Cell mediated and innate immune responses in pigs following vaccination and challenge with *Toxoplasma* parasites

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17 Abstract

18 *Toxoplasma gondii* has a worldwide distribution and can infect almost all warm blooded animals
19 including pigs and humans. This study aims to examine the immune responses induced in pigs
20 following vaccination (live S48 tachyzoites) and/or challenge with *T. gondii* oocysts, through the
21 examination of changes in levels of transcription in CD4, CD8α, IFN-γ, IL-12p35, CXCR3, MyD88.
22 The experiment involved four groups of animals; pigs in group 1 (Challenged) (Chal) were
23 challenged orally with ($1x10^3$ oocysts) on day 28 of the experiment. Pigs in group 2 (Vaccinated
24 /Challenged) (Vac/Chal) were vaccinated (S48 isolate tachyzoites) on day 0, then challenged on
25 day 28. The group 3 (Vaccinated) (Vac) animals were vaccinated (S48 isolate tachyzoites) on day
26 0 of the experiment. Finally the group 4 (control) pigs remained non-vaccinated and non-
27 challenged. All animals were culled 6 weeks post challenge. At post mortem samples of
28 retropharyngeal lymph node (RLN), mesenteric LN (MLN) and spleen were collected, RNA was
29 extracted and cDNA synthesised. The results showed significant increases in IFN-γ expression in
30 samples from groups 1 (Chal) and 2 (Vac/Chal) (RLN) and groups 1, 2 and 3 (Vac) (spleen) and in
31 MyD88 expression (RLN) in samples from groups 1, 2 and 3 compared to the group 4 (control)
32 animals. Significant increases were also observed in CD8α expression in group 1 (Chal) (RLN) and
33 groups 1 and 2 (Vac/Chal) (RLN and MLN) compared against group 4 (control) and group 3 (Vac)
34 respectively. Conversely, significant down regulation of CD4 and/or IL-12p35 transcription was
35 found in at least one sample from groups 1 (Chal), 2 (Vac/Chal) and 3 (Vac) compared to group 4
36 (control) pigs. This study demonstrates that cell mediated and innate immune responses are
37 generated in pigs following exposure to *T. gondii* parasites (oocysts or tachyzoites), key amongst
38 them appear to be IFN-γ, MyD88 and CD8α.

39

40 Key words

41 *Toxoplasma gondii*, porcine, vaccination, cell mediated immunity

1. Introduction

Toxoplasma gondii (the causative agent of toxoplasmosis) has a worldwide distribution and is capable of infecting almost all warm blooded animals, including humans, cattle, sheep and pigs (Dubey, 2008). Humans can become infected with sporulated *Toxoplasma* oocysts through the ingestion of contaminated food, water and soil (Frenkel et al., 1970). However, the ingestion of viable tissue cysts in raw or undercooked meats is also considered a major route of infection (Dubey, 1994). Should a primary human *Toxoplasma* infection occur during pregnancy, the parasite can be transplacentally transmitted from mother to foetus, which can result either in a miscarriage (Cook et al., 2000) or in neurological lesions in surviving foetuses, which can have severe lifelong consequences for the child (Jones et al., 2001).

A commercial anti-*T. gondii* vaccine (S48 Toxovax®) is available for use in sheep to protect against abortion. This vaccine has been shown in experimentally vaccinated and subsequently challenged animals not only to protect against abortion but also to reduce the numbers of parasites detectable in host tissues (Burrells et al., 2015; Katzer et al., 2014). A vaccine that could reduce the parasite burdens in pig tissues would be highly desirable, as undercooked infected pork is considered a major route of infection in humans (Dubey, 2008). However, there are currently no commercially available anti-*Toxoplasma* vaccines for use in pigs. A number of experimental vaccine approaches have been tried, these include the use of temperature sensitive mutant tachyzoites (TS-4) and tachyzoite rhoptry proteins incorporated into immunostimulating complexes (ISCOM) adjuvant, both of these vaccines significantly reduced, but did not completely prevent tissue cyst formation (Garcia et al., 2005; Pinckney et al., 1994), indicating that a vaccine could reduce the parasite burden in tissues, resulting in safer meat for human consumption.

