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Development of cross-protective *Eimeria*-vectored vaccines based on apical membrane antigens

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ABSTRACT

Recently, the availability of protocols supporting genetic complementation of *Eimeria* has raised the prospect of generating transgenic parasite lines which can function as vaccine vectors, expressing and delivering heterologous proteins. Complementation with sequences encoding immunoprotective antigens from other *Eimeria* spp. offers an opportunity to reduce the complexity of species/strains in anticoccidial vaccines. Herein, we characterise and evaluate *Et*AMA1 and *Et*AMA2, two members of the apical membrane antigen (AMA) family of parasite surface proteins from *Eimeria tenella*. Both proteins are stage-regulated, and the sporozoite-specific *Et*AMA1 is effective at inducing partial protection against homologous challenge with *E. tenella* when used as a recombinant protein vaccine, whereas the merozoite-specific *Et*AMA2 is not. In order to test the ability of transgenic parasites to

confer heterologous protection, *E. tenella* parasites were complemented with *Em*AMA1, the sporozoite-specific orthologue of *Et*AMA1 from *E. maxima,* coupled with different delivery signals to modify its trafficking and improve antigen exposure to the host immune system. Vaccination of chickens using these transgenic parasites conferred partial protection against *E. maxima* challenge, with levels of efficacy comparable to those obtained using recombinant protein or DNA vaccines. In the present work we provide evidence for the first known time of the ability of transgenic *Eimeria* to induce cross protection against different *Eimeria* spp. Genetically complemented *Eimeria* provide a powerful tool to streamline the complex multi-valent anticoccidial vaccine formulations that are currently available in the market by generating parasite lines expressing vaccine targets from multiple eimerian species.

HIGHLIGHTS

- *Eimeria tenella* harbours four different stage-specific AMA1 paralogues.
- *Et*AMA1, but not *Et*AMA2, is involved in sporozoite invasion.
- *Et*AMA1, but not *Et*AMA2, induces significant protection against *E. tenella* challenge.
- Vaccination with transgenic *E. tenella* [*Em*AMA1] parasites induces partial protection against challenge with *Eimeria maxima*.

Keywords: *Eimeria tenella*, *Eimeria maxima*, Apical membrane antigen, AMA1, AMA2, Vaccine delivery vector, Cross protection, Heterologous protection

1. Introduction

Coccidiosis is caused by apicomplexan parasites of the genus *Eimeria*. Seven species replicate in chickens resulting in acute, self-limiting, enteritis that ranges in symptoms and severity depending on the species and infective dose of parasite, and the age, gender and genotype of the host. Recovered animals develop immunity that protects them against challenge with the same species of parasite, although their productivity may have been severely compromised by the disease, and repeated rounds of natural re-infection may be needed to induce full immunity (Shirley et al., 2005). The economic impact of poultry coccidiosis is estimated at >£2 billion per annum due to productivity losses combined with the costs of prevention and/or therapy (Williams et al., 1999; Dalloul and Lillehoj, 2006). In-feed chemoprophylaxis remains the main method of control, but concerns about drug residues in the food chain, widespread drug resistance, and legislative restrictions on the prophylactic use of ionophore antibiotics in poultry, all have major impacts (Chapman, 1997; Jenkins et al., 2017b). Oral vaccination with formulations of live wild-type or attenuated parasites is highly effective, but lack of cross-protective immunity means vaccines need to include vaccine lines of all *Eimeria* spp. that pose a risk during the lifetime of the chicken. As each vaccine line requires independent passage through pathogen-free chickens, vaccines are relatively costly to make. The uptake of commercial vaccines within the global broiler sector remains low, and there is an urgent need to develop cheaper, scalable vaccines (Blake and Tomley, 2014).

Previously we have shown that transgenic populations of *Eimeria* parasites can express and deliver vaccine antigens from *Campylobacter jejuni*, infectious bursal disease virus (IBDV) and infectious laryngotracheitis virus (ILTV) to chickens (Clark et al., 2012; Marugan-Hernandez et al., 2016). This technology has the potential to streamline current chicken coccidiosis vaccines, by reducing the complexity of existing formulations (with up to eight different parasites) to a single or small number of parasite lines that express immunoprotective antigens from all the relevant species of *Eimeria*. We are now able to demonstrate the first important step in the development of such a multivalent live vaccine by showing that expression in *Eimeria tenella* of a single antigen (*Em*AMA1)

78 from *Eimeria maxima* is sufficient to induce statistically significant partial protection against challenge
79 with *E. maxima* oocysts that is broadly equivalent to that obtained using the same antigen in other
80 vaccine platforms.

81 To date no cross-protective parasite antigens have been described, but several induce
82 between 30 and 70% immune protection against homologous *Eimeria* challenge when tested
83 experimentally with diverse delivery platforms (Blake and Tomley, 2014). The most promising antigens
84 are derived from early endogenous stages of the *Eimeria* lifecycle (sporozoite and first generation
85 schizont), which correlates with the findings observed in naturally infected chickens, where these
86 stages induce the strongest anti-*Eimeria* immunity (McDonald et al., 1986, 1988). Several of these
87 antigens have critical roles in host-parasite interactions including proteins that traffic to the parasite
88 surface and beyond via the secretory microneme (MIC) organelles such as MIC2 (Sathish et al., 2011),
89 MIC3 (Lai et al., 2011), MIC4 (Witcombe et al., 2004), and apical membrane antigen (AMA)1, which
90 achieves around 45% immunoprotective capacity against homologous challenge with *E. maxima*
91 (Blake et al., 2011; Li et al., 2013) or *Eimeria brunetti* (Hoan et al., 2014).

92 AMAs are a family of proteins expressed by phylogenetically distinct classes of obligate
93 intracellular apicomplexans including species of *Plasmodium* and the zoonotic coccidian *Toxoplasma*
94 *gondii*, where they are regarded as strong vaccine candidates (Remarque et al., 2008; Zhang et al.,
95 2015). These proteins are critical for the formation and maintenance of the moving junction, a stable
96 focus of adhesion between parasite and host cell membranes through which the parasite glides into
97 its intracellular vacuole (Aikawa et al., 1978; Besteiro et al., 2009; Tyler and Boothroyd, 2011).
98 *Plasmodium falciparum* AMA1 exhibits very high levels of genetic polymorphism which poses a major
99 challenge for vaccine development, hence recent trials have included co-administration of several
100 *Pf*AMA1 allelic variants (Faber et al, 2016). In contrast, a recent study of genetic variation in *E. tenella*
101 identified little intrinsic polymorphism at the *ama1* locus ETH_00007745 (ToxoDB, (Blake et al., 2015))
102 and we now report similar findings for the locus in *E. maxima* (GenBank accession number FN813221).

103 Proteomic analysis of *E. tenella* initially identified two dominant stage-regulated AMAs
104 specific for either sporozoites (*Et*AMA1, ToxoDB Accesion number ETH_00007745) or merozoites
105 (*Et*AMA2, ToxoDB Accesion number ETH_000048600) (Lal et al., 2009; Oakes et al., 2013). More
106 recently, genomic and phylogenetic analysis defined four classes of AMA paralogues (AMA1-4) that
107 are conserved across haemosporine and coccidian apicomplexans, including *Eimeria* (Parker et al.,
108 2016). Polyclonal antiserum against recombinant EtAMA1 were shown to have inhibitory effects on
109 sporozoite invasion of cultured cells (Jiang et al., 2012), however the serum reacted on western blots
110 and IFAT with both sporozoite and merozoite antigens, suggesting either that *Et*AMA1 protein is
111 expressed by both parasite stages or that the serum recognises more than one AMA paralogue. In this
112 paper we have clarified the stage-specific nature of these two dominant AMAs expressed by *E. tenella*
113 and show that whilst sporozoite-specific *Et*AMA1 induces partial immunoprotection against
114 homologous parasite challenge, merozoite-specific *Et*AMA2 does not.

115 The focus of the present work was thus two-fold. First, to characterise and test the potential
116 of the two most abundantly expressed AMA paralogues of *E. tenella* (*Et*AMA1 and *Et*AMA2) as
117 recombinant vaccines against homologous challenge (*E. tenella*) in chickens. Subsequently, armed
118 with the information that vaccination with *Et*AMA1, but not *Et*AMA2, significantly reduces the ability
119 of *E. tenella* challenge parasites to replicate, we generated transgenic populations of *E. tenella* that
120 express the orthologous sporozoite-specific AMA1 protein from *E. maxima* and tested these as live
121 vectored vaccines, showing that they confer a similar level of partial protection against heterologous
122 challenge (*E. maxima*). These results confirm the vaccine potential of AMA antigens for coccidiosis and
123 offer new opportunities for the development of multivalent vaccines against all the *Eimeria* spp. with
124 relevance to the poultry industry.

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128

2. Materials and methods

2.1. Parasites and birds

Oocysts of the Houghton (H) and Wisconsin (Wis) strains of *E. tenella*, and the Weybridge (W) strain of *E. maxima* were propagated by regular in vivo passage through three weeks old Light Sussex or Lohmann Selected Leghorn (LSL) chickens reared under specific pathogen-free conditions (Long et al., 1976). Oocysts were recovered and sporulated, and sporozoites were purified through columns of nylon wool and DE-52, using standard methods (Shirley et al., 1995). Second generation merozoites from *E. tenella* parasites were recovered from the intestines of chickens 112 h p.i. by trypsinisation (Shirley et al., 1995). Apical organelles (micronemes and rhoptries) were isolated from sporozoites and purified by sucrose-density ultracentrifugation as previously described (Kawazoe et al., 1992).

2.2. Sequence analysis

*Et*AMA1 (ToxoDB Accesion number ETH_00007745) and *Et*AMA2 (ToxoDB Accesion number ETH_00004860) protein sequences were aligned using the Multiple Sequence Comparison in the Log-Expectation tool (MUSCLE, www.ebi.ac.uk) and edited using the BioEdit software v7·1.1. This program was also used to estimate the identity and similarity percentages through the BLOSUM62 matrix.

2.3. Isolation of nucleic acids and proteins, and synthesis of complementary DNA from oocysts

Genomic DNA (gDNA) and total RNA were extracted from oocysts using the TRIzol® reagent (Invitrogen, Paisley, UK) as detailed previously (Marugan-Hernandez et al., 2016). Protein extracts from sporozoites and merozoites were obtained following standard procedures (Tomley, 1994). Complementary DNA (cDNA) was also generated using the SuperScript II® reverse transcriptase and random hexamer primers (Invitrogen) as previously described (Marugan-Hernandez et al., 2016).

