected mice with IL2/anti-IL2 complexes induced control of the parasite and a TH1/Treg cytokine response similar to the response after Mic1.3KO vaccination. These results suggest that Treg cells may contribute to the protective response after vaccination with Mic1.3KO.

1. Introduction

Toxoplasmosis is caused by a protozoan parasite that infects human beings and other warm-blooded animals. Infections in humans are generally asymptomatic, although immunosuppressed patients may exhibit severe symptoms. Similarly, primary infection during pregnancy can lead to miscarriage and neonatal malformations. Toxoplasmosis can be

transmitted to human beings via ingestion of oocysts or via the consumption of meat products contaminated with tissue cysts (1). Effective vaccination of domestic livestock can therefore prevent human infection with *Toxoplasma gondii.* It is possible to induce strong protection by immunization with a live attenuated strain (2). Live attenuated vaccine strain models are also useful for advancing the understanding of the protective host immune response (3-8). However, the information available about the mechanism of protection involved after vaccination with a Type-I attenuated strain was obtained with non-replicating, non-persistent strains such as cps-1 (3-7) and ts4 (8). A strain known as Mic1.3KO was obtained in our laboratory by deleting the *MIC1* and *MIC3* genes (9). This strain was derived from the highly virulent Type-I RH strain, and its reduced invasion capacity *in vitro* was correlated with decreased virulence when injected into outbred Swiss OF1 mice (9). Type-I strains are characterized by the rapid dissemination of the parasite, and by a high parasite burden that results in death soon after infection by a single viable parasite in mice (10). High levels of IFNγ were produced following infection with a Type-I parasite, and mice succumbed to uncontrolled parasite growth and associated inflammation (11, 12). The Mic1.3KO deleted strain showed reduced virulence, lower levels of dissemination throughout tissues, and lower levels of IFNγ production than the parental RH strain after injection into mice (13).

In a model of lethal toxoplasmosis induced after oral administration of infection of a Type-II strain causing infection in C57BL/6 mice, overproduction of IFNγ was also responsible for mortality and was correlated with a sharp decline in the percentage of Treg cells just before death, supporting the hypothesis of defective immuno-regulation (14). Treg cells are a subpopulation of $CD4^+$ T cells, and their main function is to maintain immune homeostasis and tolerance (15). They constitutively express the interleukin-2 receptor alpha chain (IL2Rα), a surface receptor, also known as CD25, and the intracellular forkhead box-p3 transcription factor (Foxp-3) marker (16). The role of Treg has been fully described after

infection with Type II strain (14, 17-21) and has been clearly implicated in the mortality of C57BL/6 mice after oral infection in the lethal ileitis model (14). The Treg collapse is correlated with pathogenicity and only occurs in highly pathogenic conditions since oral infection of BALB/c mice with Type II strain did not induce reduction in Treg (14). However, depletion of Treg in these mice resulted in morbidity associated with high parasite burden and increased ileal pathology compared to control BALB/c mice (19), suggesting a role of Treg in protection during acute infection.

In the present study, we compared the involvement of Treg after infection with the vaccinal Mic1.3KO strain or with the parental lethal RH strain in an attempt to identify their involvement in the protection induced by vaccination. We showed a small increase in the absolute $CD4^+$ $Foxp3^+$ Treg count at the site of infection with Mic1.3KO followed by an increase in $CD4^+CD25^+Foxp3^-$ effector cells and control of the parasite. In contrast, the increase in Treg cell count in RH-infected mice was lower and the parasites were not controlled locally. Our studies showed that $CD4^+Foxp3^+$ Treg cells were involved in protection since specific expansion of these cells using IL2/anti-IL2complexes in mice infected with RH induced a reduction in parasite burden and a decrease in proinflammatory cytokines. These features were similar to those of mice infected with Mic1.3KO, supporting the role of $CD4^+Foxp3^+$ Treg cells in the protection induced by vaccination.

Materials and methods

Animals and parasites. Eight-week-old female Swiss OF1 and C57BL/6 mice were obtained from Janvier (France). All experiments using animals were approved by the local Ethics Committee (CEEA VdL) and registered under reference 2011-06-6.

Two weeks before injection, RH tachyzoites harvested from the peritoneal cavity of the mice were cultured on human foreskin fibroblast (HFF) monolayers (ATCC CRL-1634, American Type Culture Collection), as previously described (13). Mic1.3KO parasites were obtained by targeted disruption of the *MIC1* and *MIC3* genes in the ΔHX RH strain of *T. gondii,* as previously described (9). Mic1.3KO tachyzoites were also propagated by serial passage on HFF monolayers. Tachyzoites freshly harvested from a cell culture were counted using a Malassez counting chamber.

Mice were infected by intra-peritoneal (ip) inoculation of 100 freshly harvested tachyzoites diluted in 0.2 ml DMEM.