Interferon gamma (IFN-γ) is known to be involved in protection against many intracellular pathogens, including *T. gondii* (Kringel et al., 2004). Cellular responses involving CD8+ T-cells and IFN-γ (Solano Aguilar et al., 2001), along with innate immunity and humoral responses have also been shown to be involved in protection against *T. gondii* tissue cyst formation in pigs (Wang et al., 2013).

The present experiment aims to examine the immune responses generated in spleen, mesenteric lymph node (MLN) and retropharyngeal lymph node (RLN) samples collected from pigs following a vaccination with live S48 tachyzoites and a challenge with *T. gondii* oocysts (M4 isolate) as part of an experiment to determine whether vaccination is able to reduce tissue cyst formation in vaccinated animals. Differences in levels of gene transcription of CD4, CD8α, IFN-γ, IL-12p35, CXCR3, MyD88 were analysed comparing vaccinated, challenged and control animals. The markers and cytokines were chosen as they represented an overview of key cell types (CD4, CD8α (T-cells) and NK) (Mair et al., 2013) and cytokines (IFN-γ and IL-12) (Solano Aguilar et al., 2001) as well as the innate immune response (MyD88) (Dendritic cells) (Scanga et al., 2002) which are known to be important in protection against *Toxoplasma,* however there is currently little information available about their roles in pigs following vaccination and challenge with the parasite.

2. Materials and Methods

2.1. Animals, vaccination and challenge

A total of 18 mixed gender pigs (*Sus scrofa*) (Large White/Landrace cross bred) were divided into 4 experimental groups. All samples were collected from the same animals as previously described by Burrells et al., (2015). In brief animals in group 2 (Vac/Chal) (n = 5 each) were

vaccinated with $1.2x10^5$ S48 *T. gondii* tachyzoites on day 0 of the experiment. Four weeks later (day 28 of the experiment) the animals in groups 1 (Chal) and 2 (n = 5 each) were orally challenged with 10^3 M4 isolate sporulated *T. gondii* oocysts (Burrells et al., 2015). The group 3 (Vac) (n = 5) animals were vaccinated (S48 isolate tachyzoites) on day 0 of the experiment. Finally the group 4 (n = 3) (control) animals were left unvaccinated and unchallenged. All animals were culled 6 weeks post challenge (day 70 of the experiment). At post mortem examination samples of spleen, MLN and RLN were collected and snap frozen on Dry ice, then stored at -80 ^{o}C until processing for RNA extraction and cDNA synthesis.

The samples were selected as they are effective in different parts of the body, the spleen filters large quantities of blood so will collect parasites/parasite antigens in the circulatory system, the MLN drains the intestines, while the RLN is one of the lymph nodes that drains the brain/CNS, so is a good indicator for parasite dissemination.

2.2. RNA extraction and cDNA synthesis

Frozen samples of spleen, RLN and MLN (approx 1 g each) were processed for RNA extraction as previously described (Bartley et al., 2013). Briefly; following homogenisation, the samples were processed through phenol/chloroform phase separation to RNA. The final RNA pellet was resuspended in 200 µl of RNase free water. The concentration of RNA was determined by spectrophotometry (Nanodrop ND1000). Samples were then stored at -80 ^{o}C prior to cDNA synthesis and SYBR green qPCR.

2.3. Synthesis of cDNA from RNA samples

The method used to reverse transcribe cDNA from RNA has been previously described (Bartley et al., 2013). Briefly, following the manufactures instructions, a commercially available high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) was used. Following reverse transcription the cDNA was diluted to 5 ng/µl (400 µl) in DNase/RNase free water and stored at 4^{o}C prior to SYBR green qPCR analysis.

2.4. SYBR green qPCR analysis of cellular immune responses of pigs following vaccination and challenge with *T. gondii*.

To examine changes in levels of transcription that occur during a *T. gondii* infection in pigs, primers were designed against a number of cell surface markers and cytokines involved in the immune response. These included the T-cell cell surface markers CD4 and CD8α, the Th1 type cytokines IFN-γ and IL-12, the NK cell marker CXCR3 and the adaptor protein MyD88, which is involved in TLR function (Table 1). All samples were analysed by SYBR green PCR (Bartley et al., 2013) in triplicate using Fast SYBR green master mix (Applied Biosystems, Carlsbad, CA, USA). Analysis was performed using the standard reaction conditions suggested by the manufacturer (2 min at 50 °C, 10 min at 95 °C, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min). The melt curves of the PCR products was acquired through a step wise increase in temperatures from 55 to 95 °C (ABI prism 7500 using sequence detection software (SDS) (v1.2.3) (Applied Biosystems, Carlsbad, CA, USA).

