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**Evaluation of immunogenicity and protection of the Mic1-3 Knockout *Toxoplasma gondii*
live attenuated strain in the feline host.**

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1 INTRODUCTION

2

3 Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan *Toxoplasma*
4 *gondii*. Up to a third of the global human population is estimated to carry a *T. gondii*
5 infection, which can result in severe complications in immunocompromised individuals and
6 pregnant women [1 , 2 , 3]. Transmission to humans occurs mainly (i) through ingestion of
7 undercooked meat containing tissue cysts with bradyzoites, (ii) ingestion of vegetables, fruit
8 and water contaminated by sporulated oocysts from cat stool or (iii) by congenital
9 transmission from mother to foetus by tachyzoites [4-7]. As the definitive host (DH) of *T.*
10 *gondii*, the Felidae family (and mostly cats) play a crucial role in spreading the parasite in
11 the environment through the release of oocysts in their faeces, which remain infectious,
12 once sporulated, for an extended period of time [8].

13 In 1999, a 3-year vaccination trial of stray cats living around a swine farm, using a mutant
14 strain of *T. gondii* (T263), showed a clear correlation between the reduction of oocysts in the
15 environment and the decrease of *T. gondii* seroprevalence in finishing pigs [9]. This
16 experiment showed for the first time the potential to target cats for vaccination against *T.*
17 *gondii* to reduce meat contamination and indirectly protect humans. Another live
18 attenuated vaccine, based on tachyzoites of the mutant S48 strain, is commercially available
19 in Europe and New-Zealand (OVILIS® ToxoVax by MSD Animal Health) but only used in
20 sheep, to prevent *T. gondii* induced abortion [2] and economic losses. Other types of
21 vaccines have been tested in cats (eg irradiated or genetically modified strains, antigenic
22 vaccines) [10, 11] but some of these trials were challenging to apply due to environmental
23 and/or practical constraints, or did not prevent oocyst excretions by cats. Nevertheless,
24 targeting animals to protect human health should still be considered as shown by the

25 successful achievement of rabies eradication in most European countries by oral vaccination
26 of wild carnivores [12 , 13]. As such, a vaccine targeting cats to prevent environmental
27 contamination by reducing *T. gondii* oocyst excretion, is of strong interest to limit human
28 and livestock contamination [14].

29 Another live attenuated vaccine strain of *T. gondii*, called Mic1-3KO, deleted for *MIC1* and
30 *MIC3* genes (encoding for two microneme proteins Mic1 and Mic3, respectively) was
31 developed in 2005 [15]. This double gene deletion reduces the ability of the parasite to
32 penetrate host cells and prevents any reversion to virulence. This is an important difference
33 with the S48 strain which is attenuated after multiple culture passages [2, 16] and could
34 potentially revert to a virulent strain. The Mic1-3KO strain has been previously successfully
35 tested as a preventive vaccine in mice [17] and sheep [18]. Due to its efficiency as a vaccine
36 candidate in these animal species, we tested it as a subcutaneous vaccine for cats. The aims
37 of this 1st trial were to assess the immunogenicity of the Mic1-3KO vaccine strain in cats, and
38 analyse protection of the animals against challenge with a wild type *T. gondii* strain. In
39 addition, we assessed Th1 and Th2 serum cytokines following vaccination and challenge, to
40 further investigate the feline immune response induced by the Mic1-3KO vaccine-candidate.
41 Indeed, most of the mechanisms for the cell invasion capacity of the parasite, as well as host
42 immune responses, have been elucidated using *in vitro* and murine *in vivo* models,
43 intermediate hosts (IH) of the parasite [19 , 20 , 21], but very few have focused on the DH
44 immune response [22, 23]. Therefore, vaccination studies are a useful opportunity to assess
45 *in vivo* potential correlates of immune protection to *T. gondii* in the feline host.

46 As mass vaccination strategies for feral and stray cats require an orally administrable
47 vaccine, we also developed an oral formulation to allow mucosal vaccination of cats with the
48 Mic1-3KO strain and followed the same host parameters.

49

50 **MATERIALS AND METHODS**

51

52 **Animals & ethic statement**

53 Specific pathogen free domestic short-haired female cats, aged 4 months at the start of each
54 trial, were obtained from Isoquimen (Barcelona, Spain). They were housed according to
55 European laws and guidelines (Directive 2010/63/EU) on animal welfare (agreements
56 21/01/13-2 and #9859-2017050516182349v4).

57

58 ***Toxoplasma gondii* strains**

59 The vaccine-candidate strain Mic1-3KO, is a live attenuated strain of *T. gondii* (patent
60 WO2005/072754, [15]) from genotype I. The challenge was performed using tissue-cysts of
61 the wild-type 76K strain of *T. gondii* (genotype II). Tachyzoites of the Mic1-3KO strain and of
62 the same strain expressing the Green Fluorescent Protein (Mic1-3KO-GFP) were cultured in
63 HFF (Human Foreskin Fibroblast) cells, at 37°C and 5% CO₂. All *T. gondii* strains used in this
64 study were provided by Vitamfero (France). HFF Cells were maintained in complete culture
65 medium composed of DMEM, 4mM Glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin
66 (all from Lonza, Belgium) supplemented with 10% of fetal calf serum (Gibco, Thermo Fisher
67 Scientific, France). The culture medium was replaced 24h before harvesting of the parasites
68 prior to administration to the animals. Parasite counting was done using Kova-slides (Kova-
69 International, USA) under the microscope [24, 25] and the concentration of Mic1-3KO
70 tachyzoites was calculated in order to administer the desired quantity by subcutaneous
71 injection or orally.

72

73

74 ***In vitro* tests of different gastro-resistant formulas**

75 For oral administration of the Mic1-3KO strain, preliminary *in vitro* experimental testing of
76 different oral formulations were performed. Briefly, 10^7 tachyzoites/ml of the Mic1-3KO-GFP
77 were included in either water, DMEM as a positive control, or different formula (F1 to F4,
78 suppl. material & methods, table S1) then diluted 1/5 in a pH4 solution (VWR, France) and
79 incubated at 38,5°C for 4h. After incubation, the parasites were fixed with 1%
80 Paraformaldehyde (Euromedex, France) for 30min at room temperature. After two washes
81 with 1X PBS (Gibco, France), the percentage of viable tachyzoites was assessed by flow
82 cytometry on a FACS Canto2 (BD Biosciences, USA) using the GFP Mean Fluorescence
83 intensities of the parasites identified on morphologic parameters. In parallel, after 4h of
84 incubation, 100µl of the tachyzoites-containing formula were added to confluent HFF cells in
85 a 12-wells plate and culture at 37°C, 5% CO₂. Presence of intracellular parasites was checked
86 daily using an inverted microscope. The formula that showed the highest survival and
87 virulence of the tachyzoites, was then chosen for the oral vaccine.