2.4. Recombinant expression of EtAMA1 and EtAMA2

154 The extracellular domains of *Et*AMA1 (ser16-gly446, ETH_00007745 in ToxoDB) and *Et*AMA2
155 (cys15-gly452, ETH_00004860) were amplified from sporozoite or merozoite cDNA by PCR using the
156 Platinum Taq DNA Polymerase High Fidelity® (Invitrogen) and the following primers (5'-3'):
157 ATAGGATCCGAGCTGCGCAGGGCCGGCAGCA and GCGAAGCTTTTAACCGCCCCCTTTAGACTCGC for
158 *Et*AMA1, and ATAGGATCCGTGCATCAGTGCCGTGGCGGCA and
159 CGCAAGCTTTTAGCCGAAGCTAACGCCCAGGG for *Et*AMA2 (Sigma-Aldrich, Suffolk, UK). Primers
160 incorporated a *Bam*HI site at the 5' end (underlined), and a TAA stop codon and *Hind*III site at the 3'
161 end. The digested, gel purified (Qiagen, West Sussex, UK) PCR products were ligated to a pET32b (+)
162 vector that had been previously linearised by double digestion with *Bam*HI and *Hind*III (New England
163 BioLabs, Hertfordshire, UK). *Et*AMA1 and *Et*AMA2 sequences were ligated to pET32b (+) by T4 ligase
164 (Promega, Hampshire, UK), propagated in *Escherichia coli* XL1-Blue competent cells (Stratagene,
165 California, USA), purified using the QIAprep Spin Miniprep kit (Qiagen), and checked by restriction
166 analyses using *Bam*HI and *Hind*III. Recombinant *Et*AMA1 and *Et*AMA2 were produced in transformed
167 BL21(DE3)pLysS cells (Novagen, Hertfordshire, UK) and purified using HisTrap FF purification columns
168 (GE Healthcare, Buckinghamshire, UK) following standard procedures (Blake et al., 2011). Proteins
169 were dialysed extensively against PBS before use.
170
171 *2.5. Generation of polyclonal antibodies against* Et*AMA1 and* Et*AMA2*
172 Hyperimmune antisera were raised in mice (*Et*AMA1 and *Et*AMA2), chickens (*Et*AMA1 only),
173 or rabbits (*Em*AMA1) against HisTrap FF purified recombinant proteins (see Section 2.4and (Blake et
174 al., 2011)). Groups of three or four animals were immunised three times at fortnightly intervals with
175 between 10 and 50 µg of soluble antigen suspended in 100 µl of PBS and mixed with an equal volume
176 of Titremax gold (first immunisation) or Freund's incomplete adjuvant (second and third
177 immunisations) (Kawazoe et al., 1992). Animals were bled 7 days after the second and third
178 immunisations and all sera screened by western blotting against solubilised whole sporozoite and
179 merozoite proteins.

180 *2.6. Indirect immunofluorescence (IFAT)*

181 Purified *E. tenella* sporozoites were fixed in 3% paraformaldehyde in PBS for 10 min then 20
182 µL spots of the suspension were dried onto glass coverslips. Dried parasites were permeabilised by
183 immersion in ice-cold methanol for 5 min, followed by rehydration in PBS for 20 min. IFAT was carried
184 out as described (Bumstead and Tomley, 2000) using antiserum to *Et*AMA1 or *Et*AMA2. The DNA stain
185 DAPI (4', 6'-diamidino-2 phenylindole) was included in the second antibody incubation step. Coverslips
186 were air dried, mounted on Vectashield (Vector Laboratories, Peterborough, UK) and photographed
187 at ×1,000 magnification under UV light using a Leitz fluorescence microscope. Alternatively, tissue
188 sections of infected caeca containing second generation schizonts as used for merozoite harvest were
189 de-waxed and pressure cooked. All samples were treated with PBS-1% BSA to block non-specific
190 staining, exposed to primary antibody for 1 h and then, after washing, were incubated with FITC-
191 conjugated anti-chicken or anti-mouse-IgG (Sigma-Aldrich). Some samples were counterstained with
192 DAPI prior to examination. Coverslips and sections were examined with a Zeiss Axioskop microscope,
193 and pictures were taken with a cooled CCD camera using Improvision Openlab software.

194

195 *2.7. In vitro secretion assays*

196 Freshly excysted *E. tenella* H sporozoites were purified, resuspended at 10^8/ml in HAMs F9
197 media (Sigma-Aldrich) with or without 1% FCS supplementation (Sigma-Aldrich), and incubated at 41
198 ºC. Supernatants were removed at 0, 10 and 30 min post-incubation, clarified by centrifugation at
199 10,000 *g* for 10 min at 4 ºC, and then stored at -20 ºC until examined by SDS-PAGE and western blotting
200 (as described in Section 2.10). Rabbit antiserum against the *E. tenella* microneme protein 3 (*Et*MIC3)
201 was used as a positive control for secretion (Lai et al., 2011) and against the *E. tenella* heat shock
202 protein 70 (*Et*HSP70) protein as a control for inadvertent sporozoite lysis (Dunn et al., 1995; Bumstead
203 and Tomley, 2000).

204

205

206 *2.8. In vitro sporozoite inhibition assays*

207 Wells of 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) were seeded with 100 µl of
208 a suspension of Madin Darby Bovine Kidney (MDBK) cells (6 x 10^5/ml) in HAMs F-12 medium (Sigma-
209 Aldrich) and incubated at 41 ºC until the cells were settled and monolayers reached ~85% confluency.
210 Freshly hatched *E. tenella* sporozoites were suspended at 10^6/ml in HAMs F-12 supplemented with
211 dilutions of anti-*Et*AMA1 or anti-*Et*AMA2 mouse sera (1/50 to 1/800). Alternatively, freshly plated
212 MDBK cell monolayers were exposed to medium supplemented with dilutions of *Et*AMA1 or *Et*AMA2
213 recombinant protein (1 to 0.625 µg/ml). For all treatments, parasites or cells were incubated in
214 triplicate for 15 min at room temperature. A mouse antibody against parasite enolyl reductase (ENR)
215 and the recombinant thioredoxin protein were included as control treatments for each respective
216 protocol. The incubation medium was removed from each well, replaced with 200 µl of the
217 appropriate pre-incubated sporozoite suspension and the infected monolayers returned to 41 ºC.
218 Infected cultures were incubated for 48 h and the overall inhibition of parasite growth measured by
219 comparison of the incorporation of labelled uracil into untreated cultures, using well established
220 procedures (Schmatz et al., 1986). In this case infected monolayers were spiked with 1 µCi per well of
221 [3H] uracil at 24 h p.i., then returned to 41 ºC for a further 24 h at which time cells were lysed and
222 tritium incorporation was counted. Data were analysed using the Tukey's multiple comparisons test
223 from the GraphPad Prism software (version 7.02).

224

225 *2.9.* In vivo Et*AMA1 and* Et*AMA2 immunisation trials*

226 Coccidia-free Light Sussex chickens were housed within specific pathogen-free (SPF), coccidia-
227 free conditions in groups of four (experiment one) or five (experiment two). Chickens were immunised
228 at one, three and 5 weeks of age by s.c. injection with 100 µg of the corresponding vaccine antigen
229 (Table 1). The first two preparations were made up to a volume of 0.5 ml and emulsified with 0.5 ml
230 of TiterMax adjuvant (Sigma-Aldrich) except for Group 4, where 0.25 ml of each antigen was
231 emulsified separately with 0.25 ml of TiterMax adjuvant and administered together. The last

232 preparations were emulsified with Freund's incomplete adjuvant (Sigma-Aldrich). At 7 weeks of age,
233 birds were moved into single cages and each chicken was given an oral dose of 250 sporulated *E.*
234 *tenella* H strain oocysts to quantify the effect of the vaccine on parasite replication. Daily faecal
235 samples were collected from each bird from 5 to 12 days after challenge and the number of oocysts
236 excreted in the faeces calculated by counting of samples following a standard protocol (Long et al.,
237 1976). Data were analysed by one-way ANOVA with a Tukey's post-hoc test using the GraphPad Prism
238 software (version 7.02).

239

240 *2.10. SDS- PAGE and western blot*

241 Proteins were electrophoresed through NuPAGE 4–12 % Bis-Tris gels (Invitrogen) in Laemmli
242 loading buffer (Sigma) using the XCell SureLock Mini-Cell Electrophoresis System (Invitrogen), and then
243 either stained using Coomasie brilliant blue R-250 (Bio-Rad, Hertfordshire, UK) or transferred to
244 nitrocellulose membranes (GE Healthcare) in a semi-wet system using the XCell II Blot Module
245 (Invitrogen). Non-specific binding sites were blocked by overnight incubation in 3% BSA (Sigma-
246 Aldrich) in Tris-buffered saline (TBS)-Tween 0.1%, and then filters were probed with either mouse or
247 chicken antibodies, diluted in blocking solution, followed by horseradish peroxidase (HRP) conjugated
248 anti-mouse/chicken second antibodies (Merck Millipore, Hertfordshire, UK). Bound antibody was
249 visualised by enhanced chemiluminescence (ECL) using Luminata Forte Western HRP substrate (Merck
250 Millipore) and a G:BOX coupled with GeneSnap 7.12 software (Syngene, Cambridge, UK).

251

252 *2.11.* Eimeria maxima *AMA1 amplification and cloning*

253 The coding sequence of the *E. maxima* AMA1 ectodomain (*Em*AMA1, his32-phe446, GenBank
254 Accesion number FN813221.1) was amplified from the pET32b-EmAMA1 plasmid (Blake et al., 2011)
255 and flanked with *Xba*I restriction sites (underlined) by PCR using the primers (5'-3'):
256 GCTCTAGACACCAGGGTCACACAGAA and GCTCTAGAAAAGCCGCCTTCACACCG (Sigma-Aldrich). A 1,269
257 bp fragment was amplified using the Platinum Taq DNA Polymerase High Fidelity® (Invitrogen). The

258 PCR product was resolved by agarose gel electrophoresis, manually excised under UV light and purified
259 using the MinElute Gel Extraction kit (Qiagen). Then, it was cloned using the pGEM®-T Easy vector
260 system (Promega), propagated in *E. coli* XL1-Blue competent cells (Stratagene), purified using the
261 QIAprep Spin Miniprep kit (Qiagen), and sequenced (GATC Biotech, London, UK) as described by the
262 manufacturers. Sequence analysis was done using CLC Main Workbench (Qiagen).
263
264 *2.12. Plasmid constructs for transgene expression of EmAMA1 in E. tenella*
265 Three different core plasmids were used for *E. tenella* transfection as previously described
266 (Marugan-Hernandez et al., 2017). In brief, all the plasmids carried the mCitrine reporter downstream
267 of the *E. tenella* microneme protein (*Et*MIC) 1 5' promoter region, and the mCherry reporter
268 downstream of the 5'*Et*MIC2 promoter region, preceded by the *Xba*I restriction site, and flanked with
269 varying combinations of two delivery signals: the signal peptide of the *Et*MIC2 protein (SP2), and the
270 glycosylphosphatidylinositol anchor of the *Et*SAG1 protein (GPI) (Fig. 1A-C).
271 The coding sequence of the *Em*AMA1 ectodomain was cloned into the *Xba*I site of the three
272 core constructs and therefore fused to the mCherry reporter (Fig. 1). For this purpose, the pGEM-T-
273 *Em*AMA1 plasmid was digested with *Xba*I (New England BioLabs) following the manufacturer's
274 protocol, and the *Xba*I-*Em*AMA1-*Xba*I insert was purified from agarose gels as described above. The
275 three core constructs were also digested with *Xba*I (single restriction site), treated with calf intestinal
276 alkaline phosphatase (New England BioLabs) according to the manufacturer's instructions, and
277 purified from agarose gels. The ligation of the *Em*AMA1 sequence into the core plasmids was carried
278 out using the T4 ligase (Promega) as detailed by the manufacturer. The three new constructs were
279 also propagated in XL1-Blue competent cells, mini-prepped, characterized by restriction analyses to
280 determine the insertion directionality using the *Nde*I enzyme (New England BioLabs), and sequenced
281 to check that the insert reading frame had not been altered by cloning. Final plasmids were prepared
282 for transfection using a Midi Prep Kit (Qiagen), digested for linearization with *Sca*I (New England

283 BioLabs), precipitated in ethanol-sodium acetate (Marugan-Hernandez et al., 2017) and quantified by
284 NanoDrop (Thermo Scientific).
285

286 *2.13. Drug selection of mutant parasites*

287 A plasmid carrying the mutant *Toxoplama gondii* dihydrofolate reductase–thymidylate
288 synthase (DHFR-TSm2m3) gene that confers resistance to pyrimethamine (Fig. 1D) (Clark et al., 2008)
289 was also prepared for co-transfection using a Midi Prep Kit, digested for linearization with *Psi*I (New
290 England BioLabs), precipitated in ethanol-sodium acetate and quantified by NanoDrop.
291

292 *2.14. Preparation of E. tenella Wis sporozoites expressing EmAMA1 protein*

293 Transfection of freshly hatched *E. tenella* Wis strain sporozoites was carried out by restriction
294 enzyme-mediated integration (REMI) using 16-well strips and the programme EO114 of the
295 Nucleofector 4D (Lonza, Basel, Switzerland) as described previously (Marugan-Hernandez et al., 2016).
296 Briefly, 1×10^6 sporozoites were electroporated in duplicate with 10 µg of *Sca*I and *Psi*I-digested
297 plasmids (carrying the EmAMA1 sequence with different delivery signals, and the DHFR-TSm2m3
298 resistance gene, respectively) together with 6 U of the *Sca*I and *Psi*I restriction enzymes in Lonza buffer
299 P3. Shocked parasites were left for 20 min at room temperature in RPMI medium (Sigma-Aldrich), and
300 then sporozoite survival was estimated by microscopy using 0.02% Trypan blue dye (Invitrogen)
301 (Marugan-Hernandez et al., 2017). In order to obtain stable transgenic populations, duplicated wells
302 were pooled together post-transfection and used to infect two birds by the cloaca (0.75×10^6
303 sporozoites/bird). After 24 h, birds were in-feed supplemented with pyrimethamine for 6 days (150
304 ppm, Sigma-Aldrich) (Clark et al., 2008). Oocysts were harvested 7 days after infection, sporulated and
305 used for a subsequent in vivo passage after population enrichment for fluorescent parasites by
306 fluorescence-activated cell sorting (FACS) (FACS Aria III, BD, Berkshire, UK) (Marugan-Hernandez et al.,
307 2016).