To determine changes in the levels of transcription the ΔCt of each of the analytes was determined, the data was normalised against the ΔCt value for the HPRT (Hypoxanthine Phosphoribosyltransferase) gene, creating a ΔΔCt. This allowed for variations in sample quality. The mean data illustrated in Figs. 1A-F are the differences between the level of transcription of

the genes of interest and the level of HPRT transcription, so for genes where there are high levels of transcription (lower Ct values) may result in a potentially negative value being displayed, as the level of transcription for the genes of interest is greater than the level of transcription of HPRT.

2.5. Statistical analysis

The mean differences in normalised ΔΔCt values from each analyte for each tissue were compared using a one way analysis of variance (ANOVA). All calculations were performed using the minitab software (v17.1.0).

3. Results

The aim of this study was to examine changes in the levels of transcription for a number of components of the immune response in pigs following vaccination and/or challenge with *T. gondii* parasites, to help understand some of the immune mechanisms involved in controlling tissue cyst formation in vaccinated animals. The components investigated included the Th1 type cytokines IFN-γ and IL-12 (p35), the T-cell surface markers CD4 and CD8α, CXCR3 which is associated with NK cells and myeloid differentiation primary response gene 88 (MyD88) an adaptor protein, which is involved in TLR responses.

A Ct value was not always available for all samples, as for some of the analytes examined the levels of transcription were below the detection threshold of the PCR.

3.1. IFN-γ

Significant increases in mean levels of transcription of IFN-γ were seen in the RLN ($P = 0.005$ and $P = 0.006$) from group 1 (Chal) and group 2 (Vac/Chal) and spleen ($P < 0.001$, $P < 0.001$, $P < 0.001$) from group 1, group 2 and group 3 (Vac) respectively, when compared against the mean of group 4 (control) animals (Fig. 1A). The levels of IFN-γ transcription in MLN are comparable for groups 1, 2 and 3, to those seen in the RLN and spleen. Unfortunately, no statistical comparisons could be made for the MLN as the level of IFN-γ transcription of the group 4 animals was below the detection threshold of the PCR. No other statistical differences were observed in the levels of transcription when comparing the IFN-γ data from groups 1, 2 and 3.

3.2. CD8α

When the mean levels of CD8α transcription were compared, the pigs in group 1 (Chal), demonstrated significantly increased transcription of CD8α in RLN (P = 0.017) compared to the group 4 (control) animals (Fig. 1B). Group 1 (Chal) also demonstrated increased transcription of CD8α in the MLN compared to the group 4 (control) animals but these differences were not statistically significant

Groups 1 (Chal) and group 2 (Vac/Chal) demonstrated higher levels of CD8α transcription in all samples than group 3 (Vac). These differences were statistically significant in RLN (P = 0.001, P = 0.05), MLN (P = 0.015, P = 0.012) respectively. For the spleen, group 1 produced significantly higher levels of CD8α transcription than group 3 (P = 0.038).

3.3. MyD88

Significantly increased mean levels of transcription of MyD88 were seen in RLN (P = 0.011, P = 0.025 and P = 0.021)) samples from group 1 (Chal), group 2 (Vac/Chal) and group 3 (Vac) and in MLN (P = 0.031 and P = 0.05) for group 2 and 3 compared to the mean of the group 4 (control) animals (Fig. 1C). Groups 1, 2 and 3 also produced higher mean levels of transcription of MyD88 from the spleen compared to group 4; however these differences were not statistically significant. There were no statistical differences observed when comparing the levels of MyD88 transcription (RLN, spleen and MLN) from groups 1, 2 and 3.

