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Original article

Mic1-3 Knockout *Toxoplasma gondii* is a good candidate for a vaccine against *T. gondii*-induced abortion in sheep

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Abstract – This study assessed the effectiveness of a mutant strain of *Toxoplasma gondii* (RH strain) lacking the *mic1* and *mic3* genes (Mic1-3KO) against *Toxoplasma* abortion in sheep. Ewes were inoculated subcutaneously with 10^5 Mic1-3KO tachyzoïtes in three independent experiments. Following vaccination, Mic1-3KO induced a mild febrile response and serum IgG antibodies, which persisted throughout the experiments. Tissue cysts formed in the sheep, but were not, under our experimental conditions, infectious when given orally. Ewes were mated two months after vaccination and were orally challenged with the PRU strain of *T. gondii* at mid-gestation (400 oocysts in Experiments 1 and 2; 100 oocysts in Experiment 3). Challenge of vaccinated pregnant ewes resulted in a slight febrile response, whereas unvaccinated ewes developed a more severe, characteristic febrile response of longer duration. After challenge, all unvaccinated ewes aborted whereas 62%, 91% and 64% (Experiments 1, 2 and 3 respectively) of the lambs from vaccinated ewes were viable, with no clinical signs of infection. Mic1-3KO was as effective as S48, the strain used as a live vaccine for sheep (Toxovax®). A dose of 10^5 Mic1-3KO tachyzoites was sufficient to induce protection (versus a dose of 2×10^6). Both subcutaneous and intraperitoneal injections were effective. Moreover, preliminary results showed the potential of Mic1-3KO to reduce the development of tissue cysts in lambs born to vaccinated ewes. This study demonstrates that Mic1-3KO is a potent vaccine candidate.

Toxoplasma gondii **/ abortion / sheep / vaccine / Mic1-3KO**

1. INTRODUCTION

Toxoplasmosis is a cosmopolitan zoonotic disease caused by the coccidian protozoan *Toxoplasma gondii*, an obligatory intracellular parasite. *T. gondii* is capable of infecting all warm-blooded animals including humans [17].

Toxoplasmosis in animals is of great economic importance worldwide because it causes abortions and stillbirths, especially in sheep and goats [7, 8]. The most recent surveys on seroprevalence in sheep were conducted in Brazil (29.41%), southern Italy (49.9%, 28.5%) and Lithuania (42.1%) [10, 20, 37, 38]. The differences observed may be due to the frequency of felines on farms, climatic variations and age of animals.

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Following infection, sheep develop a protective Th1-type cell mediated immunity [23] and will not normally abort due to toxoplasmosis in future pregnancies [5, 29, 35]. This suggests that a strategy based on vaccination should be successful. A vaccine would also reduce or prevent formation of *T. gondii* tissue cysts, a source of contamination for humans, since sheep are animals used for food [11, 28].

A vaccine based on live S48 *Toxoplasma* tachyzoites is available (Ovilis Toxovax®, Intervet, Angers, France) and protects pregnant sheep against toxoplasmosis [6]. In ewes, 72 to 80% of lambs resulting from mothers vaccinated with Ovilis Toxovax® are viable versus 18% for lambs from unvaccinated sheep [3]. S48 is a type I strain [18], which is very virulent in mice [18, 24], but has lost the ability to form tissue cysts in intermediate hosts and oocysts in cats [1]. One strategy for developing safer vaccines against toxoplasmosis is to create specific gene-deficient strains of *T. gondii*. Genetic deletions in these strains may prevent virulence reversion in contrast to naturally attenuated strains.

The RH strain of *T. gondii* (type I) is very virulent in mice but has lost the ability to form oocysts in cats [12]. Cérède et al. [9] constructed a mutant strain of *T. gondii* RH lacking the *mic*1 and *mic*3 genes (Mic1-3KO). Disruption of these two genes impairs virulence in mice [9]. We showed that the Mic1-3KO strain confers protection against chronic and congenital toxoplasmosis in mice [27]. We aimed at demonstrating the efficacy of the Mic 1-3 Knockout *Toxoplasma gondii* in preventing abortion in sheep.

Most infections in sheep occur after birth and are associated with contamination of the environment with *Toxoplasma* oocysts derived from cat faeces [8, 14]. The predominant lineage of *T. gondii* strains isolated from sheep is currently type II [13, 15, 34]. These findings justify both the choice of oocysts and the type II strain to evaluate the efficacy of Mic1-3KO against abortion in sheep after challenge based on the natural route of infection.

2. MATERIALS AND METHODS

2.1. Animals

Thirty-six Bizet ewes aged 12 to 14 months, 54 Romanov ewes aged 12 to 14 months and 33 Sologne ewes aged 12 to 14 months, shown to be seronegative for IgG antibodies to *T. gondii* by ELISA were housed in the animal facilities at INRA (Experimental Infectiology Unit, Nouzilly, France). All animal experiments undertaken were authorised by the Direction Départementale des Services Vétérinaires (accreditation number: A37805 N°37056).

2.2. Parasites

Mic1-3KO tachyzoites (patent: WO2005/072754) were obtained by targeted gene disruption of the *mic1* and *mic3* genes in the ΔHX RH strain of *T. gondii* [9]. Tachyzoites of the live incomplete S48 strain are sold as a live vaccine for sheep and goats (Ovilis Toxovax®). Mic1-3KO tachyzoites and S48 tachyzoites (donated by J.F. Dubremetz, UMR5235, CNRS, Université de Montpellier 2, France) were grown in human foreskin fibroblast cells (HFF) at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-glutamine supplemented with 10% fetal bovine serum (FBS) and 50 U/mL penicillin/50 µg/mL streptomycin (all Invitrogen, Cergy Pontoise, France), in a 5% CO_2 atmosphere. The culture medium was replaced by serum-free DMEM 24 h before harvesting the extra cellular tachyzoites. The appropriate parasite concentration was obtained before inoculation by addition of DMEM to a volume of 1 mL.