308 Transcription of the *Em*AMA1 W strain coding sequence was determined by reverse
309 transcription PCR (RT-PCR) using cDNA isolated from the three stable transgenic populations as
310 described above and employing the primers used for *Em*AMA1 cloning. To confirm the absence of
311 gDNA in the cDNA samples, primers for the *EtActin* gene were designed across an intron such that
312 gDNA contamination of cDNA would result in amplification of two fragments (358 and 99 bp; 5' to 3':
313 TTGTTGTGGTCTTCCGTCA and GAATCCGGGGAACATAGTAG, Sigma-Aldrich; from (Marugan-Hernandez
314 et al., 2016)). The *Em*AMA1 sequence was amplified by PCR using the Platinum Taq DNA Polymerase
315 (Invitrogen), and the corresponding cDNA at 1/10 dilution. The PCR product was resolved by agarose
316 gel electrophoresis.

317 Expression of the *Em*AMA1 protein fused with mCherry was confirmed by microscopy for the
318 fluorescent reporter using the fluorescent microscope DMI3000B – DCF365FX (Leica Microsystems,
319 Milton Keynes, UK). Micrographs of sporulated transgenic oocysts were collected using the SP5
320 confocal microscope and the Leica Application Suite Advanced Fluorescence software (Leica
321 Microsystems). Image processing was performed using ImageJ software (NCBI,
322 http://rsb.info.nih.gov/ij/).

323

324 *2.15. In vivo immunisation trial of E. tenella Wis parasites expressing EmAMA1*

325 A total of 42 White Leghorn line 15I chicks (highly susceptible to *E. maxima* infection,
326 (Bumstead and Millard, 1992; Smith et al., 2002)); purchased from the National Avian Research
327 Facility, Edinburgh, UK) were split into six groups of seven in independent wire-floored cages (Table
328 2). One (Group 1, *Et*[*Em*AMA1-Ch]) and two (Group 3, *Et*[SP2-*Em*AMA1-Ch-GPI]) chicks were removed
329 prior to the start of the vaccination trial for husbandry reasons. Birds from groups 1 to 4 were
330 immunised by oral gavage at days 2, 8, 14 and 21 of age with 100, 500, 3,000, and 5,000 FACS enriched
331 transgenic *Em*AMA1 *E. tenella* sporulated oocysts, respectively, to mimic parasite recycling, whereas
332 birds from groups 5 to 6 were inoculated with sterile PBS following the same scheme. At day 30, birds
333 from groups 1 to 5 were challenged with 300 oocysts of the *E. maxima* W strain to quantify the effect

334 of the vaccine on parasite replication. Body weights and serum samples were collected at days 29 (8
335 days after the final vaccination) and 36 (6 days post-challenge) of age to determine the safety,
336 immunogenicity, and efficacy of the recombinant vectored vaccines. Also, individual faecal samples
337 were collected from days 35 to 39 (5 - 8 days post-challenge) to determine the total oocyst output
338 after *E. maxima* challenge (Shirley et al., 2005). Data were analysed by one way ANOVA with a Tukey's
339 post-hoc test using the GraphPad Prism software (version 7.02).
340

341 *2.16. Analysis of immune responses elicited after vaccination with E. tenella Wis parasites expressing*
342 *EmAMA1 and after challenge with E. maxima W*

343 Serum samples collected on day 29 were used to detect IgY responses against *Em*AMA1
344 protein by western blot. All sera (1/50 to 1/400 dilutions) were directed against nitrocellulose
345 membranes transferred with recombinant *Em*AMA1 or with *E. maxima* protein extracts as detailed in
346 Section 2.10. For *E. maxima*-based western-blots, rabbit anti-*Em*AMA1 antiserum was used as the
347 positive control to detect native *Em*AMA1 protein in oocyst extracts and compare its recognition
348 pattern with those obtained using the test serum samples.

349 In addition, serum samples collected on day 29 and 36 were used to quantify serum IL-10 and
350 IFN-γ. IL-10 ELISA was performed as described previously (Wu et al., 2016). Briefly, assay plates (Nunc
351 Immuno MaxiSorp) were coated overnight at 4 °C with 3 μg/ml of capture antibody, incubated with
352 50 μl of two-fold serially diluted standards or serum samples, and incubated with 1 μg/ml of
353 biotinylated detection antibody. The plates were incubated with streptavidin-HRP and developed with
354 1-Step™ Turbo TMB substrate solution (Thermo Scientific). The reaction was stopped by adding 50 μl
355 of 2N sulphuric acid after 10 min, and assays were read at 450 nm. IFN-γ levels were measured using
356 the IFN-γ chicken matched antibody pair (Thermo Scientific) following the manufacturer's instruction.
357 Data were analysed by one-way ANOVA with a Tukey's post-hoc test using GraphPad Prism software
358 (version 7.02). Associations between oocyst shedding and cytokine levels were estimated using the
359 Pearson correlation coefficient with the same software.

360 *2.17. Genetic characterisation of E. maxima and E. tenella apical membrane antigens*

361 Gene models for the *E. tenella* apical membrane antigens 2-4 were downloaded from ToxoDB
362 (Gajria et al., 2008; Parker et al., 2016) and used to identify candidate *E. maxima* orthologues using
363 tBLASTx with default parameters in ToxoDB. Coding sequences for *Em*AMA1 and *Et*AMA1 were
364 accessed from GenBank and ToxoDB (FN813221 and ETH_00007745). Transcription profiles were
365 accessed for all *E. tenella* genes using published RNAseq data (Reid et al., 2014; Walker et al., 2015).
366 Signatures of selection were assessed using mean Ka/Ks scores (*Eimeria acervulina*, *E. brunetti*, *E.*
367 *maxima*, *Eimeria mitis*, *Eimeria necatrix* and *Eimeria praecox* compared with *E. tenella*, (Reid et al.,
368 2014)). For *E. maxima*, additional measures of genetic diversity were calculated using 18 coding
369 sequences derived from India (*n*=3), Nigeria (*n*=2), Spain (*n*=1) and the UK (*n*=12) available under the
370 GenBank accession numbers **FN813221-2**, **LN626985-9**1 and **LT900485-LT900492**. Analyses
371 undertaken using DnaSP v5.10 (Librado and Rozas, 2009) included identification of the number of
372 variant sites (S) and numbers of non-synonymous (dN) or synonymous (dS) substitutions. Diversity
373 was defined by calculating the average nucleotide difference (k) and nucleotide diversity (π with the
374 Jukes Cantor correction). Allelic diversity was defined by calculating the number of haplotypes (H) and
375 the associated haplotype diversity (Hd). Neutrality was assessed using Tajima's D and Fu and Li's D*
376 and F* tests with the total number of mutations and significance set at $P < 0.05$. All analyses were
377 performed as described previously and compared with data published for EtAMA1 (Blake et al., 2015).
378

379 *2.18. Ethical statement*

380 This study was carried out in strict accordance with the Animals (Scientific Procedures) Act
381 1986, an Act of Parliament of the United Kingdom. All animal studies and protocols were approved by
382 the Royal Veterinary College Animal Welfare & Ethical Review Body (London, UK) and the United
383 Kingdom Government Home Office under specific project licence. The laboratory work involving
384 genetic modified organisms (GMO) was conducted under authorization GM9708.1, administered by
385 the UK Health and Safety Executive.

386 **3. Results**

387 *3.1. Expression of EtAMA genes is stage-regulated*

388 The protein sequences of *Et*AMA1 and *Et*AMA2 were compared using the BLOSUM62 matrix,
389 and displayed low identity and similarity scores (0.338 and 0.474, respectively) (Supplementary Fig.
390 S1). Samples of unsporulated oocysts (UO, 1.25×10^5/well), sporulated oocysts (SO, 1.25×10^5/well),
391 sporozoites (Sz, 1×10^6/well) and second generation merozoites (Mz2, 1×10^6/well) were probed with
392 hyperimmune mouse serum against r*Et*AMA1 or r*Et*AMA2. *Et*AMA1 was detected in the sporulated
393 oocyst and sporozoite lanes and was absent from the merozoites. Conversely, EtAMA2 was detected
394 only in merozoites (Fig. 2A). Subsequent comparison of RNAseq datasets representing UO, SO, Sz,
395 Mz2 and gametocyte (Gam) lifecycle stages confirmed these findings, with additional evidence of
396 stage-specific transcription for EtAMAs 1-4 (Table 3). Mean Ka/Ks ratios of less than 1.0 were reported
397 for AMA1 and AMA4, indicating purifying or stabilising selection.

398 Samples of sporozoites and gradient-enriched preparations of sporozoite micronemes and
399 rhoptries were probed with hyperimmune mouse and chicken sera raised against the same
400 preparation of r*Et*AMA1. The mouse antiserum recognised a single microneme protein of an apparent
401 molecular mass of ~63 kDa, whereas the chicken antiserum additionally reacted with a protein in the
402 rhoptry fraction of ~60 kDa (Fig. 2B). *Et*MIC3 antiserum demonstrated that the rhoptry fraction was
403 not significantly contaminated with micronemes, and *Et*HSP70 antiserum (a common contaminant of
404 rhoptry fractions, (Dunn et al., 1995)) demonstrated that the microneme fraction was not
405 contaminated with rhoptries.

406 Immunofluorescent antibody staining of sporozoites with mouse anti-r*Et*AMA1 showed an
407 apical localisation within the zoite (Fig. 2C), whereas mouse anti-r*Et*AMA2 showed clear apical staining
408 of mature merozoites in caecal sections harvested 114 h p.i. with *E. tenella* H strain (when second
409 generation schizogony is underway) (Fig. 2C). Some sections were counterstained with antibodies
410 against the merozoite surface antigen *Et*SAG4, to delineate the outline of merozoites within schizonts.
411 Others were counterstained with antibodies against *Et*MIC2 to delineate the micronemes.

412 Interestingly, *Et*AMA2 only partially co-localised with *Et*MIC2, indicating that it may not all be localised
413 within the microneme organelles or alternatively that the two proteins may be differentially
414 distributed within the microneme population (Fig. 2C). A similar phenomenon was previously noted
415 when co-staining for *Et*AMA1 and *Et*MIC3 (Lai et al, 2011).

416

417 *3.2. EtAMA1 is secreted by sporozoites and involved in host-cell sporozoite invasion*

418 Freshly excysted and purified sporozoites were incubated with or without FCS for up to 30
419 min. In the absence of FCS, *Et*AMA1 was secreted at a low level by sporozoites whereas FCS
420 supplementation induced rapid secretion into the culture supernatant in a manner typical of *E. tenella*
421 microneme proteins (Fig. 2D) (Lai et al., 2011). Pre-treatment of MDBK cells or *E. tenella* sporozoites
422 with, respectively, recombinant *Et*AMA1 (0.5 to 1 µg) or mouse anti r*Et*AMA1 (1/800 to 1/50 dilution),
423 resulted in significant reductions in parasite-specific uracil uptake ($P < 0.05$, Tukey's post hoc test),
424 indicative of reduced invasion and/or parasite replication. In contrast, treatment with recombinant
425 *Et*AMA2, recombinant thioredoxin, anti r*Et*AMA2 serum, or anti-ENR serum did not affect uracil
426 uptake (Fig. 2E).

427

428 *3.3. Recombinant EtAMA1 induces an immune response protective against E. tenella challenge*

429 In experiment 1, vaccination with r*Et*AMA1 induced a significant reduction in oocyst output
430 after challenge with *E. tenella* ($P < 0.05$, one way ANOVA test), reducing oocyst shedding by 77.4%
431 compared with the non-vaccinated control, whereas no significant differences were found between
432 chickens vaccinated with r*Et*AMA2 and non-immunised animals ($P > 0.05$, one way ANOVA test) (Fig.
433 3). Similarly, in experiment 2, vaccination with r*Et*AMA1 or with combined r*Et*AMA1+r*Et*AMA2 induced
434 a significant reduction in oocyst output after challenge with *E. tenella* ($P < 0.05$, one wayANOVA test),
435 reducing oocyst shedding by 76 and 66% compared with the non-vaccinated control, respectively.
436 Again, no significant differences were found between chickens vaccinated with rEtAMA2 and non-
437 immunised animals ($P > 0.05$, one way ANOVA test) (Fig. 3).

438 *3.4. Eimeria tenella can express the foreign antigen EmAMA1 and modify its trafficking when*
439 *complemented with appropriate delivery signals*

440 Three populations of transgenic *E. tenella* parasites expressing *Em*AMA1 were generated by
441 transfection and stabilised by sequential passage through chickens medicated with pyrimethamine
442 (Fig. 4A). The first round of infection resulted in shedding of 20 to 60×10^6 oocysts/bird 1 week p.i., of
443 which 1 to 2% expressed the mCitrine reporter. FACS enrichment of mCitrine expressing parasites
444 was ~95% efficient and successive in vivo passage of FACS isolated mCitrine-positive parasites under
445 pyrimethamine selection improved the transgenic proportion of each population from 1 to 2% at
446 passage 1, to 16 to 42% at passage 3 (Fig. 4A).

447 Transcription of *Em*AMA1 mRNA was confirmed by RT-PCR in all three transgenic populations
448 in the absence of gDNA contamination (Fig. 4C). In addition, expression of the *Em*AMA1 protein was
449 indicated by detection of the fused mCherry reporter by fluorescence microscopy (Fig. 4B). In the
450 three populations, FACS enriched parasites expressed mCitrine as a cytosolic protein, whereas the
451 expression pattern of mCherry differed among them. Parasites not complemented with delivery
452 signals expressed the mCherry as a cytosolic protein which co-localised with mCitrine (*Em*AMA1-Ch in
453 Fig. 4B). In contrast, populations complemented with the SP2 sequence secreted the mCherry reporter
454 into the sporocyst cavity (SP2-*Em*AMA1-Ch and SP2-*Em*AMA1-Ch-GPI in Fig. 4B). Furthermore,
455 complementation with the GPI-anchor additionally induced the anchorage of mCherry onto the
456 sporozoite surface (SP2-*Em*AMA1-Ch-GPI in Fig. 4B). In all populations both mCitrine and mCherry
457 were present within cytosolic aggregates, which in some cases were co-localised (Fig. 4B).
458

459 *3.5. Vaccination with transgenic E. tenella expressing EmAMA1 protects against challenge by the*
460 *antigen donor E. maxima without eliciting specific humoral responses, but reducing serum IL-10 levels*

461 Vaccination with live *E. tenella* parasites did not result in significant differences in weights
462 between groups prior to challenge (*P* > 0.05, one way ANOVA test) indicating no detrimental effect of
463 the vector. Similarly, no differences in body weight gain were found between groups after challenge

464 with *E. maxima* W strain, as the dose used was deliberately low in order to accurately determine the
465 vaccination effect on parasite replication ((Blake et al., 2006); $P > 0.05$, one way ANOVA test)
466 (Supplementary Fig. S2A and B). Vaccination with all three transgenic *E. tenella* populations expressing
467 *Em*AMA1 resulted in significant reductions in oocyst output after challenge with *E. maxima* ($P < 0.05$,
468 one way ANOVA test), ranging from 45 to 55% compared with the non-vaccinated and the challenged
469 groups (Fig. 5A). Pairwise comparisons did not show statistical differences between the three
470 vaccinated groups ($P > 0.05$, one way ANOVA test).

471 Serum samples collected 8 days after final vaccination (day 29) showed variable antibody
472 reactivity against r*Em*AMA1 protein within experimental groups, and no reactivity against native
473 *Em*AMA1 in *E. maxima* solubilised protein extracts, suggesting that the observed reduction in parasite
474 replication after challenge was not mediated by specific humoral responses against *Em*AMA1 (Fig. 5B).
475 Similarly, we did not detect enhanced levels of IFN-γ in serum 8 days after final vaccination (day 29)
476 or 6 days after challenge (day 36), with equally low concentrations across all groups (under 60 pg/ml,
477 $P > 0.05$, one way ANOVA test) (Fig. 5C). At day 29, all groups displayed low levels of serum IL-10 ($P >$
478 0.05, one way ANOVA test) (Fig. 5C) and at 6 days post-challenge (day 36) the average IL-10 serum
479 levels were lower in all groups immunised with transgenic parasites compared with non-vaccinated
480 and challenged birds (Fig. 5C). However this reduction was only significant in groups vaccinated with
481 *Et*[SP2-*Em*AMA1-Ch] and *Et*[SP2-Ch-GPI] parasites ($P < 0.05$, one way ANOVA test), suggesting that the
482 effect is not mediated by expression of *Em*AMA1. A positive correlation was detected between serum
483 IL-10 levels and oocyst output (64%, $P < 0.001$, Pearson correlation test, data not shown).

484

485 *3.6. Sequence diversity for the anticoccidial vaccine candidate EmAMA1 is limited and comparable to*
486 *the E. tenella orthologue*

487 Comparison of 56 *E. tenella* AMA1 coding sequences previously revealed limited antigenic
488 diversity and a largely neutral signature of selection (Blake et al., 2015). Here, comparison of 18
489 *Em*AMA1 coding sequences from parasites sampled in four countries, across three continents,

490 revealed a comparably low level of diversity (Table 4). Specifically, nine *Em*AMA1 cDNA haplotypes
491 were identified but total nucleotide diversity was low and no significant signatures of selection were
492 detected. Analysis of the 1,617 bp coding sequence alignment revealed 10 nucleotide substitutions,
493 of which three were non-synonymous. Substitutions of histidine to leucine (amino acid position 81),
494 isoleucine to methionine (position 133) and glutamic acid to aspartic acid (position 393) were
495 detected, of which the latter was located close to the glutamic acid to valine (position 386)
496 substitution described previously in the *Et*AMA1 amino acid sequence (Blake et al., 2015).
497

498 **4. Discussion**

499 In the present work we have characterised the two most highly expressed AMAs of *E. tenella,*
500 *Et*AMA1 (ToxoDB Accession number ETH00007745) and *Et*AMA2 (ToxoDB Accession number
501 ETH00004860) and demonstrate, to our knowledge for the first time, that vaccination with sporozoite-
502 specific *Et*AMA1, but not merozoite-specific *Et*AMA2, elicits highly significant protection against
503 homologous challenge with *E. tenella*. With this knowledge we went on to establish a proof of concept
504 for the use of *Eimeria* parasites as vaccine delivery vectors for heterologous coccidial antigens,
505 showing that *E. tenella* parasites expressing *Em*AMA1 antigen from *E. maxima* are able to induce
506 partial protection in all the vaccinated birds against heterologous challenge infection with *E. maxima*.
507 A polyclonal mouse serum raised against r*Et*AMA1 detected native *Et*AMA1 in sporulated
508 oocysts and sporozoites, but not in merozoites, and conversely mouse serum against r*Et*AMA2,
509 detected native *Et*AMA2 solely in merozoites. These results agree with previous proteomic and
510 transcriptomic data and confirm that these two EtAMA paralogues are tightly stage-regulated (Lal et
511 al., 2009; Reid et al., 2014). Interestingly a second antibody raised against rEtAMA1 in chickens
512 detected an additional sporozoite protein (Fig. 2B) indicating the potential for cross-reactivity of anti-
513 AMA1 antibodies. This additional protein was not identified, but it could potentially be *Et*AMA3 or
514 *Et*AMA4, both of which are expressed in the sporozoite stage (Table 3). Importantly, we showed that
515 the mono-specific mouse anti-r*Et*AMA1 serum as well as r*Et*AMA1 protein were both efficient at

516 reducing sporozoite invasion of cultured cells, in agreement with previous work (Jiang et al., 2012),
517 whereas anti-r*Et*AMA2 and -r*Et*AMA2 protein had no effect on parasite invasion, again indicating the
518 highly stage-specific nature of the two paralogues. The possession of four AMA paralogs in *T. gondii* is
519 proposed to confer exceptional molecular plasticity at the parasite–host-cell interface (Poukchanski
520 et al., 2013; Lamarque et al., 2014; Parker et al., 2016). In this work, we identified the *EtAMA2* gene
521 in merozoite-derived expressed sequence tags (ESTs) (data not shown) supporting previous
522 observations based on transcriptomic data (Reid et al., 2014), and confirmed that *Et*AMA2 protein is
523 found in merozoites by western blotting and IFAT experiments (Lal et al., 2009). Intriguingly, RNAseq
524 evidence published by others also indicates *EtAMA2* gene transcription in gametocytes, although the
525 precise function of an AMA paralogue in this stage of the parasite is unclear (Walker et al., 2015). All
526 these findings indicate that *E. tenella* parasites harbour stage-specific AMA proteins that could be
527 relevant during specific phases of the parasite cycle. Interestingly, Jiang and colleagues apparently
528 detected *Et*AMA1 protein in merozoites and gametes despite transcriptomic evidence discarding this
529 possibility (Jiang et al., 2012), which could potentially be artefactual and due to their anti-*Et*AMA1
530 serum cross-reacting with alternative *Et*AMA paralogs.

531 The results obtained in in vitro sporozoite inhibition assays were in line with what was
532 subsequently observed when r*Et*AMA1 and r*Et*AMA2 were used to immunise chickens. Birds
533 vaccinated with r*Et*AMA1, but not r*Et*AMA2, produced significantly fewer oocysts after challenge with
534 *E. tenella*, and vaccination with combined r*Et*AMA1 and r*Et*AMA2 did not induce any synergistic effect.
535 Similar promising results have been seen in previous studies where chickens were vaccinated with
536 AMA1 proteins from *E. maxima* (DNA, recombinant protein and Bacille Calmette-Guérin -vectored
537 immunisations) or *E. brunetti* (DNA immunisation) and vaccine efficacy was measured in a variety of
538 ways including improved body weight gains and reduced gut lesions after high dose challenge, or
539 reduced oocyst shedding after low dose challenge (Blake et al., 2011; Li et al., 2013; Hoan et al., 2014).

540 Given their proven ability to induce robust immunoprotective responses, and the availability
541 of established methods for vaccine administration, the use of live recombinant *Eimeria* vectors as

542 streamlined vaccines against coccidia in poultry is highly attractive. In earlier studies we demonstrated
543 that vaccination with *E. tenella* Wis strain parasites expressing the CjaA protein from *Campylobacter*
544 *jejuni* induced significant protection (86-91%) against homologous challenge infection, regardless of
545 the number of immunisations (Clark et al., 2012). Subsequently, we showed that *E. tenella* Wis
546 parasites expressing the vvVP2 protein from infectious bursal disease virus (IBDV), or glycoprotein I
547 from infectious laryngotracheitis virus (ILTV), elicited limited but specific humoral responses when
548 used as vaccines (Marugan-Hernandez et al., 2016). More recently, we carried out extensive work to
549 improve the *Eimeria* vector system by inducing higher expression of the transfected gene and
550 increasing exposure of the transprotein to the host immune system through the use of specific
551 promoters and delivery signals, respectively (Marugan-Hernandez et al., 2017). The promising results
552 obtained with *Et*AMA1 and *Em*AMA1 subunit vaccines prompted us to test the fitness of this new
553 delivery system (*E. tenella* Wis-based) to express the *Em*AMA1 protein from *E. maxima* at high levels
554 and modify the protein trafficking by inducing its secretion and tethering to the sporozoite surface. As
555 expected, all three transgenic populations transcribed the *Em*AMA1-mCherry sequence, expressed
556 the protein at high levels, and displayed differential secretion patterns (Fig. 4). In previous work we
557 showed that *E. tenella* parasites expressing the yellow fluorescent protein (YFPmYFP) can be stabilized
558 after three generations under double selection (Clark et al., 2008). In the present study, the same
559 number of in vivo passages was insufficient to obtain fully fluorescent populations, although all
560 populations displayed distinct increases in the percentage of stable transgenic parasites after three
561 generations (Fig. 4A). We have observed this to be the case for several other co-transfections (data
562 not published), and hypothesise that it could be due to the disruption of the integrated plasmids
563 during sporulation, when meiosis takes place under no drug selection.

564 Previous studies have shown that the immunological activity of AMA1 in *Plasmodium*
565 parasites relies on its correct folding (Hodder et al., 2001). While we did not check whether *Em*AMA1
566 was properly folded in transgenic populations, we assumed that the use of an *E. tenella*-based vector
567 would support appropriate folding of foreign proteins from other *Eimeria* spp., and according to our

568 data this seems to have been the case. We observed similar results in a previous study carried out
569 with birds that received single or trickle vaccinations with *E. tenella* Wis parasites expressing EmAMA1
570 under the control of the *EtAMA1* promoter and displayed a reduction in the oocyst output ranging
571 from 38.4 to 46.9% after challenge with *E. maxima* W (Supplementary Fig. S3). Furthermore, similar
572 cross-strain heterologous protection results were observed when *E. maxima* H strain sporozoites
573 transiently transfected with a bacterial artificial chromosome (BAC) construct carrying the EmAMA1
574 sequence from the *E. maxima* W strain were used as a vaccine (Blake et al., 2011). All these data offer
575 promising prospects for the future development of anti-coccidial vaccines based on *Eimeria* as a
576 vector.

577 Interestingly, all three transgenic populations induced similar levels of heterologous
578 protection after challenge with *E. maxima*, suggesting that the *Em*AMA1 protein is similarly processed
579 by the host immune system regardless of the delivery signal employed. However, sera collected after
580 vaccination were consistently not reactive against the native *Em*AMA1 protein within *E. maxima*
581 protein extracts (Fig. 5B). These findings correlate with our previous study, in which vaccination with
582 transgenic *Eimeria* parasites induced low antibody titres against the transgene (Marugan-Hernandez
583 et al., 2016), and indicate that this vector system may not be suitable when specific humoral responses
584 are needed. The role of the humoral response in conferring resistance to coccidiosis is still debated,
585 but it is clear that antibody titres do not correlate with levels of protection (Lillehoj and Ruff, 1987;
586 Wallach et al., 1992; Dalloul et al., 2003; Lee et al., 2009a, b). Our study supports this view, since all
587 vaccinated groups were able to reduce *E. maxima* replication after challenge despite the absence of
588 reliably detectable levels of antibodies specific for *Em*AMA1.

589 It is well established that *Eimeria* parasites induce significant increases in IFN-γ mRNA levels
590 in the infected tissues (Rothwell et al., 1995; Min et al., 2003), as well as protein levels in serum (Yun
591 et al., 2000) and peripheral blood leucocytes (Breed et al., 1997). However, we detected little or no
592 enhanced serum IFN-γ levels compared to the controls at either day 29 (after four oral vaccinations
593 with transgenic *E. tenella*) or day 36 (6 days after challenge with *E. maxima*). We did not expect to see

594 enhanced levels at day 29, as this was 4 weeks after the primary vaccination, but did expect to see
595 induction of serum IFN-γ at day 36, as this is reported to increase in serum at 8 days post challenge
596 with *E. maxima* (Yun et al., 2000). However, an earlier report did not detect serum IFN-γ until 20 days
597 following primary and serial exposure to *E. maxima* (Yun et al., 2000), thus the timing may vary
598 according to host strain and parasite dose. Similarly, the trickle vaccination did not affect IL-10 serum
599 levels at day 29 compared tocontrols (Fig. 5C), and again this is most likely due to the timing of
600 sampling. In contrast, all challenged birds showed increased serum IL-10 levels at day 36, including
601 birds immunised with transgenic parasites. Expressed serum IL-10 was lower in all immunised birds
602 compared to non-immunised birds, irrespective of exposure to *Em*AMA1. Increased serum IL-10 at
603 day 36 in all challenged birds may be explained by exposure to different *Eimeria* spp. at immunisation
604 and challenge (transgenic *E. tenella* and *E. maxima*), effectively indicating different primary infections.
605 The lower serum IL-10 levels in birds immunised using *Et*[SP2-Ch-GPI] suggests a low level of cross-
606 protection, which was reflected in oocyst output (17% to 27% reduction; Fig. 5B and Supplementary
607 Fig. S3). We also observed that increased serum IL-10 levels may lead to increased oocyst shedding,
608 which agrees with previous findings (Sand et al., 2016), indicating that serum IL-10 levels could be a
609 potential predictive marker of susceptibility to infection (Wu et al., 2016).

610 Previous studies have shown that fecundity of *Eimeria* parasites is reduced as the infective
611 dose increases due to a 'crowding effect', and infection with 250 oocysts is sufficient to induce the
612 highest level of oocyst shedding (Johnston et al., 2001; Williams, 2001). For this reason, measurement
613 of oocyst replication after a low-dose challenge is the most sensitive method available for measuring
614 vaccine efficacy, and is still regarded as a 'gold standard' by vaccine regulators. However, this
615 approach is not suitable for evaluation of factors such as protection against intestinal damage (lesion
616 score) and body weight gain as shown by a recent study, where infections with similar doses of *E.*
617 *maxima* only induced mild lesions and very low body weight losses (Jenkins et al., 2017a). Future trials
618 will be required to evaluate protection against more severe challenge doses.

619 The confirmation of limited genetic diversity between AMA1 alleles for *E. maxima*, with no
620 evidence of significant signatures of selection, mirrors that described previously for *E. tenella* (Blake
621 et al., 2015). Antigenic diversity within parasite populations provides the potential for immune escape,
622 with the greatest likelihood when small numbers of antigens are used in subunit or recombinant
623 vaccines to induce focused immune responses. The low levels of naturally occurring diversity
624 described for *Et*AMA1 have enhanced its candidacy for use in novel anti-coccidial vaccines. While
625 AMA1 is known to be highly polymorphic within populations of some apicomplexans such as
626 *Plasmodium falciparum* (Takala and Plowe, 2009), the low levels of polymorphism and its ability to
627 induce immune protection described for two *Eimeria* spp. suggest that AMA1 is a viable vaccine
628 candidate for all *Eimeria* spp. which infect chickens, and possibly all *Eimeria* spp.

629 Currently the commercial availability of live vaccine formulations is constrained by inherent
630 limitations in the capacity of production of live parasites, which is especially true where attenuated
631 vaccines are produced (Williams, 1998). In the broiler sector, where profit margins are very tight,
632 control measures are still highly dependent on the use of anti-coccidial prophylaxis due to the higher
633 costs of these vaccines (Sharman et al., 2010; Blake and Tomley, 2014). Therefore, there is a need to
634 reduce the cost of the available formulations in the market and make them more attractive for this
635 sector. Herein we show that *Et*AMA1 is an effective vaccine candidate, provide evidence for the first
636 time that genetic complementation of *E. tenella* parasites is a valuable tool to deliver vaccine
637 candidates from other coccidian species which infect poultry, and demonstrate that this vaccine
638 platform is able to induce significant levels of heterologous protection after complementation with
639 *Em*AMA1. Furthermore, we provide a powerful tool to develop new generation vaccines against
640 multiple *Eimeria* spp. as a means to streamline the available formulations in the market, and thus
641 reduce their cost. In this sense, successful genetic complementation of highly prolific and less
642 pathogenic species such as *Eimeria acervulina* would be highly beneficial (Zou et al., 2009).

643

644

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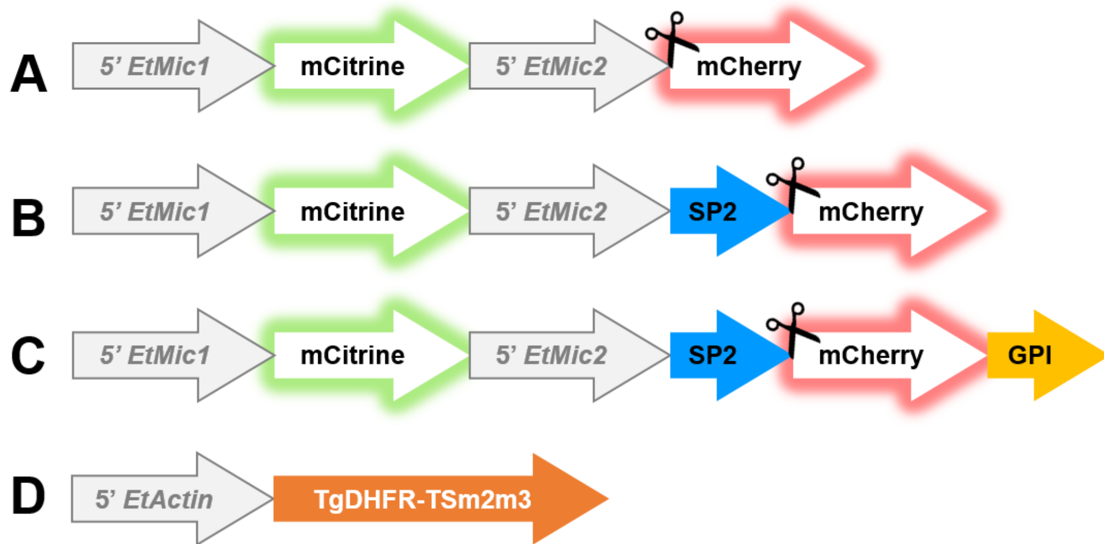
845

846 **Figure legends**



847

848 **Fig. 1.** Simplified representation of the plasmids used for *Eimeria tenella* transfection. (A-C) Core
849 constructs employed to transfect *E. tenella* parasites. Scissors represent the location of the unique
850 *Xba*I restriction site, used for transgene insertion. In transfected parasites, the mCitrine protein is
851 expressed as a cytosolic protein and used to select transgenic parasites by flow cytometry, whereas
852 mCherry, or transgene-mCherry fusion protein, is expressed as a cytosolic protein (with no delivery
853 signals), secreted into the sporocyst cavity (SP2), or secreted and anchored onto the sporozoite
854 surface (SP2+GPI) (Clark et al., 2012; Marugan-Hernandez et al., 2017). (D) A plasmid carrying the
855 mutant *Toxoplama gondii* dihydrofolate reductase–thymidylate synthase (DHFR-TSm2m3) gene was
856 used for in vivo selection of transgenic parasites resistant to pyrimethamine treatment (Clark et al.,
857 2008).

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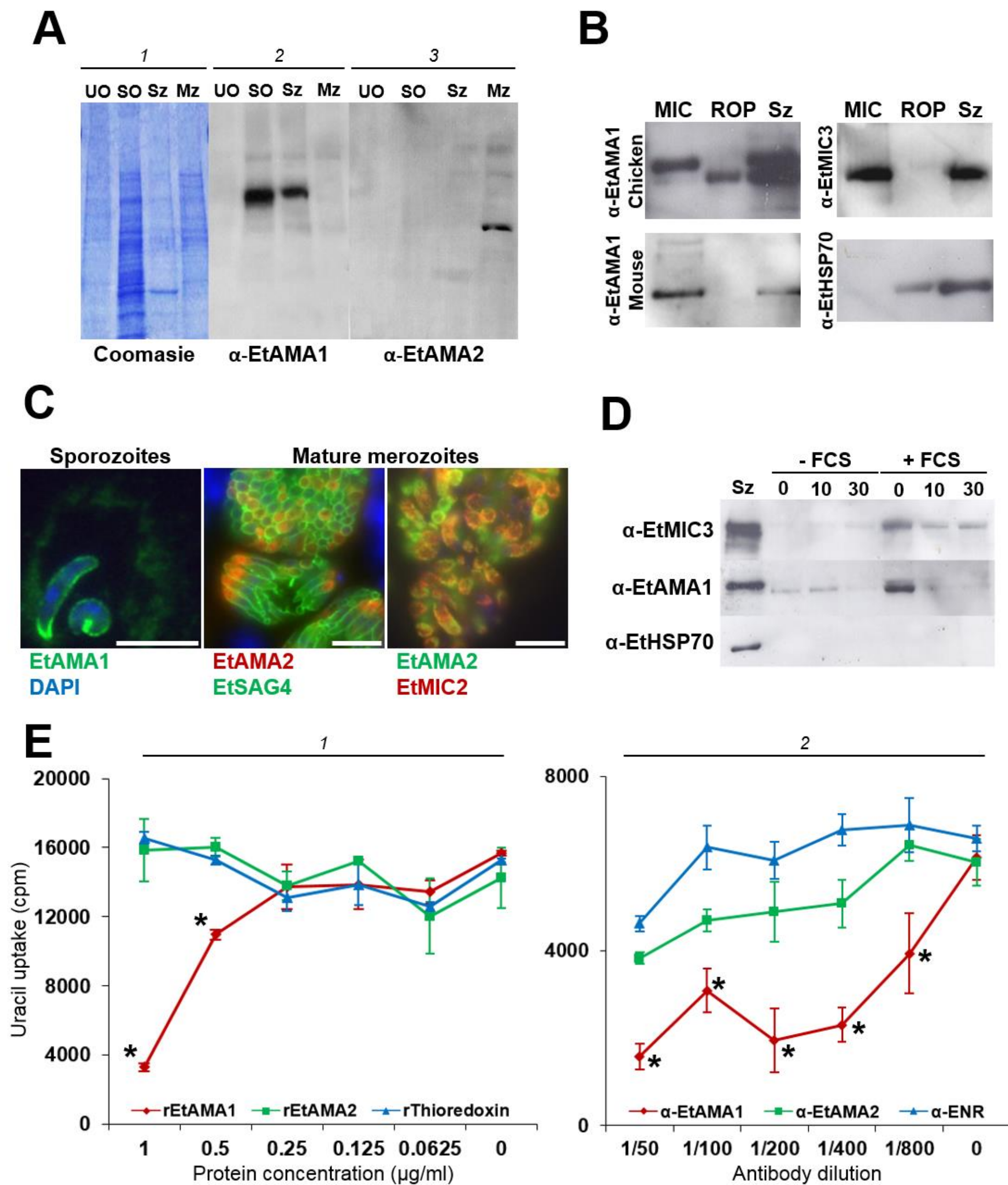

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865 **Fig. 2.** Characterisation of the *Eimeria tenella* apical membrane antigens (EtAMA) 1 and 2. (A) Protein
866 extracts obtained from unsporulated oocysts (UO), sporulated oocysts (SO), sporozoites (Sz) and
867 merozoites (Mz) resolved by SDS-PAGE and stained using Coomasie brilliant blue R-250 (1). Detection
868 of *Et*AMA1 (2) and *Et*AMA2 (3) on the same protein extracts by western blotting using specific
869 hyperimmune mouse sera. *Et*AMA1 was restricted to sporulated oocysts and sporozoites, whereas
870 *Et*AMA2 was only found in merozoites. (B) Detection of *Et*AMA1 on micronemes (MIC), rhoptries (ROP)

871 and sporozoites (Sz) by western blotting. Mouse antiserum recognised a single microneme protein
872 (~63 kDa). Antibodies raised against the *E. tenella* microneme protein 3 (*Et*MIC3) and the *E. tenella*
873 heat shock protein 70 (*Et*HSP70) confirmed that microneme fractions were not contaminated with
874 rhoptries, and vice versa. (C) Detection of *Et*AMA1 on sporozoites and *Et*AMA2 on mature second
875 generation merozoites by IFAT. Some *Et*AMA2 sections were counterstained with an antibody against
876 the *E. tenella* surface antigen 4 (*Et*SAG4) to delineate the sporozoites, and some others with an
877 antibody against the *E. tenella* microneme protein 2 (*Et*MIC2) to delineate the micronemes. *Et*AMA1
878 and *Et*AMA2 showed apical localisations within the zoites. *Et*AMA2 partially co-localised with *Et*MIC2
879 in merozoites. Bars represents 10 µm. (D) Detection of *Et*AMA1 on sporozoites (Sz) and sporozoite
880 secreted fractions (0-30) by western blotting with or without treatment with FCS (-/+ FCS) and
881 collected at 0, 10 or 30 min post-incubation. A low level of *Et*AMA1 secretion was constitutive, but
882 secretion was strongly induced after FCS supplementation. *Et*MIC3 detection was used as positive
883 control of secretion, while *Et*HSP70 detection was used to show the absence of sporozoite lysis. (E) In
884 vitro sporozoite inhibition assays using recombinant (r) *Et*AMA1 and *Et*AMA2 (1) or specific anti-sera
885 (2). All the experiments were done in triplicate. The recombinant thioredoxin protein (rThioredoxin)
886 and a mouse antibody against parasite enolyl reductase (ENR) were included as control treatments
887 for each respective protocol. Only treatment with r*Et*AMA1 or anti-*Et*AMA1 serum induced a
888 significant reduction in parasite replication. ***** indicates statistical differences ($P<0.05$).
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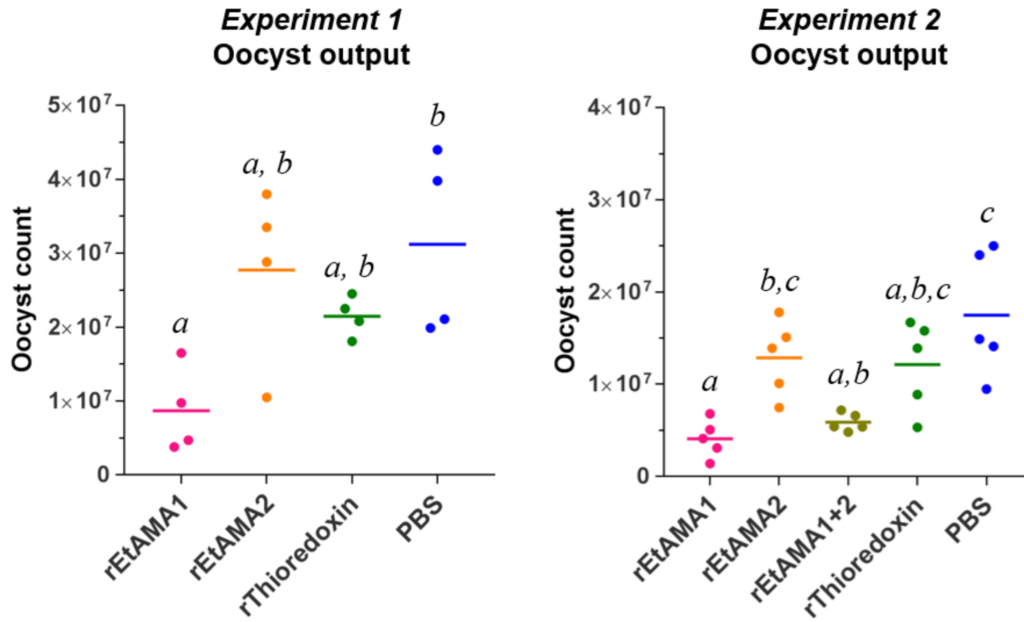

897

898 **Fig. 3.** Immunising ability of recombinant (r) *Eimeria tenella* apical membrane antigen (EtAMA) 1,
899 rEtAMA2 and their combination against challenge with *E. tenella* H parasites (Experiment 1, *n*=4;
900 Experiment 2, *n*=5). In each experiment a group of birds was vaccinated with the Trx-tag present in
901 the recombinant proteins (rThioredoxin), and an additional group was mock vaccinated using PBS.
902 Dots illustrate individual oocyst counts and bars indicate average values. Only birds vaccinated with
903 rEtAMA1, alone or in combination, displayed a significant reduction in oocyst shedding. Groups
904 marked with different letters were significantly different (*P*<0.05).

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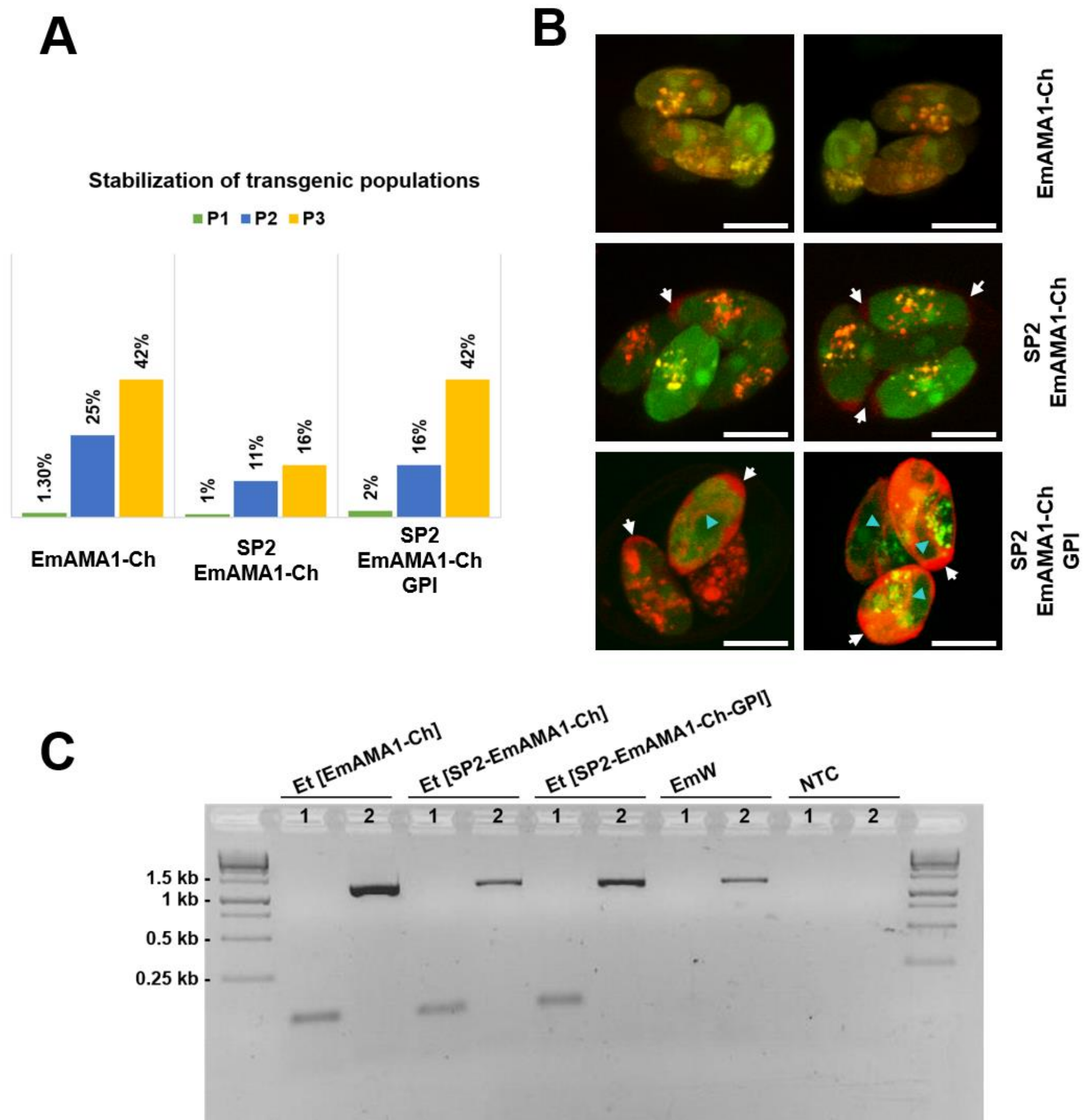

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914 **Fig. 4.** Characterisation of the three different populations of *Eimeria tenella* expressing the *Eimeria*
915 *maxima* apical membrane antigen 1 (*Em*AMA1) fused to mCherry (Ch). (A) Stabilization of transgenic
916 populations by successive in vivo passages. Percentages indicate the total proportions of transgenic
917 oocysts passage by passage (P1 to P3). (B) Fluorescent patterns observed by confocal microscopy in
918 stable transgenic populations of *E. tenella* oocysts expressing the *Em*AMA1-Ch protein. mCitrine was
919 always observed in the cytosol for all the populations, whereas mCherry was observed in the cytosol
920 (*Em*AMA1-Ch, with no delivery signals), secreted into the sporocyst cavity (SP2-*Em*AMA1-Ch, white
921 arrows), or secreted into the sporocyst cavity (white arrows) and anchored onto the sporozoite
922 surface (SP2-*Em*AMA1-Ch-GPI, white and blue arrows respectively). In all the populations both

923 mCitrine and mCherry were also present within cytosolic aggregates and in some cases were co-
924 localised. Bars represent 10 µm. (C) Detection of *Em*AMA1-Ch transcripts in cDNA isolated from stable
925 transgenic populations by reverse transcription (RT)-PCR. Presence of genomic DNA contamination
926 was discarded by the use of *EtActin* primers that amplify a region coded between two adjacent exons
927 (~0.1 kb, lane 1). A single band of ~1.3 kb was obtained from all three populations when specific
928 primers targeting *Em*AMA1 were employed (lane 2). cDNA isolated from *Eimeria maxima* W strain
929 (EmW) was included as a positive control reaction. NTC is the non-template control.
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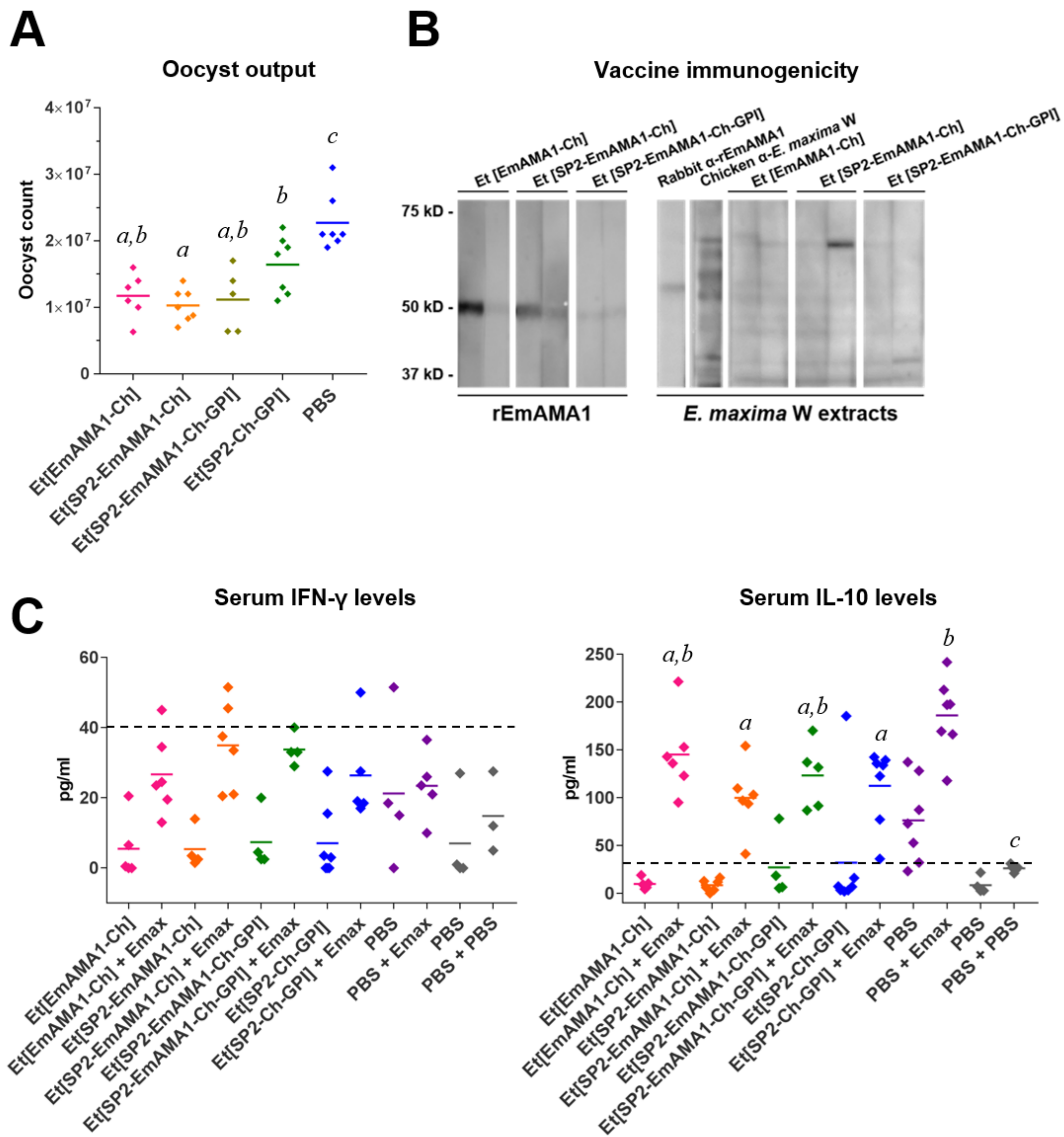

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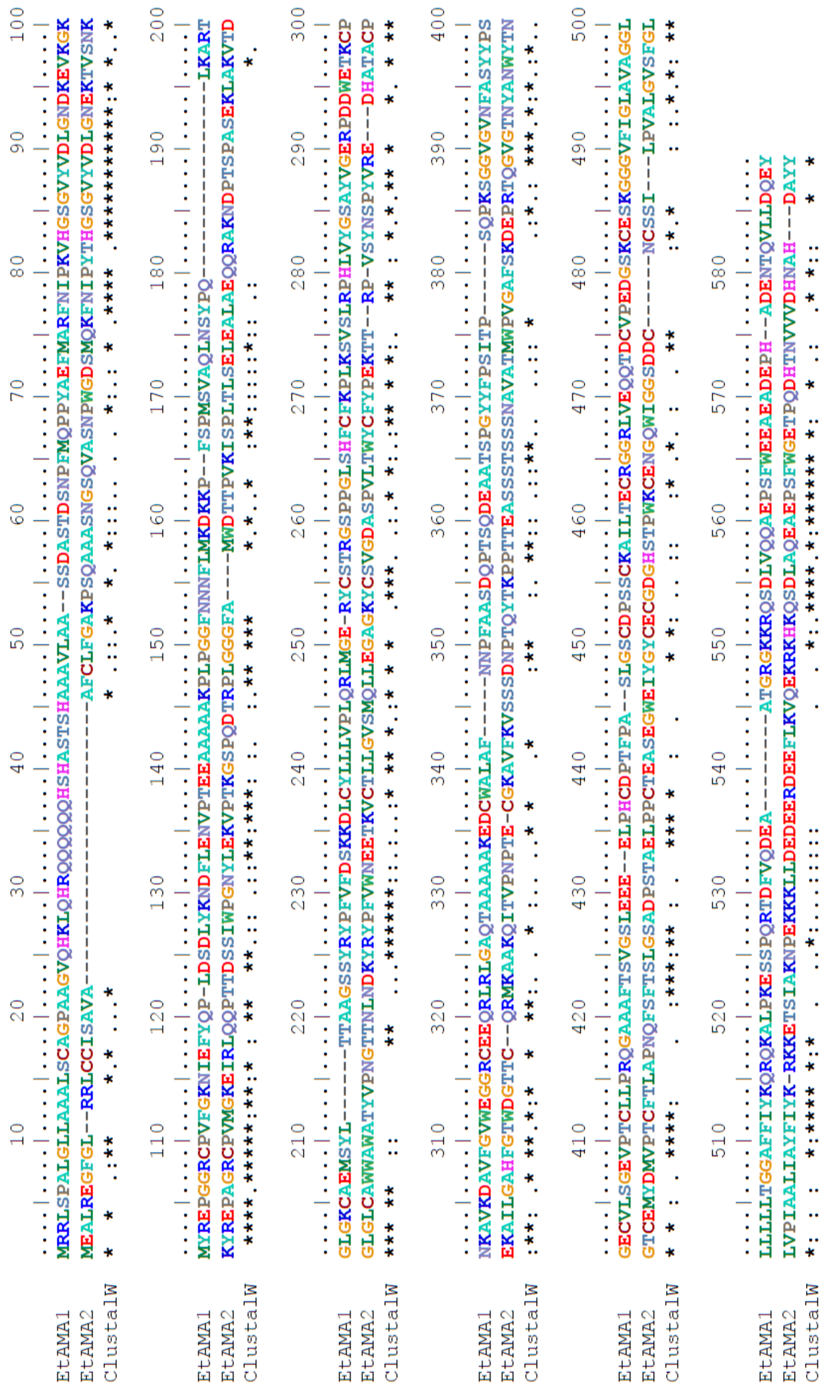
950 **Fig. 5.** Immunogenicity and efficacy of *Eimeria tenella* parasites expressing the *Eimeria maxima* apical
951 membrane antigen 1 (*Em*AMA1) under the control of different delivery signals. (A) Immunising ability
952 of *Eimeria tenella* Wis strain parasites expressing *Em*AMA1 protein fused to mCherry (Ch) and
953 expressed under the control of different delivery signals against challenge with *E. maxima* W strain
954 parasites. SP2, signal peptide from the *E. tenella* microneme 2 protein (*Et*MIC2); GPI,
955 glycosylphosphatidylinositol -anchor from the *E. tenella* surface antigen 1 (*Et*SAG1). A group of birds
956 was vaccinated with an empty vaccine vector (the most complete, carrying the SP2 and GPI signals),
957 and an additional group was not vaccinated (PBS). Diamonds illustrate individual oocyst counts and

bars indicate average values. All the birds vaccinated with *E. tenella* parasites expressing *Em*AMA1-Ch displayed a significant reduction in oocyst shedding, regardless of the delivery signal included. Groups marked with different letters were significantly different ($P < 0.05$, one-way ANOVA test). (B) IgY responses induced after vaccination with *Et*[*Em*AMA1-Ch], *Et*[SP2-*Em*AMA1-Ch], and *Et*[SP2-*Em*AMA1-Ch-GPI] by western-blot. Recombinant *Em*AMA1 (r*Em*AMA1) and *E. maxima* W protein extracts were separated by SDS–PAGE and probed with sera collected from vaccinated birds. Some birds strongly recognized the recombinant protein, but high variability within groups was observed. In contrast, none of the birds recognized native *Em*AMA1 from oocyst extracts, which could be detected with rabbit anti-r*Em*AMA1. (C) Quantification of IFN-γ and IL-10 serum levels on day 29, (8 days after the final vaccination with transgenic parasites) or at day 36 (6 days after challenge with *E. maxima* W, marked with '+ Emax' on the axis labels). Diamonds illustrate individual serum concentrations and bars indicate average values. Letters on the right plot indicate significant differences between groups after the challenge infection ($P<0.05$, one-way ANOVA test). Dotted lines depict threshold values for IFN-γ (40 pg/ml) and IL-10 (32 pg/ml) tests.