3.4. CXCR3

The levels of transcription CXCR3 were higher in the RLN, spleen and MLN samples from the animals in group 1 (Chal) and group 2 (Vac/Chal) compared to the group 4 (control) animals (Fig. 1D). However, the differences observed were only statistically significant (P = 0.009) when comparing the data from group 2 MLN against the group 4 control animals. When the levels of CXCR3 transcription in groups 1, 2 and 3 were compared, significantly increased levels of CXCR3 transcription were observed in the RLN from group 2 (P = 0.045) and the MLN from group 1 (P = 0.037) compared to group 3.. The results show that levels of transcription of CXCR3 are generally increased in pigs that received a challenge with parasites suggesting a role for NK cells in a protective immune response against *T. gondii* in pigs

3.5. IL-12 (p35)

The RLN samples from the group 4 (control) animals produced significantly higher mean levels (P <0.001) of IL-12 (p35) transcription than group 1 (Chal) (Fig. 1E), while the MLN samples from group 4 also produced significantly higher levels of transcription of IL-12 than group 2 (Vac/Chal) and group 3 (Vac) animals (P = 0.034, P = 0.025 respectively) (Figure 1E). When comparing the IL-12 data from groups 1, 2 and 3, group 1 was seen to produce significantly (P = 0.014) higher levels of IL-12 than group 2 in MLN. Levels of transcription of IL-12 (p35) are generally lower following exposure to *T. gondii* parasites and appear to be significantly down regulated in some instances.

3.6. CD4 T cells

206 The group 4 (control) animals demonstrated significantly higher mean levels of CD4 gene
207 transcription in RLN compared to group 3 (Vac) (P = 0.035) as well as in the spleen (P = 0.022, P =
208 0.004 respectively) and MLN (P = 0.05 and P = 0.015 respectively) when compared to group 2
209 (Vac/Chal) and group 3 (Vac). Interestingly significant differences were also observed when the
210 levels of transcription of CD4 from the samples of groups 1, 2 and 3 were compared. Levels of
211 CD4 transcription in the spleen of group 1 (Chal) and group 2 (Vac/Chal) were significantly higher
212 (P = 0.003 and P = 0.031, respectively) than group 3 (Vac). While CD4 transcription in the MLN
213 sample from group 2 was also significantly higher (P = 0.02) than group 3.

214

215 4. Discussion

216 The main purpose of this study was to examine the immune responses in pigs that were
217 vaccinated and/or challenged with *T. gondii*. This was done by examining changes in the levels of
218 transcription of a number of immunological analytes in pigs following exposure to *T. gondii*
219 parasites. The data from this study has shown that following either vaccination and/or challenge
220 with *T. gondii* parasites (groups 1, 2 and 3) lymph nodes (RLN and MLN) and spleen samples from
221 pigs demonstrate increases in transcription of the T-cell surface marker CD8α, the natural killer
222 (NK) cell marker CXCR3, the Th1 type cytokine interferon-γ (IFN-γ) and the adaptor protein
223 MyD88, which is involved in toll like receptor (TLR) function. These data, combined with the
224 previously described humoral response data (anti-*Toxoplasma* IgG) in these pigs (Burrells et al.,
225 2015) demonstrate the production of cellular, humoral and innate immune responses in pigs
226 following exposure to *Toxoplasma* parasites.

227 It has been well established that a cell mediated response involving cytotoxic $CD8^+$ T cells
228 (Dawson et al., 2004; Solano Aguilar et al., 2001) and innate immune responses involving NK cells

(Dotiwala et al., 2016) are important in protection against intracellular parasites (Dawson et al., 2005). During our study we demonstrated increases in CD8α transcription in RLN and MLN samples in group 1 (Chal) and group 2 (Vac/Chal), six weeks after they received an oocyst challenge, compared to the control pigs. The only significant increase in CD8α transcription was seen in the RLN from group 1 (Chal) pigs compared to the controls. This increase in immune activity also coincided with significant increases in IFN-γ and MyD88 as well as well as increased CXCR3 transcription. These increases may have been elicited by large numbers of disseminating parasites, as Burrells et al., (2015) demonstrated positive ITS1 and qPCR results from the brains of 4/5 pigs in this group. The variable levels of transcription seen in the group 2 (Vac/Chal) pigs may have been as a consequence of the lack of circulating/disseminating parasites, as evidenced by negative (0/5) ITS1 and qPCR results seen in any tissue by Burrells et al., (2015). During this current study we also demonstrate consistent increases in the transcription of CXCR3, a chemokine receptor found on porcine NK cells, with transcription of CXCR3 having been demonstrated on NKp46^{high} NK cells (Mair et al., 2013). These porcine NKp46^{+} NK cells have also been associated with high levels of production of IFN-γ (Mair et al., 2012).