RH strain tachyzoites were harvested from the peritoneal fluids of Swiss OF1 mice that had been intraperitoneally infected 3 to 4 days earlier. These were used to prepare the *T. gondii* antigen (TAg) as previously described [36].

Sporulated *T. gondii* oocysts of the PRU strain (type II strain, donated by M.L. Dardé, Laboratoire de Parasitologie-Mycologie; CHRU Dupuytren; Limoges, France) were obtained from the faeces of experimentally infected cats.

2.3. Experimental design

2.3.1. Protection against abortion

Three experiments were performed. Ewes were vaccinated subcutaneously by single injection in the

lower part of the chest behind the left foreleg or intraperitonealy, two months prior to mating. Pregnancy was confirmed by ultrasound scan at 40 days gestation.

Three groups of Romanov ewes were involved in Experiment 1: (1) non-vaccinated controls (11 ewes), (2) vaccinated s.c. with 10^5 Mic1-3KO tachyzoites (12 ewes), (3) vaccinated s.c. with 10^5 S48 tachyzoites (13 ewes). Three groups of Bizet ewes were involved in Experiment 2: (1) non-vaccinated controls (12 ewes), (2) vaccinated s.c. with 10^5 Mic1-3KO tachyzoites (12 ewes), (3) vaccinated s.c. with 2×10^6 Mic1-3KO tachyzoites (12 ewes). Three groups of Solognot ewes were involved in Experiment 3: non vaccinated controls (11 ewes), vaccinated with 10^5 Mic1-3KO tachyzoites either s.c. or i.p (two groups of 11 ewes).

All animals in Experiments 1 and 2 were infected per-os with 400 sporulated oocysts of PRU strain at mid-gestation. In Experiment 3, ewes were infected per-os with 100 sporulated oocysts of PRU strain at mid-gestation.

2.3.2. Persistence of Mic1-3KO in the brain of vaccinated ewes

Romanov ewes were inoculated s.c. ($n = 6$) or i.p. ($n = 5$) with 10^5 Mic1-3KO tachyzoites and 6 ewes were infected per-os with 400 sporulated oocysts of PRU strain. Two months after infection, the ewes were killed and the presence of the parasite in the brain was determined by Percoll density-gradient centrifugation followed by direct microscopy.

2.4. Clinical observations

Rectal temperatures were recorded at intervals for all sheep after vaccination and challenge. All animals were monitored daily until lambing was completed.

2.5. Humoral response

Blood was taken from the jugular vein before vaccination and at intervals. Sera were tested for the presence of IgG antibodies against *T. gondii* by ELISA using *T. gondii* antigen as previously described [26]. The wells were coated with TAg at 10 µg/mL. Serum samples diluted to 1:100 were added to the wells. Bound antibodies were detected with a donkey anti-sheep IgG alkaline phosphatase conjugate (Sigma, St. Quentin Fallavier, France) diluted to 1:5 000. Experimental ewes before immunisation were used as a negative control. The mean absorbance of these serum samples + 2 standard deviations (S.D.) represented the cut off point for a positive reading.

2.6. Brain cyst load

Brain tissue was homogenised in 100 mL (lamb) or 200 mL (adult) of PBS, containing 100 U/mL penicillin and 100 µg/mL streptomycin, left overnight at 4 °C, and then centrifuged in tubes of 50 mL capacity, for 15 min at 300 *g*. The sediments were washed with PBS and resuspended in 35 mL PBS and layered onto 5 mL of 0.9% NaCl/90% Percoll (Sigma). After centrifugation for 15 min at 500 *g*, the interfaces containing cysts were recovered. The upper phases were collected, washed with PBS and centrifuged for 5 min at 1 200 *g*. The resulting pellets were mixed with the interfaces containing cysts, washed again with PBS and centrifuged for 5 min at 1 200 *g*. The final pellet was resuspended in 5–7 mL PBS. The cysts were counted microscopically and the detection limit was 25 cysts per brain.

2.7. Statistical analysis

The Mann–Whitney *U*-test using GraphPad Instat 2.1 software was used to determine the significance of variations between groups.

3. RESULTS

3.1. Protective efficacy of Mic1-3KO (Exp. 1)

To assess the protective efficacy of the Mic1-3KO, Romanov ewes were immunised s.c. with 10^5 Mic1-3KO parasites or with 10^5 S48 tachyzoites [3].

3.1.1. Clinical signs after vaccination of Romanov ewes

The groups of ewes vaccinated either with 10^5 Mic1-3KO tachyzoites or 10^5 S48 tachyzoites showed a fever that began on day 2 after vaccination (Fig. 1A). On days 3 and 5, the mean temperature of the group of ewes vaccinated with 10^5 S48 (40.5 °C ± 0.81 on day 3, 41 °C ± 0.59 on day 5) was higher ($p = 0.038$ on day 3, $p = 0.007$ on day 5) than that of the group of ewes vaccinated with 10^5 Mic1-3KO (39.9 °C ± 0.57 on day 3, 40.2 °C ± 0.69 on day 5). No febrile