88

89 **Vaccination protocols**

90 For the subcutaneous (s.c.) vaccination protocol, a first group of 4 cats, called "2 injections",
91 received a first injection s.c. of 200µl serum-free DMEM containing 10^5 tachyzoites of the
92 Mic1-3KO strain at day 0 (D0), and an identical boost injection at day 35 (D35). The group
93 called "1 injection" (n=4 cats), received the same dose of the vaccine-candidate only at D35.
94 S.c. injections were done in the back of the neck, using a 1mL syringe and a 25G x 5/8 needle
95 (Beckton Dickinson, France). Both groups and one control cat (called "No vaccine") were
96 challenged orally with 1200 murine-brain tissue cysts containing bradyzoites of the 76K

97 strain at day 97 (D97) and animals were followed up to 131 days from the 1st vaccine
98 injection.

99 For the oral vaccination protocol, 10⁵ of the Mic1-3KO tachyzoites were mixed in 400µl of F1
100 and given orally to the “Vaccinated” batch. This group of cats received a second oral
101 administration of 10⁸ tachyzoites of Mic1-3KO strain in 400µl of F1 at D60. At D122, the
102 “Vaccinated” and “Non vaccinated” batches (n=4 cats/group) were challenged orally with
103 1200 bradyzoites-containing cysts of the 76K strain and animals were followed up to 161
104 days after the 1st vaccine administration.

105 Blood samples were collected weekly throughout both protocols, after intramuscular
106 tranquilization of the animals (15mg/kg ketamine+1mg/kg diazepam). After 20min of clotting
107 at room temperature, tubes were centrifuged for 10min at 2000rpm. Serum was collected
108 and frozen at -80°C until further analyses.

109

110 **Anti-*T. gondii* IgM and IgG measurement by indirect ELISA**

111 Serum anti-*T. gondii* antibodies were measured by an in-house indirect ELISA. Briefly, wells
112 were coated either with 10µg/ml of *T. gondii* antigens (76K strain , Vitamfero, France), or
113 with 1X PBS only. After three washes with 1X PBS+0.05% Montanox20 (SEPPIC, France),
114 plates were blocked with 1X PBS +1% BSA (Sigma, USA), washed again and plated with sera
115 diluted at 1/200 in 1X PBS. After overnight incubation at 4°C, plates were washed and either
116 anti cat-IgM or IgG coupled to horseradish peroxydase (Bio-Rad antibodies, France) were
117 added, at the recommended dilution in 1X PBS. After 1h at 37°C, and three washes with 1X
118 PBS+0.05% Montanox20, signal was revealed using TMB solution (Bio-Rad, France). Reaction
119 was stopped with H₃PO₄ 1M solution (Merck, Germany). Plates were read at 450nm with the
120 MultiskanFC (Thermo Scientific, USA) and analysed with SkanIt Research Edition 4.1

121 software. OD values from PBS background binding were subtracted from the signal obtained
122 with *T. gondii* specific antibodies.

123

124 **Serum cytokine measurement by ELISA**

125 Serum cytokines were measured with commercial sandwich ELISA kits specific for feline IL-2,
126 IFN γ , IL-4, and IL-5 (Bio-Techne, USA), following manufacturer's instructions. Briefly, plates
127 were coated overnight with capture antibody at room temperature, then washed three
128 times with wash buffer (1X PBS +0.05% Montanox20). After blocking with 1X PBS+1% BSA,
129 plates were washed again, sera diluted 1/50 in 1X PBS and standards were added in wells
130 according to manufacturer's instructions. After 2h, incubation plates were washed and
131 coated with specific biotinylated detection antibody, for 2h at room temperature. After
132 three washes, streptavidin coupled to horseradish peroxidase, diluted in 1X PBS as
133 recommended, was added to each well for 20min at room temperature. Plates were then
134 washed, and reaction was revealed using TMB solution. Reaction was stopped with H₃PO₄
135 1M solution and plates were read at 450nm and 540nm for background noise, according to
136 manufacturer instructions, on the MultiskanFC reader and analysed with SkanIt Research
137 Edition 4.1 software.

138

139 **Oocyst identification from cat faeces**

140 At D-7 before vaccination, and daily from D0 of both vaccination trials, cat fecal samples
141 were collected in plastic collection cups with screw lids, according to the manual of OIE for
142 fecal samples collection (OIE Terrestrial Manual 2013). Each sample was stored at 4°C for a
143 maximum of 3 days after collection, for oocysts purification and analysis. Sucrose flotation
144 technique was used to purify oocysts as described previously [24 , 25]. 10 μ l of each purified

145 sample were examined under Kova (Kova International, Netherlands). Light microscope (DMI
146 4000B, Leica, Germany) with ×200 magnification and blue ultra-fluorescent (UV) light was
147 used for oocysts detection taking advantage of autofluorescence of *T. gondii* oocysts [26].

148

149 **Multilocus nested PCR-RFLP genotyping of oocysts**

150 Purified *T. gondii* oocysts were previously diluted to a concentration of 10⁵ oocysts/mL and
151 subjected to three freeze-thawing cycles at -80°C/RT, prior to DNA extraction. During the last
152 two thaws, the tubes were sonicated to destroy the resistant wall of the oocysts. The DNA
153 was then extracted using the Mini Kit QIAamp DNA (Qiagen, USA), following the tissue
154 protocol. Strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB,
155 GRA6, c22-8, c29-2, L358, PK1 and Apico as described previously [27, 28]. PCR products were
156 digested with specific restriction enzymes and patterns were revealed on a 2.5 agarose gel,
157 using GelDoc XR and analysed with Quantity One 1-D software (Bio-Rad, France).

158

159 **RESULTS**

160

161 **Antibody production and animal protection after subcutaneous vaccination**

162 The first vaccination trial with Mic1-3KO was performed by subcutaneous injection. Seven
163 days after the 1st injection of the vaccine, both groups of cats displayed an increase in
164 specific anti-*T. gondii* IgM (Figure 1A) followed by a high production of serum anti-*T. gondii*
165 IgG, reaching a plateau 35 days post injection (Figure 1B). The 2nd injection of the vaccine
166 strain in the “2 injections” group did not show any booster effect in IgG production after the
167 2nd injection. In the control cat, the administration of the challenge strain also induced *T.*
168 *gondii* specific IgM production firstly, followed by specific IgG, reflecting the infectivity of the

169 murine brain-tissue cysts of the 76K strain. However, despite high IgG serum levels before
170 D97, the challenge induced the production of new specific IgM in vaccinated cats, but had a
171 minimal impact on IgG serum levels which remained elevated. Interestingly, the levels of IgM
172 produced after the challenge in the control cat were much higher than in the vaccinated
173 cats, and comparable to levels produced at the time of first vaccination of the “2 injections”
174 batch.

175 We then assessed if the Mic1-3KO strain could protect cats from natural infection and oocyst
176 production by daily analysis of collected cat faeces. No oocysts were found in the faeces
177 before challenge with the wild-type strain (data not shown). However, after administration
178 of the tissue cysts of the challenge strain, all cats shed oocysts with the earliest excretion
179 seen on day four post infection (Figure 1C). The period of oocyst shedding was from day 4 to
180 day 13 as expected from literature (suppl. figure S2, A).

181 Figure 1D shows the genotyping by multilocus nested PCR-RFLP, of the oocysts shed by each
182 cat included in the trial, compared to reference strains (suppl. material & methods, table S2),
183 on three of the tested loci : C29-2, SAG1 and BTUB. All cats shed oocysts of genotype II strain
184 exclusively, corresponding to the genotype of the challenge strain, as confirmed by the “No
185 vaccine” cat which could only shed genotype II oocysts.

186

187 **Design of a gastro-resistant vaccination formula for oral vaccination**

188 For oral administration of the Mic1-3KO vaccine-candidate, we had to develop different
189 gastro-resistant formulas to administer live tachyzoites to the feline intestines. Indeed, this
190 strain is only available as tachyzoites, which are highly sensitive to acidic pH of the stomach
191 [8]. For this, we took advantage of the Mic1-3KO-GFP, which allowed monitoring of the
192 parasites by flow cytometry for *in vitro* assays prior to cat administration of the Mic1-3KO

193 strain. Mic1-3KO-GFP parasites were formulated in different gastro-resistant solutions and
194 incubated in conditions mimicking intestinal passage in felines. Viability of the parasites was
195 assessed by counting the percentage of GFP+ tachyzoites after such treatments (Figure 2A
196 and 2B) using flow cytometry. Whereas about 80% of the parasite did not survive in F4, nor
197 in distilled water, 3 out of 4 formulas used (F1 to F3) were able to keep 75% of the parasites
198 alive after treatment. To confirm viability and infectivity of the remaining GFP+ parasites,
199 they were also plated onto monolayers of HFF cells after treatment, and the presence of
200 tachyzoites within cells was monitored daily post-infection (Figure 2C). The F1 formula was
201 selected for the oral vaccination trial as it allowed the best parasite viability and infectivity.

202

203 **Antibody production and animal protection after oral vaccination**

204 Once the formula selected, cats were orally vaccinated with same amount of tachyzoites as
205 for the subcutaneous vaccination trial at D0, and then with a higher quantity of tachyzoites
206 at D60 in the same formula, to boost antibody production. Murine-brain tissue cysts of the
207 76K strain was orally given to all cats, 60 days after the last vaccination. IgM levels showed a
208 high background before vaccination in both batches, however anti-*T. gondii* IgM levels in
209 serum of vaccinated cats increased after the 2nd oral administration of the vaccine strain
210 (Figure 3A), quickly followed by an increase in anti-*T. gondii* specific IgG (Figure 3B). The
211 peak of specific IgG production was reached 28 days after the 2nd administration showing
212 similar kinetics of IgG production following the subcutaneous injection, and decreased
213 slightly after 28 days. Like in the s.c. vaccination trial, the challenge strain given orally
214 induced a boost in *T. gondii* specific IgM and IgG production in the vaccinated group. The
215 non-vaccinated group induced anti-*T. gondii* IgM and IgG after the inoculation with the
216 challenge strain (Figure 3A and 3B).

217 Daily analysis of the cat faeces after oral vaccination with Mic1-3KO showed no oocysts
218 found before challenge with the wild-type strain (data not shown), as in the s.c. vaccination
219 trial. However, after administration of the murine brain-tissue cysts of the challenge strain,
220 all cats started to shed oocysts from four days post challenge up to thirteen days, similar to
221 the s.c. vaccine experiment (Figure 3C). We also genotyped the oocysts shed by multi-locus
222 nested PCR-RFLP. As in Figure 1, three of the tested loci are presented in Figure 3D. The
223 genotype II was determined for oocysts shed upon challenge in the oral vaccination trial
224 (Figure 3D, samples 1 to 4, corresponding to oocysts found in faeces of two cats in each
225 sample), as compared to reference strains.

226

227 As *T. gondii* is a parasite mainly transmitted through ingestion, we aimed to see if *T. gondii*
228 specific IgA were produced and detectable in serum. Slight increases of serum IgA were
229 measured sporadically after subcutaneous injection of the vaccine strain (suppl. figure S1, A)
230 in both vaccinated groups and also after challenge. However, we did not detect any IgA
231 production in the serum of the cat who only received the challenge strain. Similarly, no
232 induction of IgA production was seen after oral administration of Mic1-3KO tachyzoites
233 (suppl. figure S1, B), despite an apparent two fold increase in anti-*T. gondii* IgA production
234 upon challenge in the vaccinated group as compared to the non-vaccinated group. The
235 limited number of animals included in the trial and high individual variation regarding the
236 IgA measurements limit our ability to make conclusions about this increase.

237

238 **Cytokine production induced by Mic1-3KO vaccination**

239 To define a possible Th1 or Th2 signature upon vaccination with Mic1-3KO, cytokine
240 production was also studied throughout both vaccination trials (Figure 4), in the vaccinated

241 groups only. Since there was no boost effect seen in antibody production after a 2nd
242 subcutaneous injection of the vaccine, results shown in Figure 4A and 4B are focused on the
243 “1 injection” group. IL-2 and IFN γ levels in cat serum were relatively low and the
244 concentration of both cytokines remained stable throughout the subcutaneous vaccination
245 trial, under 20ng/ml (Figure 4A). As opposed to these Th1 cytokines, IL-4 levels were high in
246 serum of cats with an average range of 77,5ng/ml \pm 3,6 and IL-5 concentrations were much
247 lower (23,7 ng/ml \pm 0,8). However no changes were seen upon vaccination or challenge in
248 these Th2 cytokines (Figure 4B). Throughout the oral vaccination, as for the s.c. trial, no
249 significant changes in Th1 or Th2 serum cytokines were observed (Figure 4C and 4D). Taken
250 together, these results show no variation in cytokines classically associated with Th1 or Th2
251 immune signatures, after vaccination or challenge.

252

253 **DISCUSSION**

254 The aim of this study was to assess the immunogenicity of a new vaccine candidate against
255 *T. gondii* in cats and evaluate the protection of the vaccinated animals against the wild-type
256 parasite. This new vaccine candidate strain differs from the ones tested previously [29] as it
257 is a double-knock out for two genes *MIC1* and *MIC3*, therefore greatly limiting the possibility
258 to revert to any potential virulence [15]. The Mic1-3KO strain has been successfully used as a
259 prophylactic vaccine in mice and in sheep [17, 18]. In both murine and ovine vaccination
260 trials, the Mic1-3KO strain was administrated subcutaneously. Hence, we set up a first
261 vaccination assay in cats, with the same administration route. Specific *T. gondii* IgG could be
262 measured as soon as 10 days post-injection, reaching a plateau one month later. A second
263 injection of the vaccine did not improve this IgG production, indicating that the vaccine
264 strain is immunogenic from the 1st injection, which supports a vaccination protocol.

265 However, despite induction of high levels of anti-*T. gondii* IgG by the vaccine candidate
266 Mic1-3KO, subcutaneous vaccination did not prevent sexual replication of the challenge
267 strain in the cats' intestines and oocyst excretion.

268 Recent work on vaccination against infectious diseases such as SIV/HIV or against cancer,
269 suggested that immunization route influences the efficiency of the vaccine [30-32], i.e. to
270 protect mucosa from a foodborne pathogen, the vaccine would be more efficient if given by
271 the mucosal route. These observations, as well as the results of s.c. vaccination experiment,
272 led us to conclude that the subcutaneous administration of the Mic1-3KO strain to cats did
273 not result in any local protection at the mucosa level, despite inducing specific IgG
274 production. Moreover, an oral vaccine would be more suitable for mass vaccination of feral
275 and stray cats, living around farms, so we performed a second vaccination trial, giving the
276 Mic1-3KO orally to cats. However, the Mic1-3KO vaccine strain is only available as a
277 tachyzoite stage, which are very sensitive to acidic pH. Therefore, to administer the Mic1-
278 3KO vaccine-candidate orally, we tested different gastro-resistant formulas *in vitro*, to select
279 the formula allowing the optimal viability of infectious tachyzoites. All formulas tested
280 contained guar gum, which is commonly tested for colon-targeted drug delivery systems
281 [33], allowing the drug to resist intestinal passage and maintain its efficiency for a local
282 effect [34]. Presence of carboxymethylcellulose (CMC) and sodium bicarbonate were also
283 required to help GFP-Mic1-3KO tachyzoites to survive the acidic pH *in vitro* treatment. The
284 main useful characteristic of CMC for our study is that it is insoluble in low pH media but
285 dissolves at a higher, near neutral pH of the distal gut [35], thus protecting tachyzoites
286 through the feline stomach and duodenum. Sodium bicarbonate served as a buffering agent,
287 to help tachyzoites resist an acidic pH, and chicken aroma gave some palatability for the
288 cats. After the 1st oral administration of the Mic1-3KO strain, no IgG production was

289 observed in any of vaccinated animals. This observation raised the question of whether
290 sufficient tachyzoites survived in the stomach of cats, as opposed to our *in vitro* studies, and
291 thus were not seen by the immune system. Hence, a second administration of the vaccine
292 strain was performed at day 60 with a higher quantity of tachyzoites. The 2nd oral
293 administration induced a boost and allowed the production of a high IgG titer which
294 increased upon challenge in the vaccinated group. Taken together, these results show that
295 the Mic1-3KO is highly immunogenic for cats, whether given subcutaneously or orally. We
296 were also able to develop an oral formula preserving a majority of viable vaccine-candidates
297 and inducing antibodies against *T. gondii* antigens. This confirms that their mucosal immune
298 system responded to the vaccine-candidate antigens. Whether the live Mic1-3KO tachyzoites
299 entered the mucosa at the pharygeal-buccal level or at the duodenum level, as suggested by
300 Dubey [36, 37], was not possible to investigate in our oral vaccination trial.

301 As *T. gondii* is an intestinal parasite, we aimed to look at mucosal protection by monitoring
302 IgA after vaccination and challenge in both trials. Faeces IgA from cats are possible to
303 measure [38], however, in our hands, the heterogeneity of the diluted samples and the high
304 background obtained with our in-house ELISA with these samples, did not allow us to
305 measure locally produced anti-*T. gondii* IgA (data not shown). We thus looked at serum anti-
306 *T. gondii* IgA. Subcutaneous vaccination did not induce a high level of serum IgA but
307 challenge with the wild-type *T. gondii* strain slightly increased the concentration of specific
308 IgA. Since this increased IgA production is more evident in the animals which received only
309 one injection of the vaccine, it confirms the needlessness of the recall injection. In contrast,
310 the oral vaccination did not induce serum specific *T. gondii* IgA at detectable levels, however
311 interestingly, when challenged with the wild-type *T. gondii*, the vaccinated cats produced
312 anti-*T. gondii* IgA in a higher titer than the positive control animals, reflecting a possible

313 improved protection at the mucosal level, as compared to the vaccine strain when given
314 orally.

315 Despite high titers of specific anti-*T. gondii* IgG induced by the Mic1-3KO vaccine strain, upon
316 challenge with a virulent wild-type *T. gondii*, surprisingly all cat shed oocysts, regardless of
317 the vaccination route of the Mic1-3KO. One possible explanation is the different genotypes
318 between the two strains. Indeed, Mic1-3KO has been obtained from a genotype I strain,
319 whereas the challenge was done using the 76K strain, which is of genotype II. These two
320 genotypes display different antigens [39], even if it has been shown that the Mic1-3KO strain
321 behaves like a genotype II strain in the mouse model [40], and is protective in vaccinated
322 mice and sheep challenged with a genotype II strain [17, 18], that is mostly found in Europe
323 [41, 42]. Therefore, it is possible that some antigens of the genotype II wild type strain were
324 new to the vaccinated animals and helped the parasite to evade anti-*T. gondii* IgG induced
325 by the Mic1-3KO strain. The follow-up of IgM production in both trials could support this
326 hypothesis. Indeed, IgM titers in the serum increased and then subsided as expected. In the
327 oral vaccination trial, cats showed some anti-*T. gondii* IgM prior to vaccination or challenge,
328 which we did not see in animals of the s.c. vaccination trial. These cross-reactive IgMs are
329 quite common in humans and justify why diagnostic serologies need to be done on both IgM
330 and IgG to confirm an acute infection [43, 44]. However, after the challenge, we observed an
331 increase of IgM production in both trials. The second increase could be the sign of a primary
332 immune response against wild-type *T. gondii* antigens, specific to genotype II, which are not
333 present on the Mic1-3KO cell membrane. This hypothesis is supported by the recent
334 publication of Ramakrishnan *et al.*, where they vaccinated cats with a live attenuated
335 HAP2KO genotype II strain, obtained with CRISPR-Cas9 technology. Cats vaccinated with
336 either oocysts or tissue cysts from HAP2KO parasites were protected against challenge with

337 a wild type strain of *T. gondii* of the same genotype [11]. Another possibility for the different
338 outcomes in our experiments is that animals were vaccinated with tachyzoites, for which
339 cats are the least susceptible species. Indeed, several thousands of tachyzoites are required
340 to infect cats whereas only one bradyzoite or one oocyst are enough [8 , 45]. The parasite
341 stage can also influence the cat's seroconversion [46]. Therefore, cats in our vaccination
342 trials might have responded less intensely to the Mic1-3KO strain, in spite of high IgG
343 production.

344 To study the safety of the vaccine strain and the risk of infectivity, faeces were collected and
345 analyzed on a daily basis, throughout the length of both trials. None of the vaccinated cats
346 shed oocysts upon vaccination only. Genotyping of the oocysts shed after challenge in both
347 the subcutaneous and oral trials, showed that the oocysts were of genotype II, the same as
348 the oocysts excreted by cats which were only challenged (with genotype II 76K strain). These
349 genotyping analyses demonstrate that the Mic1-3KO is safe, cannot form oocysts and does
350 not recombine with the challenge strain in the lumen of the cat intestine. This could be due
351 to the fact that the vaccine was given two months before the challenge with the wild-type
352 strain of *T. gondii*, which prevented any genetic cross in the intestines of the cats, as for such
353 a cross to occur, a cat needs to be simultaneously infected with the different genotypes [39 ,
354 47].

355 The host immune response to *T. gondii* infection has been mainly studied in the IH murine
356 model which develops a Th1-type response with production of IL-12 by inflammatory
357 monocytes and subsequent IFN γ production by NK cells and T cells. For cats, which can be
358 both DH and IH, the immune response against *T. gondii* infection has been poorly studied.
359 Therefore, we measured IFN γ and IL-2 in for Th1 responses, and IL-4 and IL-5 for Th2
360 responses in both vaccination studies. We did not observe any significant changes in the

361 concentrations of Th1 and Th2 cytokines in cat serum, either after vaccination or challenge.
362 As effector functions of cytokines are mostly local, it would have been interesting to isolate
363 mesenteric lymph nodes and perform *ex vivo* stimulation and cell proliferation assays to
364 compare vaccinated and challenged immune responses, but this was not ethically possible to
365 do it in these vaccination trials.

366

367 **CONCLUSION**

368 The proven efficacy of the Mic1-3KO strain in mice and sheep, supported its use as a
369 promising vaccine candidate to block the parasite life cycle in its definitive host, in order to
370 prevent environmental and livestock contaminations. The vaccination trials we performed
371 showed good safety of this vaccine candidate strain in cats with no side-effects observed and
372 a good tolerance. Moreover, the Mic1-3KO strain did not sexually reproduce in the cats,
373 suggesting that it is not able to disseminate through cat faeces. Interestingly, this vaccine
374 candidate is highly immunogenic in cats, regardless of the vaccination route, as in other
375 species tested. However, antibodies produced upon vaccination were not able to stop a
376 wild-type strain of *T. gondii* to sexually reproduce in the cat intestine and to shed oocysts,
377 illustrating the unique host-pathogen interactions specific to this host. Future research
378 addressing the relationship between the cat intestinal mucosa and *T. gondii* are now
379 required to identify new targets and improve this vaccination strategy such as adding the
380 delta-6-desaturase enzyme, as suggested by a very recent publication on feline intestinal
381 organoids [48]. Blocking the *T. gondii* life cycle in its definitive host to protect animal and
382 human health would be completely in line with the OneHealth approach [49].

383

384 **AUTHORSHIP**

385

386 D.L.R. designed and performed the experiments and analysed the datas. V.Dj performed the
387 experiments and analysed the datas. S. M. provided vaccination and challenge strains of *T.*
388 *gondii*. V.Do. and S.P. provided technical and scientific help in designing the oral formulation.
389 A.C.L and C.S performed and analysed PCR-RFLP genotyping experiments. C. C., D.V., Y.V.,
390 A.G.C. and F.B. provided technical support. E.S. and R.B. partipated to the study design.
391 D.L.R. wrote the paper with the help of V.Dj. and comments from I.V. and R.B. All authors
392 have read and approved the manuscript and attest they meet the ICMJE criteria for
393 authorship.

394

395 **CONFLICT-OF-INTEREST DISCLOSURE :**

396 Animals included in the subcutaneous vaccination trial were bought by Vitamfero, of which
397 S.M. and E.S are former employees. E.S. is now an independent consultant and S.M. is
398 currently unemployed. The authors confirm that there are no financial or personal interest,
399 or belief that could affect their objectivity in reporting on the results obtained in this study.

400

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407

408

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525

526 **FIGURE LEGENDS**

527 **Figure 1 : Anti-*T. gondii* antibody production and protection of animals after subcutaneous**
528 **vaccination**

529 (A) IgM production after s.c. injection of Mic1-3KO vaccine strain (D0 and D35, blue arrows)
530 and oral challenge with type II *T. gondii* strain (D95, red arrow). (B) IgG production after s.c.
531 injection of Mic1-3KO vaccine strain (D0 and D35, blue arrows) and oral challenge with type
532 II *T. gondii* strain (D95, red arrow). *n* = 4 cats/batch (except "no vaccine" control *n*=1), errors
533 bars : SEM. (C) Presence of oocysts found in cat faeces upon challenge in the s.c. vaccination
534 trial. (note: no oocysts were found in animal faeces prior to challenge). (D) Genotyping of the
535 oocysts shed after the s.c. vaccination trial. Three loci of the ones tested by the PCR-RFLP are
536 shown (C29-2, SAG1 and BTUB). Genotype of the reference strains here (GT1, PTG, CTG) are

537 listed in suppl. Table S2. Faeces were identified for each cat, therefore each well represent
538 oocysts shed by one cat, either from the “2 injections” or from the “1 injection” batch.

539

540 **Figure 2 : Development of the gastro-resistant formulation of Mic1-3KO strain for oral**
541 **vaccination**

542 (A) Flow cytometry analysis of Mic1-3KO-GFP included in formula n°1 (F1) after 4h at 38,5°C
543 in a pH4 solution. Left panel shows the formula without (w/o) Mic1-3KO-GFP strain. Right
544 panel shows the GFP positive viable parasites after treatment (blue gate). The grey
545 population present on both panels represents the aroma included in F1. (B) Quantification of
546 live GFP positive viable parasites by flow cytometry after treatment. (C) Inverted bright field
547 microscopy images of HFF cell monolayers, at D2 and D6 post treatment with Mic1-3KO-GFP
548 strain included in F1, post *in vitro* treatment. *Bar = 10µm*.

549

550 **Figure 3 : Anti-*T. gondii* antibody production and protection of animals after oral**
551 **vaccination**

552 (A) IgM production after oral administration of Mic1-3KO vaccine strain (D0 and D60, blue
553 arrows) and oral challenge with type II *T. gondii* strain (D125, red arrow). (B) IgG production
554 after oral vaccination of the Mic1-3KO vaccine strain (D0 and D60, black arrows) and oral
555 challenge with type II *T. gondii* strain (D125, red arrow). *n = 4 cats/batch, errors bars: SEM*.
556 (C) Presence of oocysts found in cat faeces upon challenge in the oral vaccination trial (*note :*
557 *no oocysts were found in animal faeces prior to challenge*). (D) Genotyping of the oocysts
558 shed after the oral vaccination trial. Faeces were pooled for 2 cats (samples 1 to 4), sample 1
559 and 2 belonging to the vaccinated animals and sample 3 and 4 to the non vaccinated cats. In
560 between are presented references strains which genotypes are listed in suppl. Table S2

561 (MAS, TgCatBr5, TgCatBr64, TgToucan). Three loci of the ones tested by the PCR-RFLP are
562 shown (C29-2, SAG1 and BTUB).

563

564 **Figure 4 : Cytokine production after subcutaneous and oral vaccination**

565 Serum concentration of Th1 (A) and Th2 (B) cytokines throughout the s.c. vaccination trial of
566 the “1 injection” batch. Serum concentration of Th1 (C) and Th2 (D) cytokines throughout
567 the oral vaccination trial of the “vaccinated” batch, corresponding to the “1 injection batch”
568 of the s.c. vaccination trial. IL-2, IFN γ , IL-4 and IL-5 concentrations are expressed in ng/ml
569 (pg/ml x 1000). *n* = 4 cats/batch, errors bars: SEM.

570 **SUPPLEMENTARY MATERIAL**

571 **Supplementary material and methods** contains production of *T.gondii* total antigen extract
572 and anti-*T. gondii* IgA measurement by indirect ELISA. It also provides supplementary table
573 S1 (composition of gastro-resistant formulas tested) and table S2 (PCR-RFLP genotypes of *T.*
574 *gondii* reference strains used in the manuscript. **Supplementary Figure S1** shows specific
575 anti-*T. gondii* IgA production after vaccination and oral challenge and **Supplementary Figure**
576 **S2 shows** quantification of oocysts shed by cats vaccinated or not with the Mic1-3KO strain.

577

578 **SUPPLEMENTARY FIGURES LEGENDS**

579 **Supplementary Figure S1 : Specific anti-*T. gondii* IgA production after vaccination and oral**
580 **challenge**

581 (A) IgA production after s.c. injection of Mic1-3KO vaccine strain (D0 and D35, blue arrows)
582 and oral challenge with type II *T. gondii* strain (D95, red arrow). *n* = 4 cats/batch (except “no
583 Vaccine” control, *n*=1), errors bars: SEM. (B) IgA production after oral vaccination of the

584 Mic1-3KO vaccine strain (D0 and D60, blue arrows) and oral challenge with type II *T. gondii*
585 strain (D125, red arrow). *n* = 4 cats/batch, errors bars: SEM.

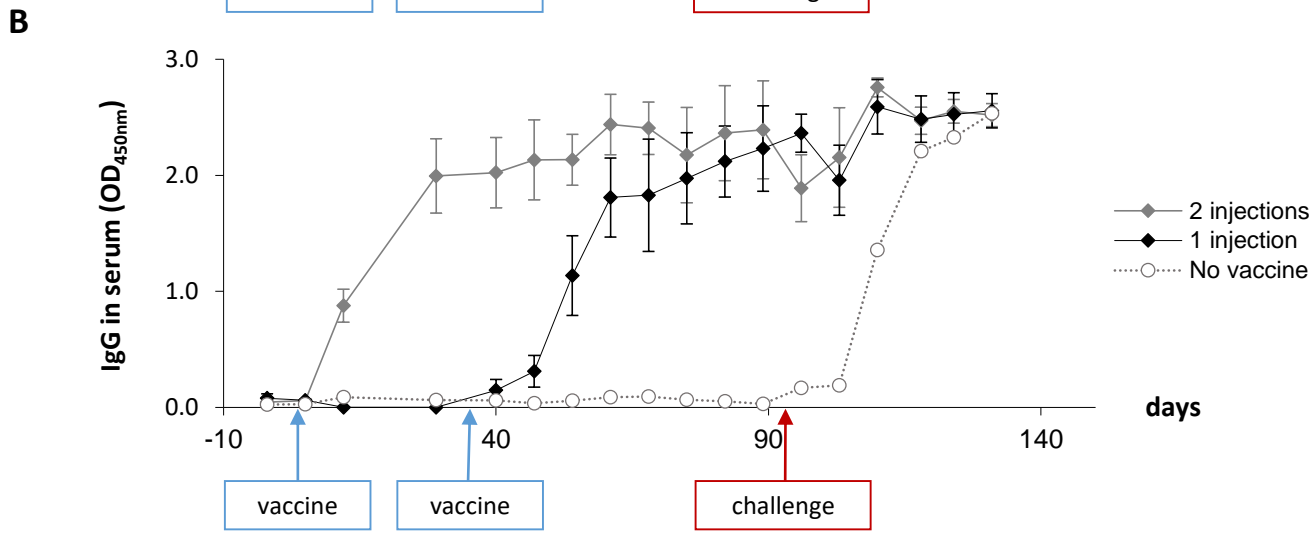
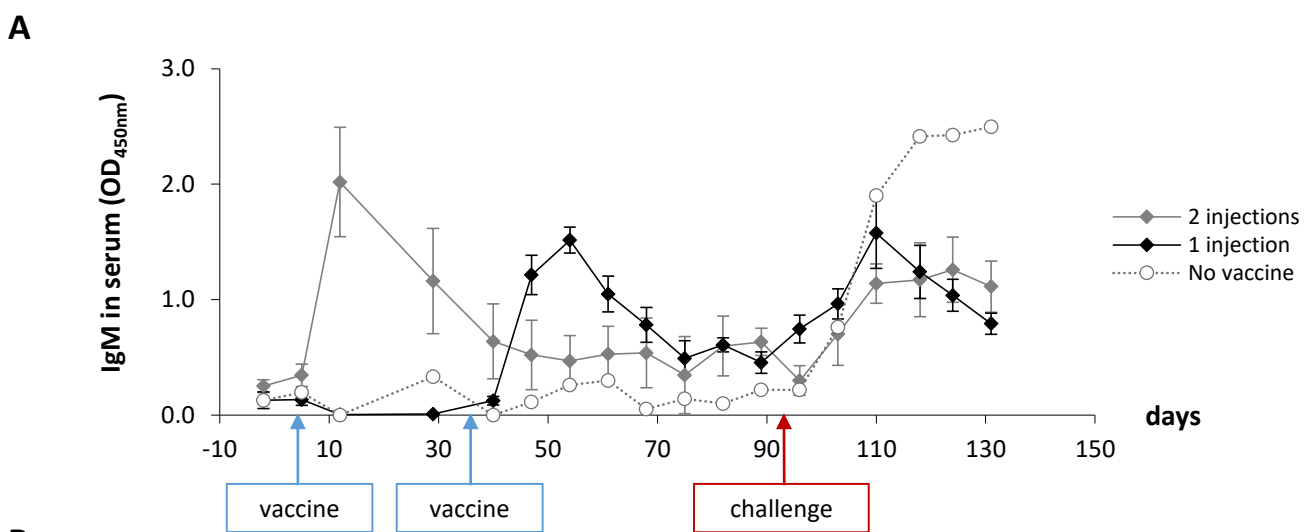
586

587 **Supplementary Figure S2 : quantification of oocysts shed by cats vaccinated or not with**
588 **the Mic1-3KO strain.**

589 (A) Average of oocysts per gram of faeces shed by cats after subcutaneous vaccination and
590 oral challenge (*n* = 4 cats/batch ; except the no vaccine control, *n*=1) (B) Average of oocysts
591 per gram of faeces shed by cats after oral vaccination and oral challenge (*n* = 4 cats/batch).

592

Figure 1 : anti-*T. gondii* antibodies production and protection of animals after subcutaneous vaccination



C

Days post challenge	1	2	3	4	5	7	7	8	9	10	11	12	13	14	15
No vaccine															
1 injection															
2 injections															

-
+

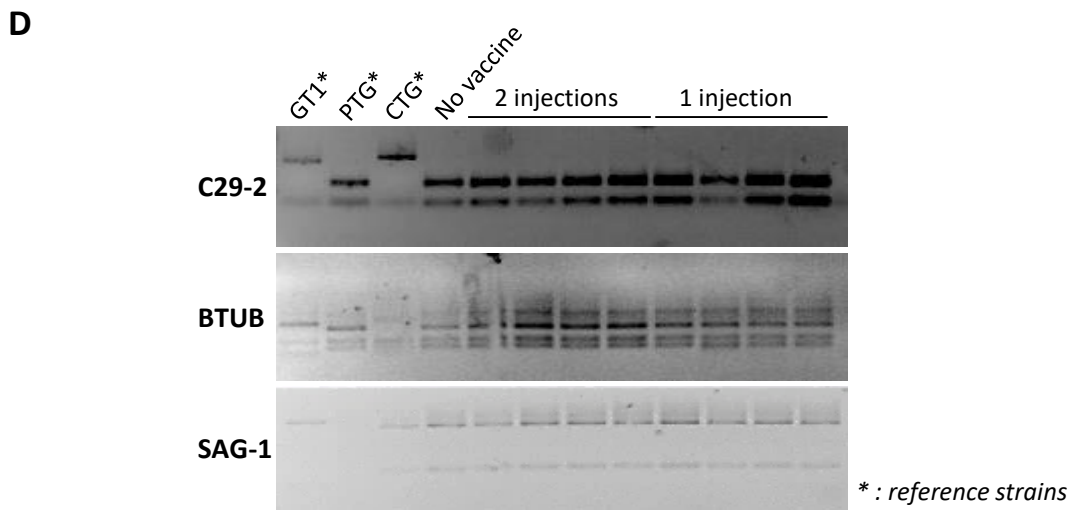


Figure 2 : development of the gastro-resistant formulation of Mic1-3KO strain for oral vaccination