Levels of transcription of IFN-γ were significantly increased in the challenged and vaccinated animals (groups 1, 2 and 3), compared to the group 4 (control) pigs. Increased production of IFN-γ has been previously documented in PBMC samples collected from pigs infected with *T. gondii* oocysts (Solano Aguilar et al., 2001), however this current study demonstrates that increased IFN-γ transcription is also being observed in the lymph nodes and spleen 6-10 weeks following vaccination and/or challenge. All of the experimental groups were comprised of both male and female pigs and there does not appear to be any bias in the levels of transcription of IFN-γ based on the gender of the individual animals (no significant differences were observed when the data

252 was compared by T-Test). Similar observations were made by (de Groot et al., 2005) who
253 showed no significant effect of gender on IFN-γ, IL-4 or IL-10 production in 2-8 week old piglets.

254 The data presented in this current study also demonstrates a significant up-regulation of MyD88
255 transcription in both the RLN and MLN samples, which is indicative of an innate immune function
256 involving TLR's. Previous studies in pigs have shown MyD88 is involved in the regulation of Th1
257 (IFN-γ) type immune responses following a challenge with *T. gondii* (Dawson et al., 2004).. If
258 $CD8\alpha^{dim/-}NKp46^{high}$ NK cells (Mair et al., 2013) are involved in the production of IFN-γ during
259 porcine *Toxoplasma* infections then this may account for the sporadic T cell (CD8α) responses
260 and the consistent increases seen in CXCR3 seen in the group 1 (Chal) and group 2 (Vac/Chal)
261 and group 3 (Vac) animals.

262 The results from this current study show reductions in levels of CD4 transcription in samples
263 from group 1 (Chal), group 2 (Vac/Chal) and group 3 (Vac) compared to the group 4 (control)
264 animals. Previous reports have documented significant decreases in the percentage of cells
265 expressing CD4 in samples of PBMC (Jungersen et al., 1999; Solano Aguilar et al., 2001), however
266 both of these reductions were seen in samples of PBMC soon after infection (1 – 2 weeks) and
267 not in lymphoid tissues (RLN, MLN and spleen) following an infection (6 weeks post challenge) as
268 we have observed in this current study. However we must bear-in-mind that during the current
269 study we only have samples from a single time point, which will not be reflective of the CD4
270 responses earlier following infection.

271 There also appears to be no evidence of IL-12 (p35) involvement in the immune response against
272 *T. gondii* parasites in pigs at 6 week post challenge. In this current study the levels of
273 transcription of IL-12 (p35) were down regulated in samples from group 1 (Chal), group 2
274 (Vac/Chal) and group 3 (Vac) compared to the group 4 (control) animals. A previous study by

Solano-Aguilar et al., (2002) demonstrated a limited involvement of IL-12 in T cell proliferation and IFN-γ production in porcine PBMC and lymphoid cells, however these were healthy non-*Toxoplasma* infected pigs (Solano-Aguilar et al., 2002). As we only have samples from 6 weeks post challenge any previous involvement of IL-12 in the initiation of an immune response would have gone undetected.