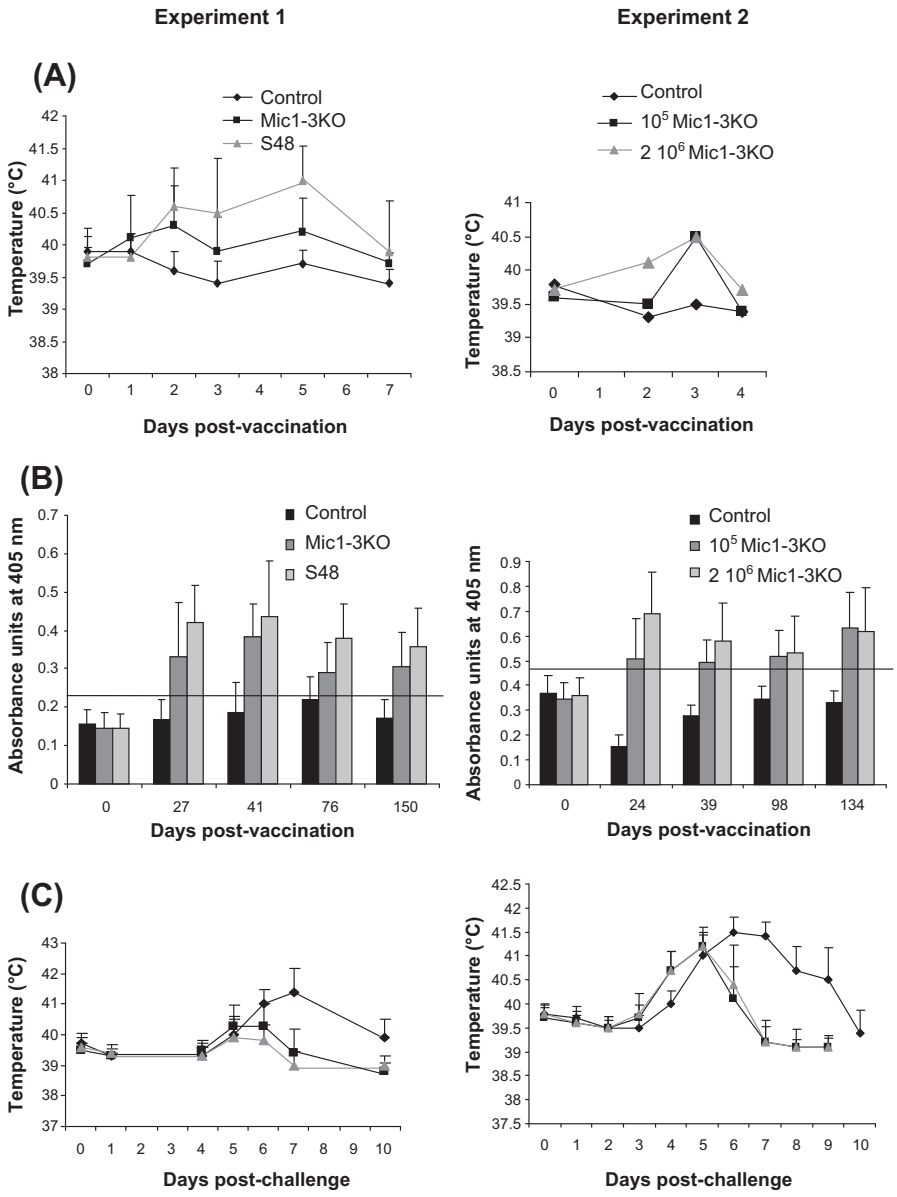

Figure 1. Experiment 1 and Experiment 2: Exp. 1, two groups of Romanov ewes received single vaccinations s.c. by single injection with either 10^5 Mic1-3KO tachyzoites or 10^5 S48 tachyzoites. Exp. 2, two groups of Bizet ewes were vaccinated s.c. by single injection with either 10^5 or 2×10^6 Mic1-3KO tachyzoites. One group in each experiment was not vaccinated (control). All ewes were challenged per-os with 400 sporulated oocysts of the PRU strain at mid gestation. (A) Mean rectal ± S.D. temperature of groups and (B) systemic IgG against *T. gondii* measured by ELISA after vaccination (mean and S.D.), line indicates positive cut off point. (C) Mean ± S.D. rectal temperature following challenge.

Table I. The number of viable and non-viable lambs born, and the mean gestation period for each group. In Experiments 1 and 2, ewes were infected per-os with 400 sporulated oocysts of PRU strain at mid-gestation. In Experiment 3, ewes were infected per-os with 100 sporulated oocysts of PRU strain at mid-gestation.

Groups	Number of ewes	Number of lambs expected	Number of viable lambs (%)	Number of non-viable lambs		Mean gestation period ± S.D.
				Early abortion*	Late abortion	
Exp. 1 (Romanov ewes)						
Control	11	11	0 (0)	9	2	98 ± 16
s.c. 10^5 Mic1-3KO	11	21	13 (62)	0	8	143 ± 4
s.c. 10^5 S48	13	25	17 (68)	0	8	146 ± 3
Exp. 2 (Bizet ewes)						
Control	10	10	0 (0)	6	4	122 ± 20
s.c. 10^5 Mic1-3KO	10	11	10 (91)	0	1	158 ± 10
s.c. $2x10^6$ Mic1-3KO	11	14	10 (71)	0	4	154 ± 8
Exp. 3 (Solognot ewes)						
Control	6	10	0 (0)	1	9	123 ± 21
s.c. 10^5 Mic1-3KO	8	11	7 (64)	0	4	137 ± 10
i.p. 10^5 Mic1-3KO	8	12	11 (92)	0	1	143 ± 6

* Abortion before 105 days gestation.

responses were detected in control ewes. All vaccinated animals appeared normal and had no loss of appetite.

3.1.2. Humoral response after vaccination

Animals in the control group did not seroconvert to *T. gondii* during the course of the experiment (Fig. 1B). Serum IgG antibodies to *T. gondii* were detected on day 27 following inoculation with either Mic1-3KO tachyzoites or S48 tachyzoites ($p = 0.0004$, $p < 0.0001$ respectively) and persisted throughout the experiment (day 150) with no significant differences between the two immunised groups.

3.1.3. Clinical signs after challenge with 400 oocysts of the PRU strain

The most severe temperature responses were observed in the control group (Fig. 1C), reaching a peak on day 7 (41.4 °C). Ewes vaccinated with 10^5 Mic1-3KO or S48 tachyzoites showed a fever that began on day 5 after challenge and lasted for 2 days before returning to normal by day 7. The maximum temperatures, 40.3 °C and 39.9 °C respectively, were not significantly different ($p = 0.18$).

3.1.4. Outcome of pregnancy after challenge at mid gestation

After challenge, all the ewes in the control group aborted their lambs with a mean gestation time of 98 days (Tab. I). Nine lambs were aborted before 105 days gestation (early abortion, less than 15 days after infection) and 2 lambs were aborted after 105 days gestation (late abortion). Ewes vaccinated with 10^5 Mic1-3KO tachyzoites produced 13 of 21 viable lambs with 8 aborting after 105 days gestation (late abortion). Ewes vaccinated with 10^5 S48 tachyzoites produced 17 of 25 viable lambs with 8 aborted lambs after 105 days gestation (late abortion). No significant difference was observed in expected lambs per ewe in the two vaccinated groups (KO group: 1.91 ± 0.70; S48 group: 1.92 ± 0.49) or in the viable lambs born per ewe (KO group: 1.18 ± 1.08; S48 group 1.31 ± 0.94). The mean gestation times for the ewes that received 10^5 Mic1-3KO or S48 tachyzoites prior to pregnancy were 143.4 and 146 days respectively. The mean gestation time was not significantly different between the two vaccinated groups but was longer than the mean gestation time of the control group ($p < 0.0001$). There was

no difference between the mean weights of viable lambs from the ewes vaccinated with 10^5 Mic1-3KO tachyzoites (2692 ± 788 g) and of viable lambs from the ewes vaccinated with S48 tachyzoites (3042 ± 420 g) ($p = 0.085$). In conclusion, the two vaccines were equally effective.

3.2. Protective efficacy of two vaccination doses of Mic1-3KO (Exp. 2)

3.2.1. *Clinical signs after vaccination of Bizet ewes*

Following vaccination with 10^5 Mic1-3KO tachyzoites, a mild febrile response was observed on day 3 (Fig. 1A). Ewes vaccinated with 2×10^6 Mic1-3KO tachyzoites showed a febrile response that began on day 2 and lasted for 1 day before returning to normal by day 4. The mean temperature reached a peak of 40.5 °C for both groups. All animals appeared normal and had no loss of appetite.

3.2.2. *Humoral response after vaccination*

Animals in the control group did not seroconvert to *T. gondii* during the course of the experiment (Fig. 1B). Serum IgG antibodies to *T. gondii* were detected on day 24 following inoculation with either 10^5 or 2×10^6 Mic1-3KO tachyzoites ($p < 0.0001$) and persisted throughout the experiment (day 134) with no significant difference between the two vaccinated groups.

3.2.3. *Clinical signs after challenge with 400 oocysts of the PRU strain*

The highest temperature responses were observed in the control group (Fig. 1C) on days 6 and 7 (41.5 °C and 41.4 °C, respectively). Ewes vaccinated with 10^5 or 2×10^6 Mic1-3KO tachyzoites developed a fever that began on day 4 after challenge and lasted for 1 day before returning to normal by day 7. For both groups, the mean temperature reached a peak of 41.2 °C.

3.2.4. *Outcome of pregnancy after challenge at mid gestation*

All the ewes in the control group aborted their lambs after challenge, with a mean gestation time of 122 days (Tab. I). Six lambs were aborted before 105 days gestation (early abortion) and four lambs were aborted after 105 days gestation (late abortion). Ewes that received 10^5 Mic1-3KO tachyzoites produced 10 of 11 live lambs, one lamb was aborted after 105 days gestation (late abortion). Ewes that received 2×10^6 Mic1-3KO tachyzoites produced 10 of 14 live lambs with four aborted lambs after 105 days gestation (late abortion). There was no significant difference in the number of viable lambs per ewe between the two vaccinated groups (10^5 Mic1-3KO tachyzoites: 1.00 ± 0.471; 2×10^6 Mic1-3KO tachyzoites: 0.91 ± 0.83). The mean gestation times for ewes receiving 10^5 or 2×10^6 Mic1-3KO tachyzoites prior to pregnancy were 158 and 154 days respectively. These were not significantly different but they were greater than the mean gestation time of the control group ($p < 0.0001$). There was no difference in mean weight ($p = 0.19$) of viable lambs from ewes vaccinated with 10^5 Mic1-3KO tachyzoites (3410 ± 776 g) and viable lambs from the ewes vaccinated with 2×10^6 Mic1-3KO tachyzoites (2950 ± 535 g). The two vaccine doses were equally effective.

3.3. Evaluation of the infection rate of viable lambs (Exp. 3)

A lower challenge dose was used (100 oocysts instead of 400) to have viable lambs in the control group. Furthermore, two vaccination routes were used; Solognot ewes were vaccinated either s.c. or i.p. with 10^5 Mic1-3KO tachyzoites.

3.3.1. *Clinical signs after vaccination of Solognot ewes*

A mild febrile response was observed on day 3 (Fig. 2A) in the group of ewes vaccinated s.c. (39.7 °C ± 0.692, $p = 0.0024$, versus the control group). The group of ewes vaccinated

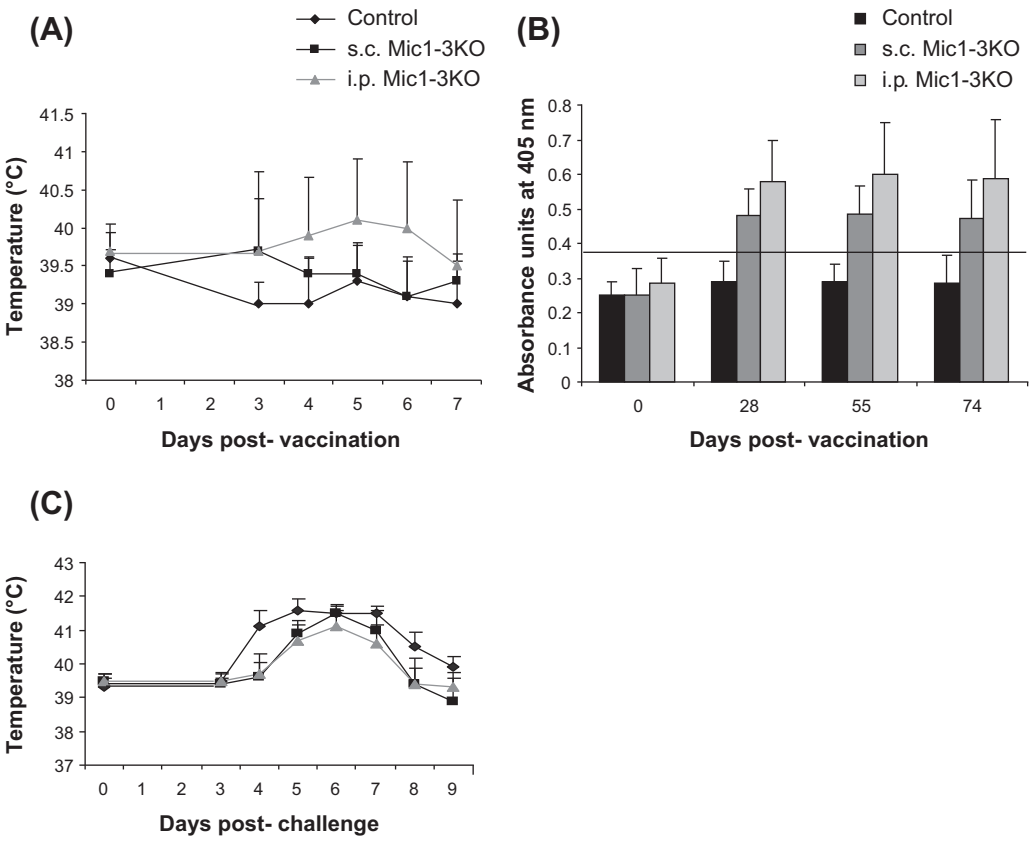

Figure 2. Experiment 3 (Solognot ewes): two groups of ewes were vaccinated by single injection with 10^5 Mic1-3KO tachyzoites either s.c. or i.p. and were challenged per-os with 100 sporulated oocysts of the PRU strain at mid gestation. One group was infected but not vaccinated (control). (A) Mean ± S.D. rectal temperature of groups and (B) systemic IgG against *T. gondii* measured by ELISA after vaccination (means and S.D.). The solid line indicates a positive cut off point. (C) Mean ± S.D. rectal temperature following challenge.

i.p. developed a fever that began on day 4 after vaccination and reached a peak of 40.1 °C on day 5 (40.1 °C ± 0.813, $p = 0.006$, versus the control group) before returning to baseline levels by day 7. No febrile responses were detected in control ewes. All vaccinated animals appeared normal and had no loss of appetite.

3.3.2. Humoral response after vaccination

Animals in the control group did not seroconvert to *T. gondii* during the course of the experiment (Fig. 2B). Serum IgG antibodies to *T. gondii* were detected on day 28 following either s.c. or i.p. inoculation with 10^5 Mic1-3KO tachyzoites ($p < 0.0001$ versus the control group) and persisted throughout the experiment (day 74). The mean serological response was lower following s.c. inoculation on days 28 and 55 than that following i.p. inoculation ($p = 0.04$ and $p = 0.019$, respectively).

3.3.3. Clinical signs after challenge with 100 oocysts of the PRU strain

Vaccinated groups developed a fever that began on day 5 after challenge, with maximum

temperatures on day 6 (Fig. 2C), with no significant difference between the groups. For both vaccinated groups, temperatures returned to baseline levels on day 8 whereas the febrile response persisted until day 9 for the control group.

3.3.4. Outcome of pregnancy after challenge at mid gestation

The challenge dose was severe since it caused 100% fetal mortality in the control group (Tab. I). However, only one lamb was aborted before 105 days of gestation (early abortion) and the other nine lambs were aborted later (mean gestation period: 120 ± 19). Ewes that received s.c. 10^5 Mic1-3KO tachyzoites produced 7 of 11 viable lambs with four aborted lambs after 105 days gestation (late abortion). Ewes that were inoculated i.p. produced 11 of 12 viable lambs with one aborted lamb after 105 days gestation. There was no significant difference in the number of viable lambs per ewe (s.c. inoculation: 0.75 ± 0.834; i.p. inoculation: 1.38 ± 0.916) between the two vaccinated groups. Furthermore, no significant difference was observed in mean gestation period (s.c. inoculation: 137 ± 10; i.p. inoculation: 143 ± 6) or in lamb body weight (s.c. inoculation: 2884 ± 986 g; i.p. inoculation: 2845 ± 999 g).

3.3.5. Cyst load in brain tissue of lambs

No cysts were detected in the brain of 6 of 7 viable lambs born to ewes inoculated s.c. or in the brain of 11 of 11 viable lambs born to ewes inoculated i.p. This indicates that these lambs had < 25 cysts per brain. However, one viable lamb from ewes inoculated s.c. had 562 cysts. The number of cysts was analysed in 4 of 10 dead lambs produced in the control group and in 3 of 4 produced in the group inoculated s.c. Cysts were detected in the brains of 4 of 4 dead lambs from the control ewes (25, 150, 225 and 300 cysts per brain), in 3 out of 3 dead lambs from the ewes inoculated s.c. (980, 780 and 275 cysts per brain) and in the dead lamb from the ewes inoculated i.p. (1025 cysts per brain).

Table II. Brain cysts load in vaccinated sheep.