Cell culture conditions and cytokine quantification. For cytokine detection, splenocytes were recovered and purified as described (13, 21) and stimulated for 72h with 10 µg/ml *Toxoplasma* extract (TE) or with purified anti-CD3 (clone 145-2C11) (eBioscience) at 1µg/ml. The cells ($5x10^5$) were seeded into 24-well plates in 1 ml RPMI 1640 containing 5% FCS, and supernatants were collected 24, 48 and 72h after activation.

Peritoneal exudate cells (PECs) were obtained from uninfected mice (day 0 controls), and from infected mice on days 4, 7, and 11 pi by peritoneal lavage with 5 ml of ice-cold PBS, as already described (13), and centrifuged to collect supernatants. The supernatants were kept frozen at –20°C until assayed for cytokines. Cytokines (IFNγ, IL2, IL10, IL12p40, IL12p70 and IL23) and chemokines (CCL2, CCL3 and CCL20) were quantified in the serum, cell culture supernatants and peritoneal washes by ELISA using Ready-set-Go Kit (eBioscience) and R/D duoset kits.

Cell surface staining and intracellular staining. PECs and splenocytes were obtained from mice on day 0 (non-infected controls), and on 4, 7, and 11 days post-infection (dpi). Cells

were washed once in PBS, and counted to determine total viable cell numbers by trypan blue exclusion. Standard procedures were used to stain 2-5×10^5 PECs and 10^6 splenocytes in 5% FCS in PBS and Mouse Fc Block (BD Biosciences) as previously described (21). Antibodies for the detection of CD4 (GK1.5), CD8 (eBioH35-17.2), CD25 (PC61), CD69 (H1.2F3), Ki-67 (SolA15) and Foxp3 (FJK-16s) were purchased from eBioscience.

For IL10 detection, splenocytes were seeded into 6-well plates at 10^6/ml in a final volume of 5 ml RPMI 1640 containing 5% FCS, and stimulated for 18h with 10 µg/ml TE. The culture medium was then removed and replaced by fresh culture medium containing PMA (50 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5µg/ml) for a further 4h period. Antibodies for the detection of IL10 were purchased from BD Biosciences.

Cell acquisition was undertaken with a BD FACSCalibur cytometer and analyzed using Cellquest software (BD Bioscience).

In vivo Treg expansion. IL2/anti-IL2 complexes were prepared as described in the literature (14, 20) with minor modifications. rIL2 and the anti-IL2 monoclonal antibody (mAb) (clone JES6-1A12) were obtained from eBioscience. IL2 (1µg) was mixed with anti-IL2 mAbs (10µg) and incubated for 15 min at room temperature prior to ip injection on 0, 3, and 5 dpi.

Statistical analyses. Differences between the groups were compared using Mann-Whitney or Kruskal-Wallis non-parametric tests followed by Dunn's post test using GraphPad Prism software. All statistical tests were two-sided and a value of $P<0.05$ was considered statistically significant in all tests. Some analyses were performed with StatXact software (Cytel Studio) using non-parametric exact tests with strata to take into account variability between repetitions of the same experiment.

144 All data in the graphs are expressed as the median plus the range (unless otherwise
145 specified).
146
147 **Results**
148 **Lack of control of the parasite was not associated with decreased IFNγ**
149 **production in mice infected with RH.** Parasite burden was monitored in the peritoneum by
150 direct counting on days 4, 7, and 11 after inoculation of 100 tachyzoites of both strains (**Fig.**
151 **1A**). On day 4, few parasites were found in either group ($\leq 0.02x10^6$), and the difference
152 between the two groups was not significant. However, on day 7 parasite counts were
153 significantly increased in the RH-injected group ($16x10^6$) but not in the Mic1.3KO group
154 ($0.002x10^6$). In the Mic1.3KO group, the parasite counts remained very low even at 11 dpi,
155 and the number of parasites decreased between days 4 and 11. Taken together, these results
156 suggest that after day 4 the parasite was being controlled at the local level in the Mic1.3KO
157 mice in contrast to the RH mice, where the parasite count increased exponentially until the
158 animals died. This high parasite count at 7dpi was correlated with a high cell mortality rate
159 (between 25 and 90% determined by trypan blue exclusion) in PECs recovered from the RH
160 mice. Similar results were obtained when using inbred C57BL/6 mice in which lower parasite
161 counts were found in the Mic1.3KO-infected group compared to the RH group
162 (Supplementary Table 1), suggesting that these mice were as resistant to the infection with the
163 vaccinal strain as outbred mice.
164 We also measured serum levels of systemic IFNγ at 4, 7 and 11 dpi (**Fig. 1B**). In control naive
165 mice, IFNγ was below the detection threshold (data not shown). The serum levels of IFNγ
166 rose significantly following infection. In the RH-infected group, the level increased
167 significantly between days 4 and 7 and paralleled parasite multiplication. In the Mic1.3KO
168 group, levels also increased between days 4 and 7, and subsequently remained at similar

levels until day 11. Taken together, these results suggest that mortality in the RH group may not have originated from a lack of IFNγ production but, as for Type II infected C57BL/6 mice from uncontrolled IFNγ production (14) or a lack of parasite control, or both.