Previous studies have attempted to use a number of different vaccine formulations to inhibit tissue cyst formation in pigs. These have included using either live RH isolate tachyzoites, temperature sensitive mutant-4 (TS-4), tachyzoite- excreted–secreted antigens (ESAs) and crude rhoptry antigens prior to a *T. gondii* oocysts challenge (Garcia et al., 2005; Pinckney et al., 1994; Wang et al., 2013). All of these vaccines failed to prevent tissue cyst formation, though they did all reduce parasite numbers, compared to non-vaccinated/challenged animals. In this current study we have shown that prior exposure to live S48 tachyzoites significantly limits (even potentially completely inhibits) parasite dissemination in animals challenged with oocysts and appears to inhibit tissue cyst formation, as is evidenced by the negative 529 bp *T. gondii* qPCR, and *T. gondii* ITS1 PCR results from the tissues (brain, chop, loin, left tricep, left semitendinosus, diaphragm, heart, tongue and masseter) collected from the group 2 (Vac/Chal) pigs (Burrells et al., 2015). When tissues from group 2 pigs were used in a mouse bioassay, no mice (0/45) inoculated returned positive results for the presence of *T. gondii* (Burrells et al., 2015). All of the animals in both groups 1, 2 and 3 were shown to have sero-converted, to be producing anti-*Toxoplasma* IgG within 14-21 days after first exposure to the parasites, while the group 4 (control) animals remained sero-negative throughout the experiment (Burrells et al., 2015) This current data combined with the findings of Burrells et al., (2015) suggests that a live attenuated vaccine could be used to significantly reduce parasite burdens in pigs making for safer meat for

human consumption. This could be particularly important in the continuing climate of increased consumer demand for outdoor reared “free range” pork.

5. Conclusion

The results from this current study show that exposure of pigs to live *T. gondii* parasites (S48 tachyzoites) elicits a strong cell mediated immune response against a subsequent oocyst challenge with *T. gondii* parasites. The data presented here demonstrates that at six weeks post challenge this immune response appears to involve both cell mediated and innate immune responses (CD8α T-cells and NK cells along with IFN-γ and MyD88), along with strong anti-*Toxoplasma* IgG responses (Burrells et al., (2015) which appear to be sufficient to limit the spread of *T. gondii* parasites in pigs.

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Conflict of Interest:

The authors declare that they have no conflict of interest

Ethical approval:

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Experiment number - E28/11).

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385

386

Figure Caption

Fig. 1A-F. ΔΔCt values for A - IFN-γ, B – CD8α, C – MyD88, D – CXCR3, E- IL-12 and F- CD4 normalised against HPRT, from samples of Mesenteric Lymph Node, Retropharyngeal Lymph Node and spleen from:

[A] – Results significantly different (p=<0.05) compared to Group 4 (control)

[B] – Results significantly different (p=<0.05) compared to Group 3 (Vac)