984 **Supplementary figure legends**



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986 **Supplementary Fig. S1.** Sequence alignment of the *Eimeria tenella* apical membrane antigens 1
987 (*Et*AMA1, ToxoDB Accession number ETH_00007745) and 2 (*Et*AMA2, ToxoDB Accession number
988 ETH_00004860). Asterisks (*) indicate fully conserved residues. Colons (:) and periods (·) indicate
989 conservation between groups of strongly or weakly similar properties, respectively.

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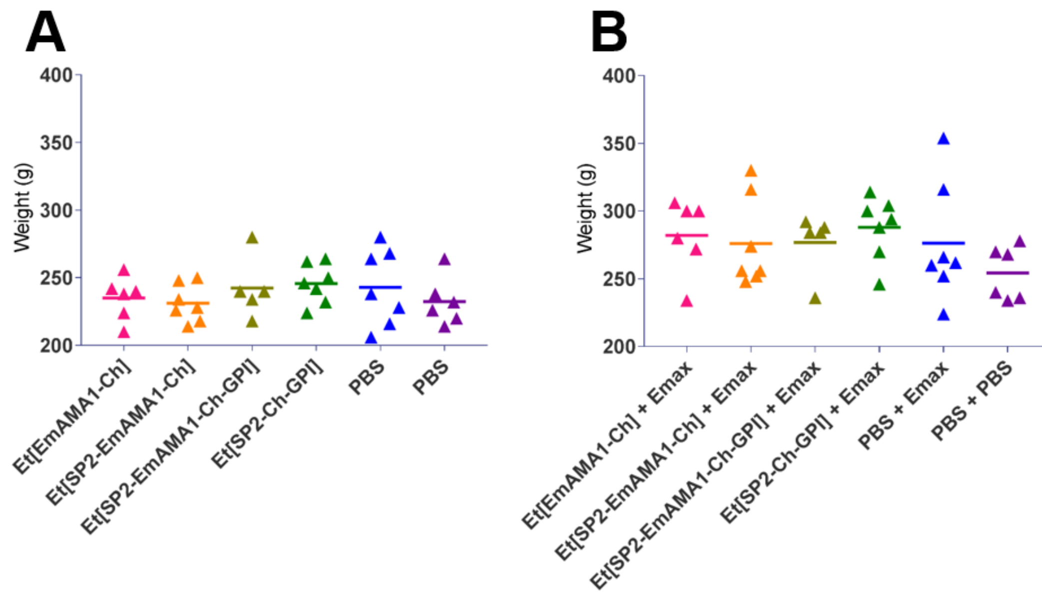

991

992 **Supplementary Fig. S2.** Safety of transgenic vaccines based on genetically complemented *Eimeria*
993 *tenella* parasites expressing the *Eimeria maxima* apical membrane antigen 1 (*Em*AMA1).fused to
994 mCherry (Ch) and under the control of different delivery signals (SP2, signal peptide from the *E. tenella*
995 microneme 2 protein (*Et*MIC2); GPI, glycosylphosphatidylinositol -anchor from the *E. tenella* surface
996 antigen 1 (*Et*SAG1)). Individual weights from birds vaccinated with transgenic parasites expressing
997 *Em*AMA1 (*Et*[*Em*AMA1-Ch], *Et*[SP2-*Em*AMA1-Ch], *Et*[SP2-*Em*AMA1-Ch-GPI]), birds vaccinated with the
998 empty vector (*Et*[SP2-Ch-GPI]), and non-vaccinated birds (PBS) 7 days after the final vaccination (A) or
999 6 days after challenge infection ('+ Emax', B). Triangles illustrate individual weights and bars indicate
1000 average values. No differences were found between groups (*P*>0.05).

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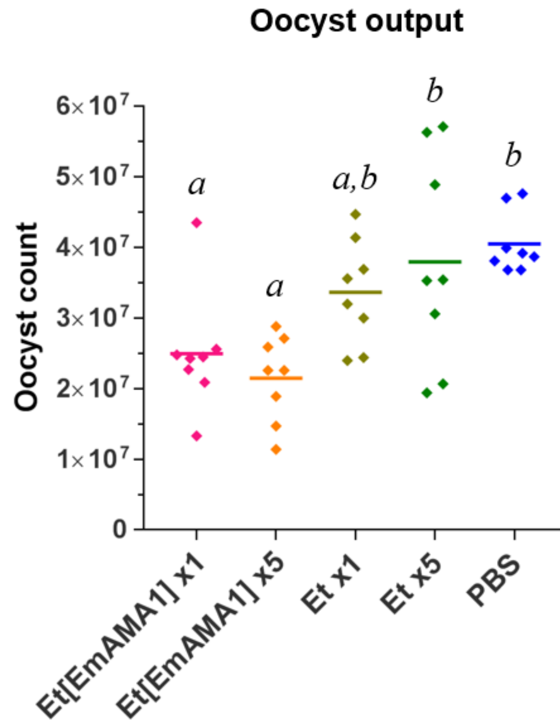

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1009 **Supplementary Fig. S3.** Immunising ability of *Eimeria tenella* Wis strain parasites expressing the

1010 *Eimeria maxima* apical membrane antigen 1 (*Em*AMA1) under the control of the *E. tenella* AMA1

1011 promoter (*Et*[*Em*AMA1]). A total of 40 3 weeks old Light Sussex chickens were split into five groups of

1012 eight in independent wire-floored cages and housed within, coccidia-free conditions. Birds were dosed

1013 by oral gavage with *Et*[*Em*AMA1] oocysts one (x1) or five times (x5), or with wild type *E. tenella* Wis

1014 strain (*Et*) oocysts one (x1) or five times (x5), or with PBS. Three weeks after the last vaccination, birds

1015 from all groups were challenged with 300 oocysts of the *E. maxima* W strain, and individual faecal

1016 samples were collected from 5 to 9 days post-challenge to determine the total oocyst output. Dots

1017 illustrate individual oocyst counts and bars indicate average values. All the birds vaccinated with

1018 *Et*[*Em*AMA1] parasites displayed a significant reduction in oocyst shedding, regardless of the number

1019 of doses received. Groups marked with different letters were significantly different (P<0.05).

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1022 **Table 1.** Experimental design for the vaccine trials using recombinant (r) *Eimeria tenella* apical

1023 membrane antigens 1 (*Et*AMA1) and 2 (*Et*AMA2).

Experiment	*n*	Vaccine	Immunisation protocol	Age at *Et*H challenge
1	4	PBS	500 µl at 1, 3 and 5 wks	7 wks
	4	rThioredoxin (protein tag)	100 µg at 1, 3 and 5 wks	7 wks
	4	r*Et*AMA1	100 µg at 1, 3 and 5 wks	7 wks
	4	r*Et*AMA2	100 µg at 1, 3 and 5 wks	7 wks
2	5	PBS	500 µl at 1, 3 and 5 wks	7 wks
	5	rThioredoxin (protein tag)	100 µg at 1, 3 and 5 wks	7 wks
	5	r*Et*AMA1	100 µg at 1, 3 and 5 wks	7 wks
	5	r*Et*AMA2	100 µg at 1, 3 and 5 wks	7 wks
	5	r*Et*AMA1 + r*Et*AMA2	100 µg at 1, 3 and 5 wks	7 wks

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1026 Wks, weeks; *Et*H, *Eimeria tenella* Houghton strain.

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1028 **Table 2.** Experimental design for the vaccine trials using *Eimeria tenella* parasites (*Et*) expressing the

1029 *Eimeria maxima* apical membrane antigen 1 (*Em*AMA1).fused to mCherry (Ch) and under the control

1030 of different delivery signals (SP2, signal peptide from the *E. tenella* microneme 2 protein (*Et*MIC2);

1031 GPI, glycosylphosphatidylinositol -anchor from the *E. tenella* surface antigen 1 (*Et*SAG1)).

Group	*n*	Vaccine	Immunisation protocol	Age at *Em*W challenge (days)
1	7	*Et*[*Em*AMA1-Ch]	100 oocysts at day 3	30
2	7	*Et*[SP2-*Em*AMA1-Ch]	500 oocysts at day 9	30
3	7	*Et*[SP2-*Em*AMA1-Ch-GPI]	3,000 oocysts at day 15	30
4	7	*Et*[SP2-GPI], empty vector	5,000 oocysts at day 22	30
5	7	PBS	PBS at days	30
6	7	PBS	3, 9, 15 and 22	No challenge

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1033 *Em*W, *Eimeria maxima* Weybridge strain.

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Table 3. *Eimeria* apical membrane antigen (*AMA1-4*) gene identities (ID), transcription profiles and evidence of selective pressure (mean Ka/Ks ratios for six *Eimeria* spp. which infect chickens compared to *Eimeria tenella*). *Eimeria tenella* gene-specific transcription profiles are presented as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) normalised against average FPKM for the full relevant dataset.

	E. maxima gene ID	*E. tenella* gene ID	*E. tenella* transcription profiles (FPKM)								Selective pressure?
			UO_1[c]	UO_2[c]	SO[c]	Sz[c]	Sz[d]	Mz2[c]	Mz2[d]	Gam[d]	Mean Ka/Ks[c]
AMA1	FN813221[a]	ETH_00007745[b]	0.010	0.006	6.89	25.47	33.67	0.028	0.169	0.057	0.12
AMA2	EMWEY_00006480[b]	ETH_00004860[b]	0.000	0.000	0.041	0.015	0.029	4.21	0.510	4.612	na
AMA3	?	ETH_00017730[b]	0.035	0.031	2.64	4.16	16.05	0.005	0.026	0.074	na
AMA4	EMWEY_00022320[b]	ETH_00013620[b]	0.001	0.002	2.58	2.39	2.60	1.97	2.08	3.04	0.11

UO, unsporulated oocyst; SO, sporulated oocyst; Sz, sporozoite; Mz2, second generation merozoite; Gam, gametocyte.

[a]GenBank Accesion Number.

[b]ToxoDB Accesion Number.

[c]Data derived from Reid et al. (2014).

[d]Data derived from Walker et al. (2015).

1060 **Table 4.** Summary of genetic diversity for the *Eimeria maxima* and *Eimeria tenella* AMA1
1061 orthologous coding sequences. Data for *E. tenella* are reproduced from Blake et al. (2015).

	E. maxima	*E. tenella*
n	18	56
Continents sampled	3	5
S	10 (9)	13 (13)
dN	3	10
dS	7	3
K	3.49	4.04
π Jukes Cantor	0.0028	0.0032
H	9	8
Hd	0.922	0.771
Tajima's D	0.722 (ns)	1.259 (ns)
Fu and Li's D*	0.953 (ns)	1.513 (0.05)
Fu and Li's F*	1.026 (ns)	1.688 (0.05)

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1063 *n,* the number of sequences tested; S, the number of variant sites detected, with the number of
1064 parsimony-informative variant sites shown in parentheses; dN, the number of non-synonymous
1065 variant sites; dS, the number of synonymous variant sites; k, the average number of pairwise
1066 differences; π, nucleotide diversity, calculated with the Jukes Cantor correction; H, the number of
1067 sequence haplotypes detected; Hd, the haplotype diversity; ns, not significant.

1068 Tajima's D and Fu and Li's D* and F* tests were used to assess the extent or neutrality of signatures
1069 of selection with significance (*P*) shown in in parentheses where relevant.