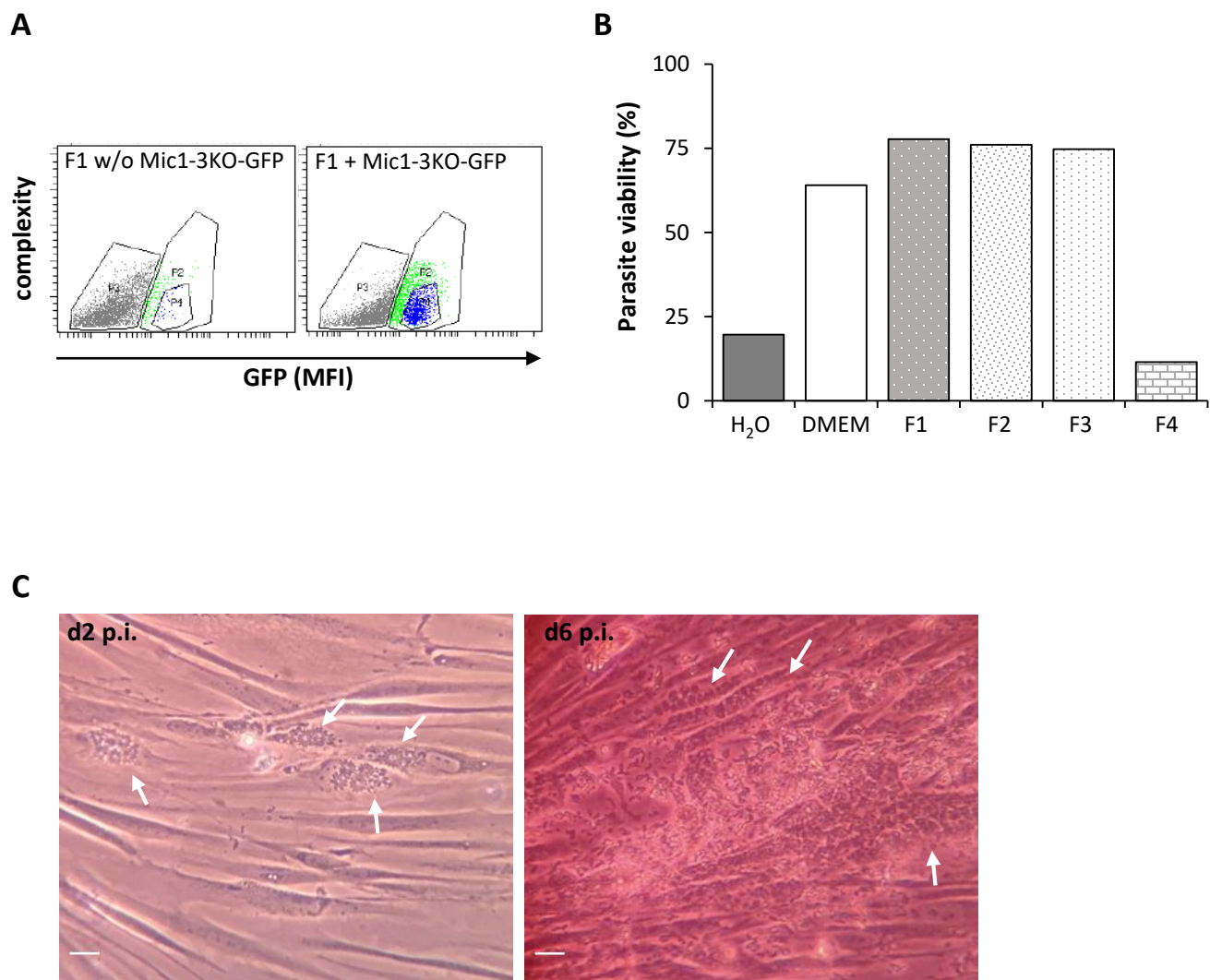


Figure 3 : anti-*T. gondii* antibodies production and protection of animals after oral vaccination

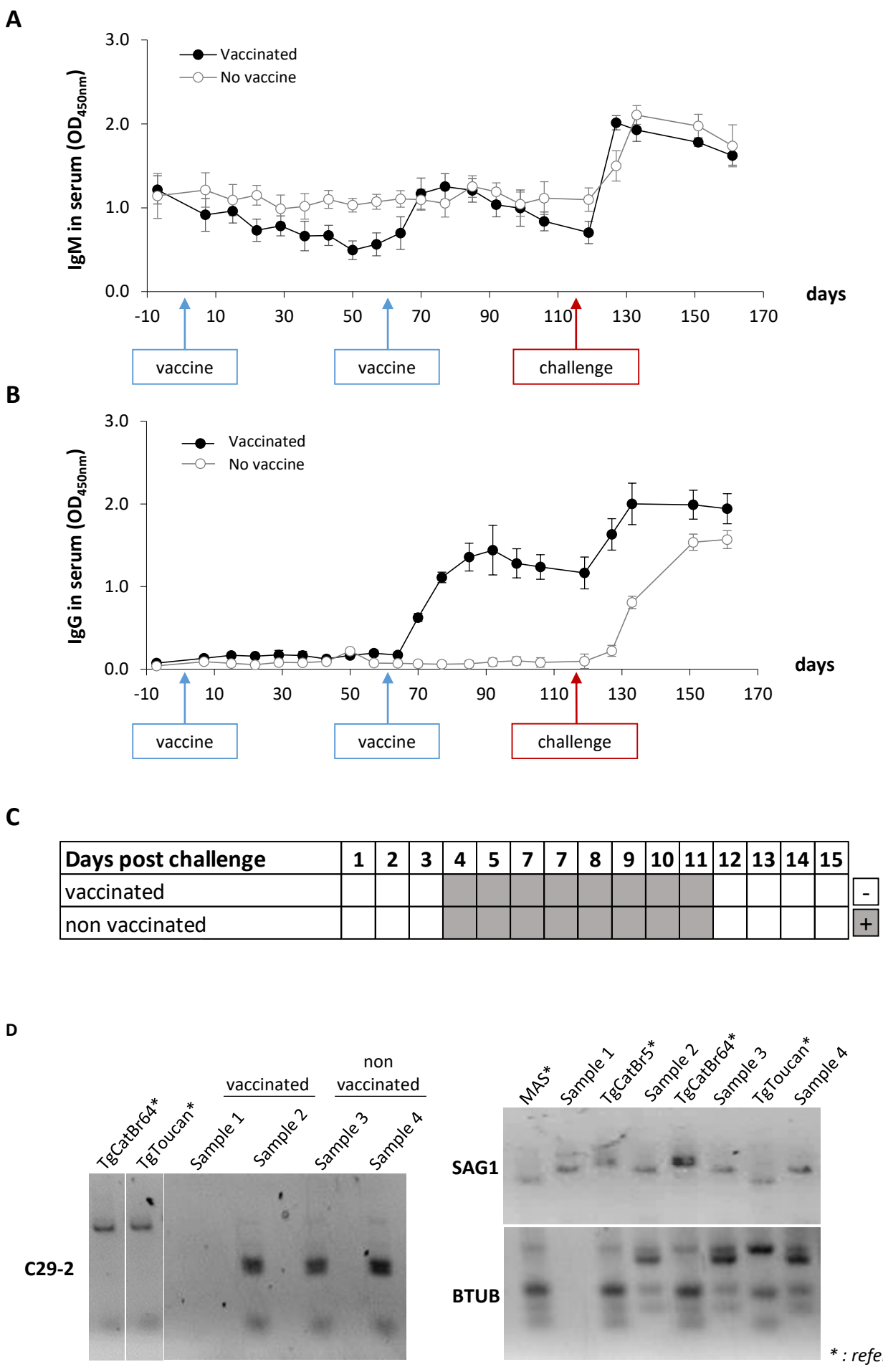


Figure 4 : Cytokine production after subcutaneous and oral vaccination