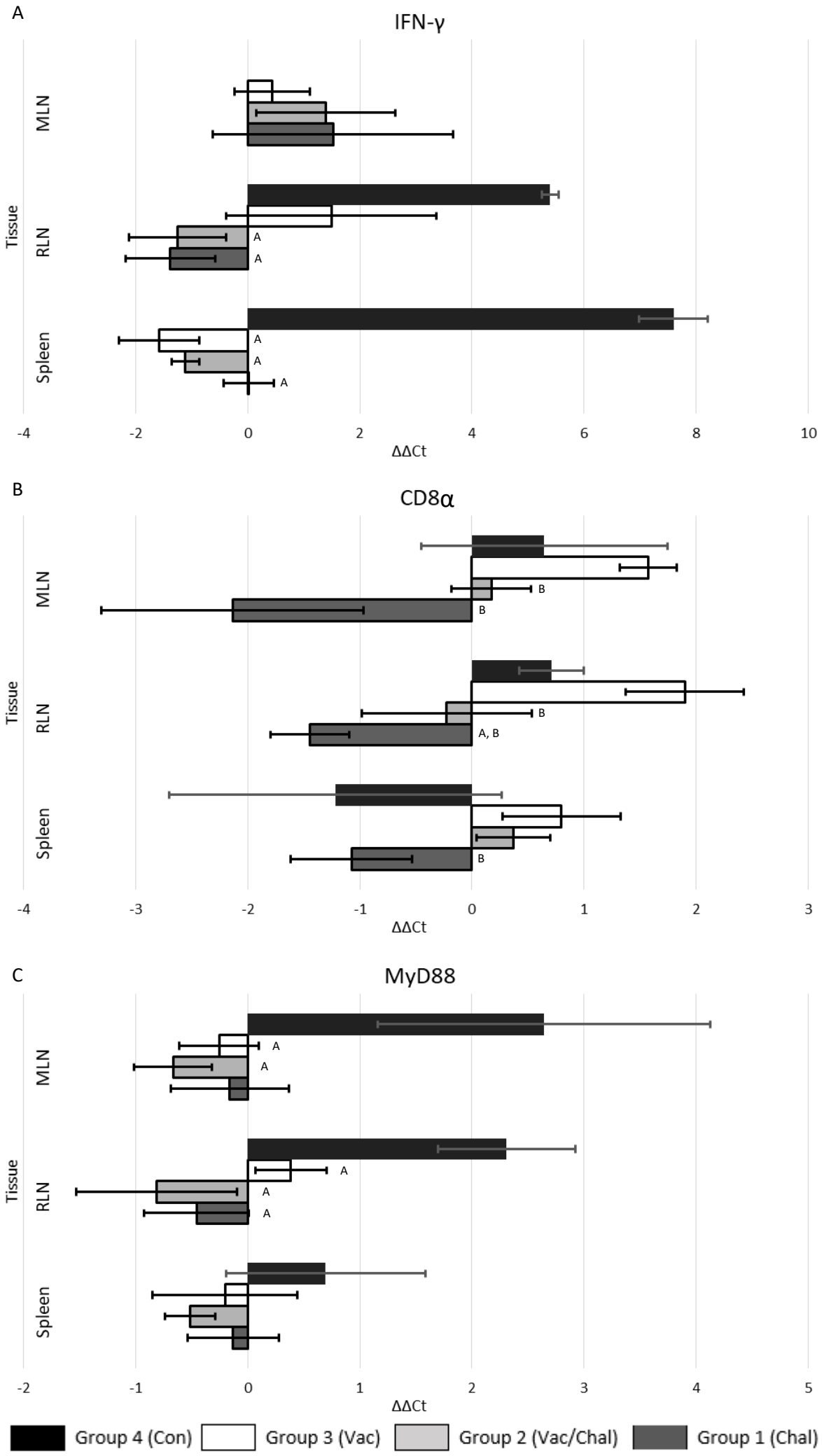
Table

Table 1. Primers for SYBR green qPCR analysis of spleen and lymph node samples collected from pigs vaccinated / challenged with *Toxoplasma gondii*.