Oocyst infection (PRU) 400 per-os	Mic1-3KO 10^5 s.c.	Mic1-3KO 10^5 i.p.
70*	60	40
50	< 25*	25
67	< 25	60*
150	45	83*
300	< 25	< 25
340	105*	

* Bioassayed in mice (see Discussion).

3.4. Persistence of Mic1-3KO in the brain of vaccinated ewes

Like the wild type parasite (RH strain), Mic1-3KO is able to form tissue cysts in mice that are not, in our experimental conditions, infectious when given orally [30]. Following *T. gondii* infection, tissue cysts are readily found in sheep tissues, in particular in the brain and heart [16]. Ewes were inoculated s.c. (n = 6) or i.p. (n = 5) with 10^5 Mic1-3KO tachyzoites and six ewes were infected per-os with 400 sporulated oocysts of the PRU strain. The ewes were monitored for febrile and serological responses (data not shown). Two months after infection, the ewes were killed and microscopy was used to detect the parasite in the brain (Tab. II).

Tissue cysts were detected in all ewes infected with oocysts of the PRU strain (163 ± 127 cysts per brain), in 3 of 6 ewes vaccinated s.c. with Mic1-3KO (70 ± 31 per brain) and in 4 of 5 ewes vaccinated i.p. (52 ± 25). No cysts were detected in the brain of the other ewes, which indicates that these ewes had less than 25 cysts per brain.

4. DISCUSSION

We demonstrated that ewes vaccinated with the Mic 1-3 Knockout *Toxoplasma gondii*, developed protection against *T. gondii* abortion. To date, successful vaccination strategies have relied on using a live vaccination approach.

This allows correct processing and presentation of the antigen to the sheep immune system. In three separate experiments, the Mic1-3KO tachyzoites readily infected seronegative sheep. A mild febrile reaction was observed following inoculation, a clinical sign consistent with infection of susceptible sheep with *T. gondii* [2, 4, 29]. Following s.c. injection of 10^5 S48 tachyzoites the febrile response was more severe and lasted longer. S48 is lethal for mice; very low doses [24] are fatal, whereas a high mortality rate is only observed with $\geq 10^6$ Mic1-3KO parasites [30]. The Mic1-3KO parasites did not multiply at the site of injection after i.p. injection of mice, and the parasite burden in mouse tissues was less than that caused by the parental RH strain [30]. In sheep, after s.c. injection, S48 tachyzoites were shown to multiply locally, producing parasitemia and were not detected by bioassay 6 weeks after infection [1, 39]. As tissue cysts were detected in mice vaccinated with Mic1-3KO parasites [30], we analysed the persistence of Mic1-3KO parasites two months after vaccination in the brain of vaccinated ewes. Tissue cysts were detected in 7 of 11 vaccinated ewes. Three positive (105, 60 and 83 cysts per brain) and one negative (less than 25 cysts per brain) concentrated brain homogenates from vaccinated ewes were bioassayed in mice together with one concentrated brain homogenate from a ewe infected with oocysts (70 cysts per brain). Half of each concentrated brain homogenate was injected into naive recipient mice by the i.p. route and the other half was given orally to other naïve mice (data not shown). All the mice inoculated i.p. or per-os with cysts from the ewe infected with oocysts became seropositive and cysts were detected in the brain. Cysts from 1 of 3 positive vaccinated ewes were infectious by the i.p. route (mice became seropositive and cysts were detected in their brain) but not by the oral route. All other mice were seronegative and brain cysts were not detected. The observation that mice inoculated i.p. with positive concentrated brain homogenates from vaccinated ewes did not all display positive serology was probably attributable to the lower pathogenicity rather than the low cyst frequency. We found that Mic1-3KO cysts from vaccinated ewes as well as those from vaccinated mice [30] were not infectious by the oral route. The lower pathogenicity but also the low number of cysts may explain the lack of infectivity by the oral route [30]. Further studies are needed to clarify these points.

The parental RH strain has lost the ability to form oocysts in cats [12], however in order to make Mic1-3KO acceptable for vaccination, it is necessary to analyse oocyst production in feces of cats by feeding tissue cysts of Mic1-3KO to cats.

Antibodies to *T. gondii* in sera were detected in all Mic1-3KO vaccinated groups on days 24–28 following inoculation, persisting throughout the experiments. Since *T. gondii* protective immunity in sheep involves cellular responses [23, 25], diluted whole blood samples collected three weeks after s.c. inoculation with 10^5 or 2×10^6 Mic1-3KO tachyzoites (Exp. 2), were exposed to *T. gondii* antigen in vitro (data not shown). Increased proliferation of PMBC was seen in a proportion of animals from each vaccinated group (7 of 12 in the group vaccinated with 10^5 tachyzoites; 3 of 11 in the group vaccinated with 2×10^6 tachyzoites) with wide variation within each group. These results indicate that a cellular response was stimulated. Ewes with a proliferative response gave birth to viable lambs, except one ewe with a low proliferative response. It would be interesting to do further work to determine if this in vitro test could be a predictive marker of effectiveness.

The immune response as well as the febrile responses observed were sufficient to determine whether immunisation of sheep with Mic1-3KO parasites prior to pregnancy protects against a challenge in mid-gestation with *T. gondii* oocysts. In three independent experiments and in three different strains of ewes (Romanov, Bizet and Solognot), Mic1-3KO tachyzoites conferred protection against abortion. A higher rate (91%) of viable lambs from vaccinated Bizet ewes compared to the two other strains (62% and 64%) was observed. Further studies are required to establish whether the effectiveness of the vaccine is dependent on the strain of sheep. Congenital transmission of *T. gondii* has been found to be infrequent in Scottish black ewes persistently infected [35]

whereas other studies based on Charolais ewes suggest that persistent infection of sheep by *T. gondii* may not prevent foetal infection [31, 32, 40]. However, further investigation is needed to assess whether some sheep have a particular genetic susceptibility to *T. gondii*.

All ewes from the control groups aborted whatever the challenge dose. Vaccinated ewes developed consistently lower febrile responses to challenge than unvaccinated ewes. Lower febrile responses may be used as an indicator of vaccine efficacy [3, 29]. Some ewes in the control groups aborted in the acute phase of infection. These early abortions were shown to occur before the placenta or the fetus had been invaded by *T. gondii* [33] and were probably due to pyrexia and hormonal regulation [19]. Moreover, after challenge with 400 oocysts, the number of expected lambs was consistently lower in the control groups than in the vaccinated groups suggesting resorption of the fetus. After the lower dose of 100 oocysts, the number of expected lambs was similar in the control and vaccinated groups and only one early abortion was recorded in the control group. The first objective was to assess the efficacy of Mic1-3KO. Hence, a positive control was included consisting of a group of ewes vaccinated s.c. with 10^5 S48 tachyzoites which were shown to provide protection [3]. Under the same experimental conditions, both strains were equally effective regarding the number of viable lambs per ewe and the mean gestation period. These results are similar to those previously obtained by Buxton et al. [3]. A vaccination dose of 10^5 Mic1-3KO tachyzoites is sufficient to induce a protective immune response versus a vaccination dose of 2×10^6 Mic1-3KO parasites. In view of the natural penetration of the intestinal tract by *T. gondii*, induction of both systemic and mucosal immune responses would be of interest. The feasibility of accessing the mucosal immune system via the serosa by administration of an intraperitoneal antigen has been explored with a *Salmonella typhimurium* vaccine [21, 22]. Sologno ewes vaccinated with 10^5 Mic1-3KO parasites by the i.p. route developed a febrile response of longer duration than ewes vaccinated by the s.c. route and had a higher level of anti-*T. gondii* antibodies on days 28 and 55 than the s.c. group. These results suggest a higher immunogenicity by the i.p. route than the s.c. route. However, the increased protection against abortion provided by the i.p. route versus the s.c. route was not found to be significant. We did not analyze the mucosal immune response. Meat is the major source of *T. gondii* infection in humans [28], thus, a vaccine against *T. gondii* should prevent the formation of tissue cysts in mutton or lamb. After challenge at mid gestation with 100 oocysts, no cysts (less than 25 cysts) were detected in the brain of viable lambs born to i.p. vaccinated ewes and cysts were only detected in one viable lamb born to s.c. vaccinated ewes. These preliminary results are encouraging, and the i.p. route may be more effective than the s.c. route. Furthermore, a repeated vaccination, whatever the route could be more effective.

Mic1-3KO tachyzoites may induce lifelong immunity as described for natural infection. The duration of immunity in sheep as well as the efficacy against challenge are still to be studied.

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REFERENCES

[1] Bos H.J., Development of a live vaccine against ovine toxoplasmosis, in: Smith E. (Ed.), Toxoplasmosis, NATO A SI Series, vol. H78, 1993, pp. 231–243.

[2] Buxton D., Ovine toxoplasmosis: a review, J. R. Soc. Med. (1990) 83:509–511.

[3] Buxton D., Thomson K., Maley S., Wright S., Bos H.J., Vaccination of sheep with a live incomplete strain (S48) of *Toxoplasma gondii* and their immunity to challenge when pregnant, Vet. Rec. (1991) 129:89–93.

[4] Buxton D., Thomson K.M., Maley S., Wright S., Bos H.J., Experimental challenge of sheep 18 months after vaccination with a live (S48) *Toxoplasma gondii* vaccine, Vet. Rec. (1993) 133:310–312.

[5] Buxton D., Thomson K.M., Maley S., Wastling J.M., Innes E.A., Panton W.R., Nicoll S., Primary and secondary responses of the ovine lymph node to *Toxoplasma gondii*: cell output in efferent lymph and parasite detection, J. Comp. Pathol. (1994) 111:231–241.

[6] Buxton D., Innes E.A., A commercial vaccine for ovine toxoplasmosis, Parasitology (1995) 110: S11–S16.

[7] Buxton D., Protozoan infections (*Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis* spp.) in sheep and goats: recent advances, Vet. Res. (1998) 29:289–310.

[8] Buxton D., Maley S.W., Wright S.E., Rodger S., Bartley P., Innes E.A., *Toxoplasma gondii* and ovine toxoplasmosis: new aspects of an old story, Vet. Parasitol. (2007) 149:25–28.

[9] Cérède O., Dubremetz J.F., Soête M., Deslée D., Vial H., Bout D., Lebrun M., Synergistic role of micronemal proteins in *Toxoplasma gondii* virulence, J. Exp. Med. (2005) 201:453–463.

[10] Clementino M.M., Souza M.F., Andrade Neto V.F., Seroprevalence and *Toxoplasma gondii*-IgG avidity in sheep from Lajes, Brazil, Vet. Parasitol. (2007) 146:199–203.

[11] Cook A.J., Gilbert R.E., Buffolano W., Zufferey J., Petersen E., Jenum P.A., et al., Sources of toxoplasma infection in pregnant women: European multicentre case-control study, European Research Network on Congenital Toxoplasmosis, B.M.J. (2000) 321:142–147.

[12] Dardé M.L., Bouteille B., Pestre-Alexandre M., Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications, J. Parasitol. (1992) 78:786–794.

[13] Dubey J.P., Sundar N., Hill D., Velmurugan G.V., Bandini L.A., Kwok O.C.H., et al., High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA, Int. J. Parasitol. (2008) 38:999–1006.

[14] Dubey J.P., Toxoplasmosis in sheep-The last 20 years, Vet. Parasitol. (2009) 163:1–14.

[15] Dumètre A., Ajzenberg D., Rozette L., Mercier A., Dardé M.L., *Toxoplasma gondii* infection in sheep from Haute-Vienne, France: seroprevalence and isolate genotyping by microsatellite analysis, Vet. Parasitol. (2006) 142:376–379.

[16] Esteban-Redondo E., Maley S.W., Thomso K., Nicoll S., Wright S., Buxton D., Innes E.A., Detection of *T. gondii* in tissues of sheep and cattle following oral infection, Vet. Parasitol. (1999) 86:155–171.

[17] Fayer R., Toxoplasmosis update and public health implications, Can. Vet. J. (1981) 22:344–352.

[18] Fazaeli A., Ebrahimzadeh A., A new perspective on and re-assessment of SAG2 locus as the tool for genetic analysis of *Toxoplasma gondii* isolates, Parasitol. Res. (2007) 101:99–104.

[19] Fredriksson G., Buxton D., Uggla A., Kindahl H., Edqvist L.E., The effect of *Toxoplasma gondii* infection in unvaccinated and iscom-vaccinated pregnant ewes as monitored by plasma levels of 15-ketodihydroprostaglandin F2 alpha, progesterone, and oestrone sulphate, Zentralbl. Veterinarmed. A (1990) 37:113–122.

[20] Fusco G., Rinaldi L., Guarino A., Proroga Y.T., Pesce A., Giuseppina de M., Cringoli G., *Toxoplasma gondii* in sheep from the Campania region (Italy), Vet. Parasitol. (2007) 149:271–274.

[21] Husband A.J., Intestinal immunity following a single intraperitoneal immunisation in lambs, Vet. Immunol. Immunopathol. (1980) 1:277–286.

[22] Husband A.J., Novel vaccination strategies for the control of mucosal infection, Vaccine (1993) 11:107–112.

[23] Innes E.A., Panton W.R., Sanderson A., Thomson K.M., Wastling J.M., Maley S., Buxton D., Induction of $CD4^+$ and $CD8^+$ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function, Parasite Immunol. (1995) 17:151–160.

[24] Innes E.A., Toxoplasmosis: comparative species susceptibility and host immune response, Comp. Immunol. Microbiol. Infect. Dis. (1997) 20:131–138.

[25] Innes E.A., Lundén A., Esteban I., Marks J., Maley S., Wright S., et al., A previous infection with *Toxoplasma gondii* does not protect against a challenge with *Neospora caninum* in pregnant sheep, Parasite Immunol. (2001) 23:121–132.

[26] Ismael A.B., Sekkai D., Collin C., Bout D., Mévélec M.N., The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis, Infect. Immun. (2003) 71:6222–6228.

[27] Ismael A.B., Dimier-Poisson I., Lebrun M., Dubremetz J.F., Bout D., Mévélec M.N., Mic1-3 Knockout of *Toxoplasma gondii* is a successful vaccine against chronic and congenital toxoplasmosis in mice, J. Infect. Dis. (2006) 194:1176–1183.

[28] Jongert E., Roberts C.W., Gargano N., Förster-Wald E., Petersen E., Vaccines against *Toxoplasma gondii*: challenges and opportunities, Mem. Inst. Oswaldo Cruz (2009) 104:252–266.

[29] McColgan C., Buxton D., Blewett D.A., Titration of *Toxoplasma gondii* oocysts in non-pregnant sheep and the effects of subsequent challenge during pregnancy, Vet. Rec. (1988) 123:467–470.

[30] Moiré N., Dion S., Lebrun M., Dubremetz J.F., Poisson I.D., Mic1-3KO tachyzoite a live attenuated vaccine candidate against toxoplasmosis derived from a type I strain shows features of type II strain, Exp. Parasitol. (2009) 123:111–117.

[31] Morley E.K., Williams R.H., Hughes J.M., Terry R.S., Ducanson P., Smith J.E., Hide G., Significant familial differences in the frequency of abortion and *Toxoplasma gondii* infection within a flock of Charollais sheep, Parasitology (2005) 131:181–185.

[32] Morley E.K., Williams R.H., Hughes J.M., Thomasson D., Terry R.S., Duncanson P., et al., Evidence that primary infection of Charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings, Parasitology (2008) 135:169–173.

[33] Owen M.R., Clarkson M.J., Trees A.J., Acute phase toxoplasma abortions in sheep, Vet. Rec. (1998) 142:480–482.

[34] Owen M.R., Trees A.J., Genotyping of *Toxoplasma gondii* associated with abortion in sheep, J. Parasitol. (1999) 85:382–384.

[35] Rodger S.M., Maley S.W., Wright S.E., Mackellar A., Wesley F., Sales J., Buxton D., Role of endogenous transplacental transmission in toxoplasmosis in sheep, Vet. Rec. (2006) 159:768–772.

[36] Sharma S.D., Mullenax J., Araujo F.G., Erlich H.A., Remington J.S., Western Blot analysis of the antigens of *Toxoplasma gondii* recognized by human IgM and IgG antibodies, J. Immunol. (1983) 131:977–983.

[37] Stimbirys A., Bagdonas J., Gerulis G., Russo P., A serological study on the prevalence of *Toxoplasma gondii* in sheep of Lithuania, Pol. J. Vet. Sci. (2007) 10:83–87.

[38] Vesco G., Buffolano W., La Chiusa S., Mancuso G., Caracappa S., Chianca A., et al., *Toxoplasma gondii* infections in sheep in Sicily, southern Italy, Vet. Parasitol. (2007) 146:3–8.

[39] Wastling J.M., Nicoll S., Buxton D., Comparison of two gene amplification methods for the detection of *Toxoplasma gondii* in experimentally infected sheep, J. Med. Microbiol. (1993) 38:360–365.

[40] Williams R.H., Morley E.K., Hughes J.M., Duncanson P., Terry R.S., Smith J.E., Hide G., High levels of congenital transmission of *Toxoplasma gondii* in longitudinal and cross-sectional studies on sheep farms provides evidence of vertical transmission in ovine hosts, Parasitology (2005) 135:169–173.