The total numbers of PECs and splenocytes were checked on 4, 7 and 11 dpi and compared to those of naive mice in response to Mic1.3KO and to RH (data not shown). Both infected groups displayed a slightly higher total PEC count after infection than the control naive group. This difference was significant only at day 11 for the Mic1.3KO-injected group. The number of spleen cells had increased in both infected groups at 4 dpi compared to the naive group, although the difference was significant only for the RH group. In the Mic1.3KO group, this increase was significant at 7 and 11 dpi. At 7dpi there were significantly higher spleen cell counts in the Mic1.3KO group compared to the control and RH groups. In the RH-infected group, the cell counts were also significantly lower than at 4 dpi. This suggests that a significant immune response was induced on day 4 after infection in both infected groups.

Kinetics of $CD4^+Foxp3^+$ Treg after infection. After infection with a lethal dose of a Type II strain of *T. gondii*, numbers and frequencies of $CD4^+$ $Foxp3^+$ Treg cell have been reported to be reduced at the site of infection and systemically in C57BL/6 mice, and this decrease is correlated with high IFNγ production (14). We therefore followed the $CD4^+$ $Foxp3^+$ Treg cells after infection at local (peritoneum) and systemic (spleen) sites. Three populations ($CD25^+Foxp3^+$, $CD25^-Foxp3^+$, and $CD25^+Foxp3^-$) were observed within the $CD4^+$ gated population in both groups. The percentage of total $Foxp3^+$ cells in the peritoneum at 4 dpi was slightly but non-significantly higher in the RH group than in the naive mice (**Fig. 2A**). Moreover, there was no significant difference in the numbers (data not shown) or percentages of $Foxp3^+$ cells (**Fig. 2A**) between the two infected groups at this time. The total percentage of $Foxp3^+$ cells at 4dpi was significantly greater in the Mic1.3KO group than in the naive group (**Fig. 2A**). The kinetics of both effector ($CD4^+CD25^+Foxp3^-$) and regulator

(CD4$^+$Foxp3$^+$) cells were followed until 11 dpi in the Mic1.3KO group (**Fig. 2B and C**). This analysis could not be performed for the RH group because there were high levels of cell mortality at 7 dpi, and the permeabilization step further increased cell mortality to 100% (data not shown). The relative number of regulatory Foxp3$^+$ cells at day 4 was significantly higher in the Mic1.3KO group than in the naive group (**Fig. 2B**) (25.9x10^4 versus 1.8x10^4 for the naive group). This increase was transient since there was no significant difference between the groups at 7 and 11 dpi.

Similar results were obtained using C57BL/6 mice (Fig. S1). C57BL/6 mice infected with RH showed reduced percentages of Foxp3$^+$ cells at the local level compared to the mice infected with Mic1.3KO.The percentages of Foxp3$^+$ cells in the spleen were similar

The numbers and percentages of effector cells at day 4 were slightly and non-significantly higher in the Mic1.3KO group than in the naive mice (**Fig. 2C**). However, the numbers of CD4$^+$Foxp3$^-$CD25$^+$ cells increased exponentially between days 0 and 11 (**Fig. 2C**) and were significantly different from day 0 at 7 and 11 dpi. This high CD4$^+$Foxp3$^-$CD25$^+$ cell count at the local level was correlated with low parasite counts in Mic1.3KO-infected mice.

The total percentage (**Fig. 2D**) and absolute number of the Foxp3$^+$ population in the spleen (not shown), did not differ significantly between the two groups at 7dpi. However, significantly higher percentages of CD25$^+$Foxp3$^+$ cells were found in the RH-infected group. The ratio of Foxp3$^+$ expressing CD25 cells compared to the total percentage of Foxp3$^+$ cells was similar between the naive and the Mic1.3KO groups (0.61 and 0.60, respectively) but was significantly higher for the RH group (around 0,71) compared to both the naive and Mic1.3KO-infected groups (**Fig. 2E**).

We investigated the expression of phenotypic markers associated with activation (CD69) and proliferation (Ki-67). Cells were analyzed directly *ex vivo* from the peritoneal cavity and spleen by flow cytometry on day 7 (Tables 1 and 2).

There was no significant difference in percentages of $CD4^+$ and $CD8^+$ in PECs between the control and infected groups (Table 1). The percentage of $CD69^+$ cells in PECs was significantly higher in the RH group compared to the naive group (19.9 versus 5.8 %) at 7dpi (Table 1). The percentage of $CD69^+$ cells in splenocytes was also significantly higher in the RH group (11.8%) compared to both naive (3.7%) and Mic1.3KO groups (1.9%). The kinetics of the expression of CD69 on splenocytes were monitored in both groups up to 11 dpi (for the Mic1.3KO group alone), and this higher level was only seen at 7 dpi in the RH group (data not shown). The level had not increased at 11 dpi in the Mic1.3KO group, suggesting that CD69 expression may be correlated with the mortality observed in the RH group.

Ki-67 expression was used to follow the proliferation of PECs and splenocytes after infection with both strains. Ki-67 expression was significantly higher in PECs of both infected groups compared to the naive control group (16.4% in the control group versus 62.8 and 52.7% in the RH and Mic1.3KO groups, respectively). The difference between the two infected groups was not significant. Similar results were obtained for splenocytes, with higher percentages of Ki-67^+ cells in both infected groups (14.0% in the control group versus 24.3 and 37.6% in the RH and Mic1.3KO groups, respectively), but the difference between control and RH groups was not significant.

The expression of CD25 was monitored in the $CD4^+$ and $CD8^+$ splenocytes and compared to that in the naive group (Table 2). Both the $CD4^+$ and $CD8^+$ populations expressed CD25 at 7 dpi. Moreover, the percentage of $CD25^+$ cells was not significantly different between the naive and infected groups. However, the difference between the two infected groups was significant for the $CD4^+$ population and was correlated with the higher expression of CD25 on $Foxp3^+$ cells observed in the RH group.

Although proliferation rates were similar in both groups, the RH group showed features of uncontrolled inflammation, with expression of high levels of CD69 molecules.

Induction of pro-inflammatory chemokines and cytokines after infection. To determine whether protection may be due to equilibrium between effector and regulator cells, chemokines and cytokines associated with TH1/Treg were quantified at the local level (IP) and in the sera of both infected groups at 7 dpi.

Chemokine levels were quantified in the peritoneal washes and in the sera at 7dpi. In the sera, only CCL2 was above the detection threshold and the level was higher in the RH group (**Fig. 3A**). Levels of CCL2, CCL3 and CCL20 in the peritoneal washes were significantly higher in the RH group than in the Mic1.3KO group (**Fig. 3B, C and D**).

Systemic (sera) and local (IP) IL12 and IL23 levels were also quantified at 7dpi (**Fig. 4**). The levels of the IL12p40 subunit were significantly higher for the RH group in the peritoneal washes (**Fig. 4A**) and in the serum (**Fig4 B**). IL12p70 was undetectable in the peritoneal washes and the levels of the biological form of this cytokine were not significantly different between the two groups (**Fig. 4C**). IL23 was detected at low levels in the sera of the RH group mice but not in the Mic1.3KO mice (**Fig.4D**). IL23 was below the threshold of detection in the peritoneal washes.

As IL12p70 directs the differentiation of naive T cells in TH1 cells and IL23 is linked to TH17 differentiation and pathogeny, cytokines related to TH1 and TH17 cells were then quantified.

Seric IFNγ levels were significantly higher in the RH group than in the Mic1.3KO group (**Fig. 4E**). Slightly higher levels of IFNγ in the peritoneal washes were observed for the RH group but the difference from the Mic1.3KO group was not statistically significant (**Fig.4F**). IL6 levels were also significantly higher in the sera and peritoneal washes in the RH group compared to the Mic1.3KO group (**Fig. 4G and H).** IL17A levels were at the detection limit for the RH group and below the detection limit for the Mic1.3 KO group (data not shown).

Taken together, these results suggest that the responses of the RH group were more inflammatory and revealed a TH1/TH17-like response, in contrast to the Mic1.3KO group that showed a more regulated TH1/Treg-like response.

To evaluate TH1/Treg response further, Ag-specific IFNγ production was measured in spleen cell supernatant following 48h restimulation with soluble *Toxoplasma* antigens (TE) (**Fig. 5A**). The IFNγ levels were below the detection threshold on day 4 in both groups (data not shown). At day 7, the levels were very high in the RH group and significantly different between the two groups. Cells from the RH group produced higher levels of IFNγ than those from the Mic1.3KO group, with or without TE stimulation.

Interleukin-10 (IL-10) plays an important role in the suppressive function of antigen-specific Tregs, and IL-10 production by Tregs has been described as a prominent Treg suppressor mechanism (22). IL10 levels in the splenocyte supernatants were significantly different in both groups (**Fig. 5B**). Splenocytes of animals from the RH group produced less IL10 than those from the Mic1.3KO group. However, for both groups IL10 production was dependent on Ag restimulation. To investigate the cellular source of IL10, spleen cells were cultured with TE in order to track the IL10-secreting cells by flow cytometry. There was no significant difference between the two groups in the percentage of IL10-secreting cells (median value 10.5 for the RH group and 7 for the Mic1.3KO group). We were unable to detect IL10 in the $Foxp3^+$ population. Moreover, the main sources of IL10 in both groups were the $CD4^-$ cells, since the percentages of $CD4^+$ cells amongst IL10-producing cells in two different experiments was 28% and 30% for the RH and the Mic1.3KO groups, respectively.

IL2 is a cytokine that is essential for Treg cell development and survival (23) and is also involved in the immunosuppression induced during the acute phase of toxoplasmosis (24, 25). IL2 levels were quantified in spleen culture supernatants after activation with anti-CD3 (data not shown). IL2 levels were significantly lower in the RH group than in the naive mice at

7dpi. The high levels of IFNγ in the RH group supernatants indicated that the cellular cytokine secretion capacity was not impaired.

Taken together, these results suggest that, despite a strong cellular response and high levels of production of IFNγ, the parasite burden was not controlled in the RH group, in contrast to the Mic1.3KO group, in which the cellular response and the parasite burden were controlled.

Induction of Treg in vivo in the RH-infected group induced better control of the parasites and reduced the level of inflammatory cytokines.

Administration of a complex consisting of a low dose of IL2 and anti-IL2 JES6-1 specifically amplifies Treg *in vivo* (26). To evaluate further the role of $CD4^+Foxp3^+$ cells in protection, RH-infected mice were treated with IL2/anti-IL2 complexes, and the cytokine secretion and parasite burden were evaluated at 7 dpi. Treatment with IL2/anti-IL2 complexes significantly increased the frequency of $Foxp3^+$ cells in the spleen. The percentages of $CD4^+Foxp3^+$ splenocytes in control and IL2/anti-IL2 treated mice were 1.6±0.5% and 3.7±1.6% (in the first experiment) and 1.3±0.4% and 3.5±0.3% (in the second experiment), respectively. Mice treated with IL2/anti-IL2 complexes showed a diminished number of parasites in the peritoneal cavity compared to control mice injected with PBS with $0.5x10^6$ in the IL2-treated group versus $5.5x10^6$ in the group injected with PBS (corresponding to median values from two independent experiments with 5 to 6 mice per group per experiment) correlated with increased numbers of cells (data not shown).

CCL2, and CCL3 levels were higher in the control group compared to the IL2 treated group (**Fig.6 A and B**) at the local level (IP). IFNγ and IL6 levels in the serum and IP were also significantly higher in the control group compared to the IL2-treated group (**Fig.6 C to F**). IL17A was detectable at low levels at the local level only in the control group (data not shown).

Specific IFNγ and IL10 secretion by splenocytes after restimulation with the antigen was also significantly higher in the IL2-treated group than in the control group (**Fig. 7A and B**). These findings suggest that the cytokine response after IL2 treatment may be switched from an inflammatory response to a more controlled response.

In addition, mice vaccinated with Mic1.3KO and treated with the anti-IL2 receptor antibody PC61, that is known to deplete Treg preferentially, revealed features similar to RH-infected mice, including higher parasite count, morbidity and inflammatory cytokine levels (data not shown).

Taken together, these results indicate that mice infected with RH and treated with IL2/anti-IL2 complexes showed features similar to mice vaccinated with Mic1.3KO (reduced parasite count, more regulated TH1 response), suggesting a role for Treg in the protection developed with this vaccine strain.

Discussion

The aim of this study was to investigate the role of T regulatory cells (Treg) $CD4^+Foxp3^+$ in the protection induced by an attenuated replicating Type I Mic1.3KO strain. Type-I strains are uniformly lethal except after attenuation, leading to lower replication and dissemination in the host and the development of long-term immunity, as for Type II strains. Several studies have been performed to examine the role of Treg using a C57BL/6-susceptible mouse strain (14, 17-18, 20) and a Type II *Toxoplasma* strain. Decreased $Foxp3^+$ Treg counts at local and systemic sites were associated with such mortality in C57BL/6-susceptible mice after lethal oral infection with the Type II strain (14). This decrease occurred very shortly before the animals died and was correlated with virulence. When the dose and the route of infection were changed and the mice recovered (14, 18), the decrease was only transient. In the present study we investigated the $Foxp3^+$ Treg after infection with the attenuated Mic1.3KO parasite

342 in comparison with the parental RH Type-I strain from which it is derived. In contrast to the
343 results obtained after infection with Type II strain (14), the mortality induced by infection
344 with the parental RH strain was not associated with decrease in $CD4^+Foxp3^+$ Treg numbers
345 and frequencies. Neither the number nor the frequency differed from the naive control group.
346 Lack of Treg cell reduction seemed to contradict the uncontrolled immune response observed
347 in the RH group and previous reports (14; 18) showing that a decrease in Treg cells was
348 associated with mortality. However, we cannot exclude the possibility that this decrease
349 appeared just before death or that it was only significant at the local site. Oldenhove *et al.*
350 showed a highly significant reduction in $Foxp3^+$ Treg frequency both in the intestine (local)
351 and spleen at 10dpi. However, the reduction in Treg splenocytes was only significant at
352 12dpi, which was the day the mice died.
353 In our experiment, a weak increase in Treg cells was observed at the local site after infection
354 with the vaccinal strain. This increase was small and transient and was followed by increases
355 in $CD4^+CD25^+$ T effector cells correlated with parasite control. This increase in Treg cell
356 number was limited to local sites, since both the numbers and percentages of $CD4^+Foxp3^+$T
357 cells remained unchanged in spleens until 7 dpi in both infected groups compared to naive
358 control mice. However, the percentage of $CD4^+$ $Foxp3^+$ splenocytes expressing CD25 in the
359 RH group was slightly higher than in the Mic1.3KO group on day 7. Tenorio *et al.* (24)
360 reported that, after infection with a Type II strain of *T.gondii*, Treg cells were activated and
361 showed increased expression of CD25 and increased percentages of Treg expressing CD25.
362 Treg expanded *in vivo* following IL2 injection also expressed higher levels of CD25 (27).
363 The lower increase in Treg cells in the RH group at the local site of infection may explain the
364 difference between the Mic1.3KO and RH groups. In BALB/c resistant mice, Treg cells are
365 thought to reduce immunopathology since depletion of these cells induces morbidity due to
366 increased production of pro-inflammatory cytokines and higher parasite burden after oral

infection (19). In this study, mice of both the resistant BALB/c and susceptible C57BL/6 strains had higher $CD4^+Foxp3^+$ counts at the local level 6 days post-infection than naive mice, and the absolute count for the resistant strain was higher than that in the susceptible strain. This suggests that differences in Treg counts may account for the differences in mortality. This may also have been true in our experiment, since the Treg count was lower for the RH group than for the Mic1.3KO group.

Treg expansion/recruitment and accumulation at local sites of infection have been extensively described in various parasite infections, especially in chronic infections (28).

After infection, the microenvironment may also influence the ability of Tregs to determine the outcome of an immune response towards tolerance or immunity. TH17 and Treg differentiation is closely related and in the presence of proinflammatory cytokines TGFβ–induced Foxp3 expression is reduced, favoring the differentiation of TH17 (29).

Although IL17A was undetectable in our study, several cytokines or chemokines induced in TH17 differentiation or induced by IL17 were preferentially expressed in the RH group. CCL2, CCL3 and CCL20 levels were higher in RH-infected mice than in Mic1.3KO-infected mice, both at the local level and at the systemic level. CCL20 is produced by TH17 cells (30) and IL17 stimulates production of CCL2 and IL6 (29). CCL2 is produced early after infection and plays an essential role in resistance to acute toxoplasmosis by recruitment of $Gr1^+$ monocytes to the site of injection (31). Dal Secco *et al.* (32) also showed that Tregs inhibit CCL2 and CCL3 production by DC *in vivo* and limit the recruitment of inflammatory cells.

Higher levels of IL6 and IL23 were found in the RH group compared to the Mic1.3KO group. Both cytokines are involved in TH17 differentiation. Moreover, TH17 cells that differentiate in the presence of IL23 are pathogenic (33). The pathological role of IL17 has been shown in ocular toxoplasmosis. Sauer *et al.* (34) demonstrated that the balance between TH17 and TH1 responses was crucial for the outcome of infection. They also showed a deleterious TH17

response at the local level after primary infection which was reduced after reinfection. TH2/Treg responses were enhanced in the reinfection condition (35).

Contrary to the TH1/TH17-type signature after RH infection, the cytokine signature in our study after Mic1.3KO infection was of TH1/Treg type.

We observed a higher level of IL10 in the spleen cell supernatant of the Mic1.3KO group. IL10 is an immunosuppressive cytokine and the common feature of all effector Tregs is the expression of IL10 (22). Most of the IL10-secreting splenocytes of both infected groups (75%) were $CD4^-$, and within the CD4 population the secreting cells were $Foxp3^-$. This result is consistent with previous results reported by Jankovic *et al*. (36), who showed that the main producer of IL10 during *T. gondii* infection within a CD4 population was the $Foxp3^-$ $IFN\gamma^+$ cell population.

IL2 is essential for Treg cell development and survival (23) and is also known to inhibit TH17 and to favor Treg differentiation. A decrease in the IL2 level was observed in the RH group after anti-CD3 activation *in vitro* compared to the control naive group and the Mic1.3KO group. The low level of IL2 in the RH group may have resulted from greater consumption of IL2 rather than from lower IL2 release, since cells are not impaired in IFNγ production. This low IL2 level may therefore be correlated with the higher expression of CD25 by $Foxp3^+$ cells. This reduced IL2 level in cells from animals infected with *T.gondii* has been described by others (14, 19, 24). A reduced number of Treg cells during the acute phase of *T.gondii* infection is a consequence of a reduced IL2 availability (14, 19). IL2 is also involved in the transient immunosuppression observed during acute toxoplasmosis (24, 25).

IFNγ levels were higher in the sera, peritoneal cavity and spleen cell supernatant of the RH group. However, the production of IFNγ by splenocytes of the RH group was not antigen-dependent and was not increased by antigen restimulation. These findings suggest that splenocytes from the RH group were in an activated state, and the expression of CD69 by

417 these cells further supports this hypothesis. Expression was not observed until day 7, one to
418 three days before the mice died. CD69 expression was not observed in the Mic1.3KO group
419 even at day 11. Expression of $CD69^{+}$ T cells is known to be localized at sites of chronic
420 inflammation and at sites of active immune response *in vivo* (37). The role of CD69 has not
421 yet been elucidated, and previous findings have suggested that CD69 is an activating
422 molecule, although recent studies have also reported regulatory functions. CD69 regulates
423 immune and inflammatory responses by acting as a brake on the differentiation of TH17
424 effector cells (38).
425 In summary, the protective response after infection with the attenuated Type I-derived
426 Mic1.3KO may originate from a TH1 regulated by an appropriate Treg response. The
427 mortality induced by infection with the parental RH strain may have been due to a TH1/TH17
428 type response.
429 When the balance was in favor of Treg by using IL2/anti-IL2 complexes we demonstrated
430 that treated mice infected with RH showed similar features (lower parasite burden and
431 TH1/Treg cytokines and chemokines) to mice vaccinated with Mic1.3KO. These complexes
432 work by inducing and expanding peripheral Treg cells (27). Treatment of C57BL/6 mice with
433 IL2/anti-IL2 during *T.gondii* infection with a lethal dose of Type II cysts was shown to
434 prevent the loss of Treg and reduce the morbidity but it induced higher parasite burden in the
435 brain (14). The lower parasite burden that we observed at the local site in the Treg-induced
436 group is surprising, and seems contradictory. However, a higher parasite burden has also been
437 reported in lamina propria tissue of Treg-depleted BALB/c mice after oral infection (19). The
438 author argued that this high parasite burden may be an indirect effect of the high levels of pro-
439 inflammatory cytokines at the local site causing tissue destruction. This may also have been
440 true in our experiments since RH infection induced high levels of IFNγ at the infection site.
441 Alternatively, very high and constitutive production of IFNγ may lead to the exhaustion of the

response as a result of negative feed-back or by saturation of the IFN receptor leading to impairment of the IFNγ-induced response. This could also be related to a failure to induce Ag-specific IFNγ production that may be ineffective in parasite control.

In conclusion, this study provides evidence that Treg cells may have contributed to protecting mice after infection with an attenuated, replicating Type-I strain of *T. gondii*. It is important to determine whether Treg cells have a beneficial or detrimental role in protection against toxoplasmosis in order to design an efficient vaccine. Although a complex mechanism may underlie the protection induced by an attenuated Type-I strain, we showed that a change in the absolute number of Treg cells may be crucial in determining whether animals are protected or whether they will succumb.

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Table 1. PECs of RH group mice presenting activated phenotype after infection

	Percentage of positive cells			
Groups	**CD4$^+$**	**CD8$^+$**	**CD69$^+$**	**Ki-67$^+$**
Naive	8.1±4.1	0.82±0.74	5.8±4.2	16.4±3.4
RH	15.4±2.4	2.8±1.1	19.9±5.1[a]	62.8±3.5[a]
Mic1.3KO	16.1±3.1	3.3±2.5	11.0±3.3	52.7±7[a]

PECs obtained at 7 dpi were stained with different mAbs as indicated and analyzed by cytometry. Results are presented as means± SD and are representative of two independent experiments with 4 to 6 mice per group. [a] The difference between the infected groups and the control group (P<0.05) test was significant; [b] the difference between the two infected groups using Kruskal-Wallis test followed by Dunn's post test was significant (P<0.05).

599 Table 2. Splenocytes of RH group mice presenting activated phenotype after infection
600
601

	Percentage of positive cells				
Groups	**CD69$^+$**	**CD25$^+$**	**CD4$^+$CD25$^+$**	**CD8$^+$CD25$^+$**	**Ki-67$^+$**
Naive	3.7±2.7	3.8±0.6	2.8±0.6	0.9±0.6	14.0±5.5
RH	11.8±5[a,b]	6.5±0.8[b]	3.4±1.1[b]	1.5±0.6	24.3±3.4
Mic1.3KO	1.9±1.2	2.0±0.7	1.2±0.7	0.8±0.6	37.6±4.9[a]

602

603 Splenocytes obtained at 7 dpi were stained with different mAbs as indicated and analyzed by
604 cytometry. Results are presented as means± SD and are representative of three independent
605 experiments with 4 to 6 mice per group. [a] The difference between the infected groups and the
606 control group (P<0.05) was significant; [b] the difference between the two infected groups
607 using Kruskal-Wallis test followed by Dunn's post-test was significant (P<0.05).

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611 Figure legends

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614 FIG 1 Lack of parasite control in the RH group uncorrelated with low IFNγ secretion. (A)
615 Parasites were counted directly after peritoneal lavage at the times indicated post-infection
616 with 100 RH or Mic1.3KO tachyzoites (A). Results are expressed as the median plus range.
617 Cumulative data from four different experiments are shown (4 to 6 mice per group per time
618 point per experiment). Sera (B) were recovered at the times indicated post-infection, and the
619 IFNγ levels were quantified. Results are representative of three independent experiments with
620 5 or 6 mice per group per time point injected with 100 tachyzoites of RH or Mic1.3KO, (*,
621 $P<0.05$; **, $P<0.01$; ***, $P<0.001$ Kruskal-Wallis followed by Dunn's post-test.).

622

623 FIG 2 $CD4^+Foxp3^+$ Treg cells recruited/expanded before $CD4^+$ $Foxp3^-$ $CD25^+$ Teff cells at
624 the local site after infection with Mic1.3KO but not at the systemic level. PECs recovered at
625 the times indicated post-infection and splenocytes at 7 dpi were stained with anti-CD4 anti-
626 CD25 and anti-Foxp3 mAbs, and analyzed by flow cytometry. (A) Percentage of total $Foxp3^+$
627 population within the $CD4^+$ cells recovered at 4dpi. * $P<0.05$ using Kruskal-Wallis test. For
628 calculation of the count (B and C), the absolute number of lymphocytes was evaluated by
629 multiplying the total PEC count by the percentage of the lymphocyte gate compared to the
630 whole population. Treg cells were defined as $Foxp3^+CD25^+$ and $Fopx3^+CD25^-$ (B), and Teff
631 cells as $Foxp3^-CD25^+$ (C) within the $CD4^+$ population. The data presented are representative
632 of two independent experiments with 5 mice per group, per injection time point. * $P<0.05$,
633 ***P <0.001 Kruskal-Wallis followed by Dunn's post-test.

(D) Percentage of the total Foxp3$^+$ and CD25$^+$Foxp3$^+$population within the CD4$^+$ splenocytes at 7dpi with RH or Mic1.3KO tachyzoites. Results are representative of four independent experiments with 4 to 6 mice per group. *, P<0.05 using Kruskall Wallis followed by Dunn's post test. (E) Percentage of Foxp3$^+$ expressing CD25 compared to total Foxp3$^+$cells. Results are cumulative data from 4 different experiments. *, P<0.05 using Kruskall Wallis followed by Dunn's post test.

FIG 3 Higher chemokine levels in the RH-infected group. Chemokines (CCL2, CCL3, CCL20) were quantified in the serum (A) and peritoneal washes (B-D) at 7dpi with RH or Mic1.3KO tachyzoites. Control naive mice were below the detection threshold for all chemokines. The data presented are representative of five independent experiments with 5 to 6 mice by group. ** P<0.01, ***P<0.001 using Wilcoxon-Mann Whitney exact test with strata.

FIG 4 Higher pro-inflammatory cytokine levels in the RH-infected group. IL12p40, IL12 p70 and IL23 were quantified by ELISA in peritoneal washes (A) (only for IL12p40) and in the sera (B-D) at 7dpi. IFNγ and IL6 levels were quantified by ELISA in peritoneal washes (E, G) and the sera (F, H) at 7dpi. Control naive mice were below the detection threshold for all cytokines. The data presented are representative of four independent experiments with 4 to 6 mice per group. ** P<0.01, ***P<0.001 using Wilcoxon-Mann Whitney exact test with strata.

FIG 5 Strong systemic, non-specific IFNγ production and low IL10 production induced by infection with RH. Splenocytes were recovered 7 dpi with RH or Mic1.3KO tachyzoites, and stimulated with medium or with parasite extract (TE). Culture supernatants were collected

after a 48-h stimulation period. IFNγ (A) and IL10 (B) levels were determined by ELISA. The data presented are representative of four independent experiments with 4 to 6 mice per group.*, $P<0.05$; **, $P<0.01$ using Kruskall-Wallis test followed by Dunn's post test.

FIG 6 Higher chemokine and pro-inflammatory cytokines levels in RH-infected control group compared to RH-infected and IL2-treated group. The chemokines CCL2 (A) and CCL3 (B) were quantified in peritoneal washes at 7dpi. IFNγ (C, D) and IL6 (E, F) levels were quantified by ELISA in peritoneal washes (C, E) and in sera (D, F) at 7dpi. The control and IL2 groups were injected by ip route with PBS or IL2/anti-IL2 Ab, respectively. Results are expressed as the median plus range. The data presented are representative of 2 independent experiments with 5 to 6 mice per group. *$P<0.05$ Wilcoxon-Mann Whitney exact test with strata on the cumulative data of the two experiments.

FIG 7 Systemic, antigen specific IFNγ and IL10 production after treatment with IL2/anti-IL2 in RH-infected mice. Splenocytes were recovered at 7 dpi with RH tachyzoites, and stimulated for 72h with TE. Culture supernatants were collected after a 48-h stimulation period. The data presented are representative of 2 independent experiments with 5 to 6 mice per group. The control and IL2 groups were injected by ip route with PBS or IL2/anti-IL2 Ab, respectively. Values were corrected by subtraction of level in control wells without antigen. IFNγ (A) and IL10 (B) were measured by ELISA.* $P<0.05$ Wilcoxon-Mann Whitney exact test with strata on the cumulative data on the two experiments.