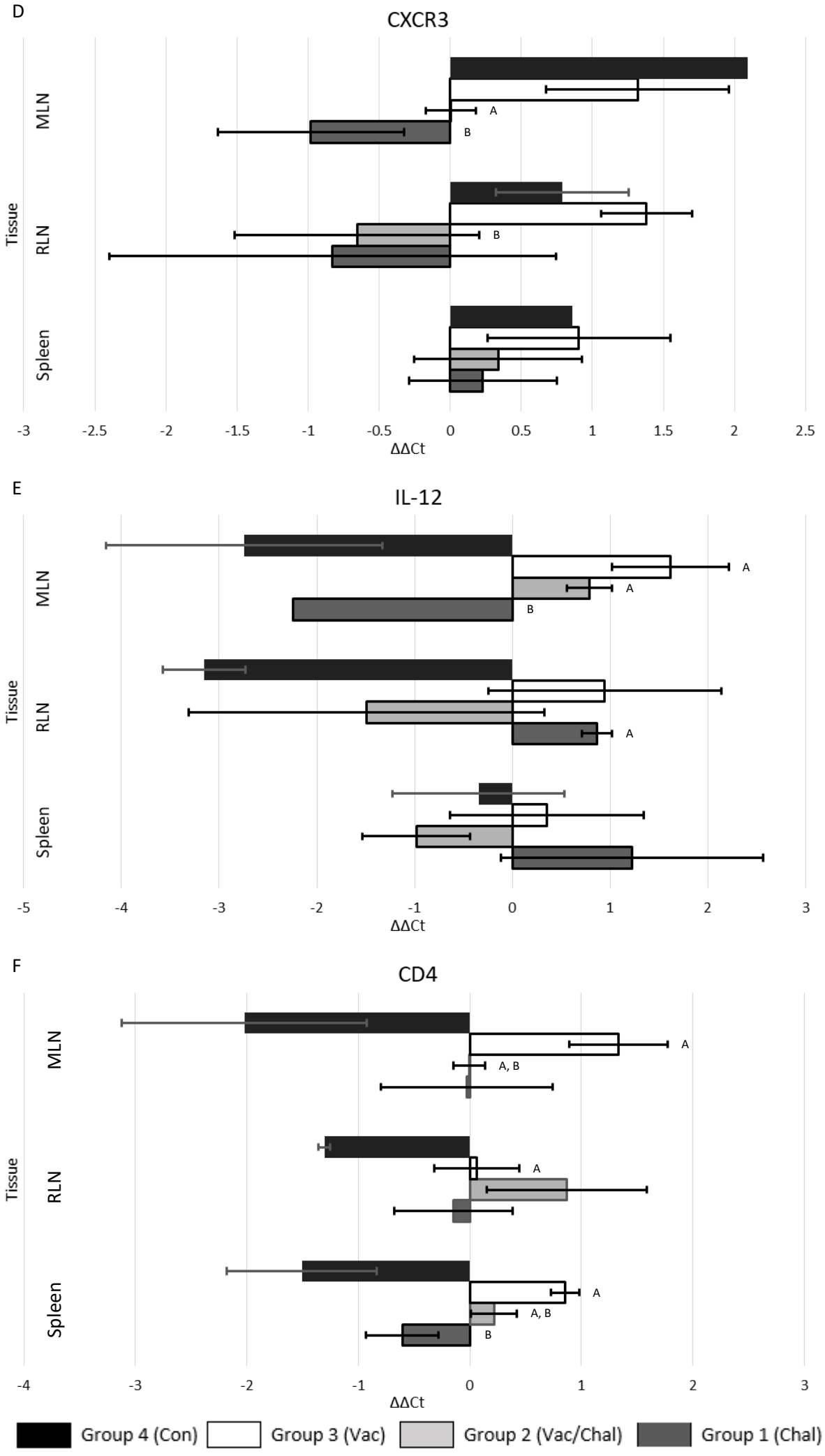
Target	Primer Name	Sequence	Product (bp)	Tm Value	GENBANK Accession Number
Hypoxanthine phosphoribosyltransferase	HPRT-For	5'-CTTTGCTGACCTGCTGGATT-3'	114	60.4	CV870598
	HPRT-Rev	5'-CCCGTTGACTGGTCATTACA-3'		59.4	
T-cell surface glycoprotein CD4	CD4-For	5'-AGCAGAGGGGAAGAGAGACC-3'	123	60.0	NM001001908
	CD4-Rev	5'-AGGAACAGGTGCCTCAGAGA-3'		60.0	
CD8 antigen alpha	CD8α-For	5'-AGCTGTTCCTGGCTCTACCA-3'	133	60.0	AY517855
	CD8α-Rev	5'-TGTCATTGGCCTTGTAACCA-3'		60.0	
Interferon-gamma	IFN-γ-For	5'-TCAGCTTTGCGTGACTTTGT-3'	150	59.6	X53085
	IFN-γ-Rev	5'-CACAATTCCAATTCAGCATCA-3'		59.5	
Interleukin-12 p35 subunit	IL-12p35-For	5'-CCTCCAAACTAGCGACCTCA-3'	150	60.4	L35765
	IL-12p35-Rev	5'-CTGAGATGGTCCAGGTGGTT-3'		60.0	
C-X-C chemokine receptor type 3	CXCR3-For	5'-CTGGTGGACACCCTCATGTA-3'	150	59.4	AJ851240
	CXCR3-Rev	5'-GAACTTGACACCCACGAAGG-3'		60.5	
Myeloid Differentiation Primary Response Protein 88	MyD88-For	5'-CCTGCTGATGCTTTGAGGTC-3'	146	60.9	EU056736
	MyD88-Rev	5'-AGAGGCAGATGAGAGGTGGA-3'		59.9	

Footnote – Tm – melting temperature

Figure



A – Results significantly different (p=<0.05) compared to Group 4 (control)
B – Results significantly different (p=<0.05) compared to Group 3 (Vac)



A – Results significantly different (p=<0.05) compared to Group 4 (control)
B – Results significantly different (p=<0.05) compared to Group 3 (Vac)

***Conflict of Interest**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: