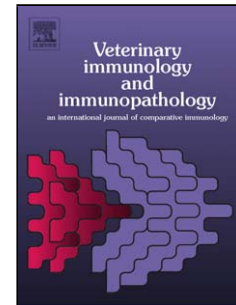


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1 **In vitro effect of vitamin E on lectin-stimulated porcine peripheral blood mononuclear cells**

2

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13

14

15

16

17 **Abstract**

18

19 In order to analyze the effect of vitamin E on Th1 and Th2 cytokine production, porcine peripheral
20 blood mononuclear cells (PBMC) were isolated from healthy pigs (n= 8) and cultured with either 0,
21 10, 50, or 100 μ M of vitamin E (α -tocopherol). PBMC were stimulated with PHA for either, 24 h to
22 determine: a) the concentration of tocopherol incorporated into the cell membrane, b) cytokine
23 production and c) Th1 and Th2 regulators gene expression; or 72 h to determine the proliferation of
24 PBMC. Vitamin E was incorporated into the PBMC in a dose dependent manner, giving as a result
25 a high proliferation of cells irrespective of the dose of vitamin E used. Regarding cytokine
26 production, vitamin E consistently decreases the mRNA expression and the percentage of cells
27 producing IL-10. Vitamin E did not influence the production of IFN- γ but the lowest level of
28 vitamin E (10 μ M) was sufficient to maximally increase the proportion of cells producing IL-2, to
29 diminish IL-4, and discreetly increase the mRNA expression of TBX21 vs. GATA3. In conclusion,
30 our results revealed that vitamin E is able to suppress IL-10 production and to influence the
31 production of IL-2, IL-4, and maybe TBX21. Vitamin E clearly has immunomodulatory effects,
32 though further work *in vivo* to determine the physiological nature of these effects is warranted.

33

34

35 Keywords: Vitamin E, cytokines, swine, TBX21, T-bet, GATA3

36

37

38 1. Introduction

39

40 CD4 T lymphocytes orchestrate the adaptive immune response through the production of cytokines.

41 The cytokines produced by CD4 T cells can be divide into Th1 (IFN- γ), Th2 (IL-4) (Mosmann,

42 1992) or regulatory T cells (Treg; IL-10 and TGF- β) (Belkaid, 2007). Recently, a new subset with

43 immune regulatory effects has been identified, Th17 (IL-23) (Bi et al., 2007). After activation by

44 APC, naïve T cells engage in complex differentiation events before developing into Th1, Th2, Th17

45 or Treg cells. Signals T cells received from APC drive their differentiation and provide the basis for

46 the design of vaccines and/or adjuvants.

47

48 IL-12 is considered the main inducer of Th1 (Brombacher et al., 2003). In addition, IFN- γ and IFN-

49 α also enhance the generation of Th1. In contrast, IL-4 promotes Th2 whereas TGF- β and IL-10 are

50 the main inducers of Treg cells. The development of Th1 cytokines is regulated by the transcription

51 factor T-bet, whereas GATA3 regulates Th2 response. Treg regulation is more complex, but

52 expression of the transcription factor Foxp3 is closely related to this phenotype (Hori et al., 2003).

53

54 Cytokines production of Th1 and Th2 has been described in pigs, humans and rodents. Th1

55 cytokines are characterized by IFN- γ production in pigs infected with bacterial, viral and parasitic

56 pathogens and in healthy pigs (Azevedo et al., 2006; Dawson et al., 2005; Dawson et al., 2004; de

57 Groot et al., 2005). Also, the expression of transcription factor TBX21 (also known as T-bet) has

58 been associated with the induction of Th1 responses (Dawson et al., 2004). IL-4 is not always the

59 most important Th2 cytokine and the functions of IL-13 overlap considerably with those of IL-4,

60 and IL-13 takes over the function of IL-4 in a number of cases (Azevedo et al., 2006; Bautista et al.,

61 2007; Dawson et al., 2005; de Groot et al., 2005), however GATA3 expression is not associated

62 with Th2 response. Recently, Tregs has been described in pigs, which are characterized by the

63 production of IL-10 (Kaser et al., 2008). TGF- β -producing Tregs (also known as Th3) have not

64 been reported in pigs yet. However, data from our laboratory have shown its presence in pigs
65 (Silva-Campa et al., in preparation).

66

67 Nutritional immunology seeks to increase or modulate the immune response through manipulation
68 of the level of dietary nutrients. Many reports have described how vitamins can modulate cytokine
69 production after *in vitro* or *in vivo* supplementation (Adolfsson et al., 2001; Boonstra et al., 2001;
70 Dawson et al., 2006; Han et al., 2006; Han et al., 2000; Hernandez et al., 2008; Li-Weber et al.,
71 2002; Ma et al., 2005; Pinelli-Saavedra, 2003; Wang et al., 2007). Vitamin A enhances Th2
72 cytokines and improves the immune response against gastrointestinal parasites (Dawson et al.,
73 2006; Wang et al., 2007). Vitamin D also polarizes Th2 by increasing expression of GATA3 and *c-*
74 *maf*, leading to the secretion of IL-4, IL-5 and IL-10 and inhibiting IFN- γ (Boonstra et al., 2001).
75 Vitamin E is also an immunoregulatory nutrient; it increases the production of IL-2 in several
76 species and decreases IL-4 (Adolfsson, 2001; Han et al., 2006; Han et al., 2000). Controversy exists
77 with regards to the ability of vitamin E to increase IFN- γ (Han et al., 2000; Malmberg et al., 2002).
78 Vitamin E also induces the production of IL-10, when evaluated in lymphocytes stimulated with
79 dendritic cells (Tan et al., 2005). The effects of vitamin E on the regulatory genes T-bet and
80 GATA3 have not been evaluated.

81

82 Previous reports have involved vitamin E as an important immunomodulator in pigs. It has been
83 described that vitamin E can increase the proliferation of PHA-stimulated PBMC, the antibody
84 production and phagocytosis (Larsen and Tollersrud, 1981; Pinelli-Saavedra, 2003; Pinelli-
85 Saavedra et al., 2008). Also, it has been described that vitamin E inhibits inflammatory cytokine
86 responses (Webel et al., 1998), but its effects on Th1 or Th2 polarization are unknown. In this work,
87 the effects of vitamin E supplementation on cytokine production of porcine PBMC were evaluated.
88 Different concentrations of vitamin E were tested and proliferation, cytokine production and
89 expression of TBX21 and GATA3 were determined.

90 2. Material and Methods

91

92 2.1. Experimental design

93

94 In order to analyze the effect of vitamin E on Th1 and Th2 cytokines, porcine PBMC were isolated
95 from two-month-old pigs (n= 8), cultured with different concentrations of vitamin E, (α -tocopherol;
96 0, 10, 50, and 100 μ M), and stimulated with PHA for either, 24 h to determine: a) the concentration
97 of vitamin E incorporated into the cell membrane, b) cytokine production and c) Th1 and Th2
98 regulators gene expression; or 72 h to determine the proliferation of PBMC. HPLC analysis was
99 carried out to quantify vitamin E, cytokine production was analyzed by intracellular staining using
100 flow cytometry (FACS), cytokine mRNAs were semi-quantified by conventional RT-PCR, Th1 (T-
101 bet) and Th2 (GATA3) regulatory gene expression was analyzed by real time PCR, and
102 proliferation was evaluated with carboxyfluorescein diacetate-succinimidyl ester (CFSE) and FACS
103 analysis.

104

105 2.2. Antibodies and Reagents

106

107 Mouse monoclonal antibodies (mAbs) specific for porcine IL-2 (IgG2b, clone No. 100312), IL-4
108 (IgG1, clone No. 99613), IL-10 (IgG2A, clone No. 148806), and IFN- γ (IgG2b, clone No. 154007)
109 were purchased from R&D Systems (Pullman, WA, USA), phycoerythrin (PE)-conjugated goat-anti
110 mouse IgG (SouthernBiotech) was used as the secondary antibody. All primers were purchased
111 from Sigma GENOSYS, Sigma (Table 1). Primers and probes for GATA3 and T-bet were kindly
112 supplied by Dr. Harry Dawson (Table 1). Vitamin E as α -tocopherol (Cat. No. T3251) was
113 purchased from Sigma (St Louis, MO, USA), and CFSE from Molecular Probes (Eugene, OR,
114 USA).

115

116 2.3. *Animals*

117

118 Healthy conventional Landrace/Yorkshire hybrid two-month-old pigs were housed at the metabolic
119 unit of CIAD, A.C. Water and food were provided *ad libitum*. Feed supplied the minimal
120 requirement (30 mg/kg fed) of vitamin E to avoid deficiencies. Pigs were kept according to the
121 International Guidelines for Animal Care.

122

123 2.4. *Preparation of vitamin E for supplementation*

124

125 A stock solution of vitamin E was prepared by dissolving α -tocopherol in absolute ethanol. To
126 optimize cellular uptake, the stock solution was then mixed with complement inactivated fetal
127 bovine serum (FBS; 16000-044, GIBCO, Gland Island, NY, USA) at a final concentration of 2.31
128 mM and incubated at 37°C for 1 h in the dark with intermittent vortexing. For supplementation of
129 PBMC, solutions of vitamin E in RPMI-1640 (R4130, Sigma) with 10% of FBS were prepared and
130 the final concentration of vitamin E was 10, 50 or 100 μ M.

131

132 2.5. *PBMC proliferation assay*

133

134 Fifteen ml of blood was collected into heparin-coated blood collection tubes (Becton-Dickinson),
135 diluted 1:2 with RPMI-1640 (Sigma), underlaid with Ficoll-Hypaque (Amersham Biosciences,
136 Uppsala, Sweden), and centrifuged at 500 \times g for 20 min. PBMC were collected from the interface,
137 washed three times in RPMI-1640 and cell viability was determined by the trypan blue dye
138 exclusion method. For analysis of PBMC proliferation, cells were stained with CFSE. Briefly, 1 ml
139 of PBMC suspension (1×10^7 cells/ml) was incubated with 7.5 μ M of CFSE (CFSE was prepared
140 from a 5 mM stock solution dissolved in dimethyl sulfoxide) in culture medium for 10 min at room
141 temperature in the dark. After incubation, 10 ml of culture medium supplemented with 10% heat-

142 inactivated fetal bovine serum, 50 mM 2-mercaptoethanol (M7522, Sigma), 100 UI penicillin/ml
143 and 100 µg streptomycin/ml (P4458, Sigma) was added to the cells. Subsequently, cells were
144 centrifuged at 400 g for 20 min, suspended in 4 ml (2.5×10^6 cells/ml) of supplemented culture
145 medium and cell viability was determined.

146

147 Proliferation assays were performed with 2.5×10^5 CFSE-treated PBMC supplemented with 0, 10,
148 50, or 100 µM of vitamin E and stimulated with PHA (10 µg/ml; Sigma). Cells were seeded into
149 96-well culture plates (3596, Corning, NY, USA) in 200 µl final volume of supplemented RPMI-
150 1640 culture medium and incubated for 72 h at 37 °C in a humid atmosphere containing 5% CO₂.
151 At the end of the culture period, 10,000 cells were acquired on FACSCalibur equipped with
152 CellQuest software (Becton-Dickinson, San Jose, CA, USA), data were analyzed using CellQuest®
153 software (Becton-Dickinson) or WinMDI (<http://facs.scripps.edu/software.html>) and the blast cells
154 were gated by forward scatter (FSC) and side scatter (SSC) characteristics. A histogram based on
155 the fluorescence intensity of unstimulated CFSE-stained cells, which lay within the blast scatter
156 gate, was made in order to differentiate dividing cells of lower intensity. The initial gate (R0)
157 included the undivided cell population and the subsequent gates (R1 to R7) enclosed populations
158 with progressive two-fold decreases in fluorescence intensity. Cell proliferation was determined as
159 follows: % divided cells = $100 * (R1+R2+\dots+Rn / R0 + R1 + R2 +\dots+ Rn)$

160

161 *2.6. Flow cytometry analysis of intracellular cytokine production*

162

163 Detection of intracellular cytokine production in PHA-stimulated PBMC by two-color stain
164 approach was done. Cells were cultured for 24 h as described above in the proliferation assay. Four
165 hours before cell harvesting, 10µg/ml Brefeldin-A (Cat. No. B-7651, Sigma) was added to inhibit
166 new cytokine release. Cells were washed with phosphate-buffered saline (PBS; 0.15 M) containing
167 0.2% bovine serum albumin (BSA, Cat. No. A9418, Sigma), and fixed with 4% paraformaldehyde

168 for 10 min at 4 °C, washed, and permeabilized with 0.1% saponin in PBS and 10% BSA, and gently
169 shaken in the dark for 15 min at room temperature. Cells were stained with mAbs against swine IL-
170 2, IL-4, IL-10, or IFN- γ for 30 min at 4 °C. Thereafter, cells were washed twice with 0.5 %
171 saponin/PBS and incubated with PE-conjugated goat-anti mouse IgG for 30 min. Finally, 10,000
172 cells were counted by flow cytometry and analyzed using CellQuest® software or WinMDI
173 (<http://facs.scripps.edu/software.html>). To analyze intracellular cytokines, the blasts were first gated
174 by their physical properties (FSC and SSC), then a second gate was drawn based on the
175 fluorescence characteristics of the gated cells. Background staining was assessed using samples
176 incubated with PE-conjugated second-step antibody only.

177

178 *2.7. RT-PCR for porcine cytokines.*

179

180 Porcine cytokines were analyzed in PHA-stimulated PBMC supplemented with different
181 concentrations of vitamin E. After 24 h, PBMC were washed once in PBS and the pellet was
182 resuspended in TRIzol (15596-018, Invitrogen, Carlsbad, CA, USA) for total RNA extraction
183 according to manufacturer's protocol. Total RNA was resuspended in 20 μ l of DEPC-treated water
184 (1302688, Invitrogen) for further analysis. For cytokine analysis, reverse transcription was done
185 using Superscript II reverse transcriptase (18064-022, Invitrogen) in a total volume of 20 μ l,
186 following manufacturer's recommendations. The cDNA was stored at -20 °C until used in PCR
187 amplification. PCR reactions were done in a 50- μ l reaction using 10 mM Tris HCl, 50 mM KCl (pH
188 8.3), 3 mM MgCl₂, 0.8 mM each dATP, dTTP, dCTP and dGTP, 20 μ M of each primer, 0.25 U *Taq*
189 DNA polymerase (all products from Invitrogen), and 2 μ l of cDNA. The PCR reaction was carried
190 out for 35 cycles at 94 °C for 3 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final
191 elongation at 72 °C for 10 min. Each PCR product (10 μ l) was run on 1.2% agarose gels and stained
192 with ethidium bromide for UV visualization. To compare the relative mRNA expression level of
193 each cytokine, the PCR products from supplemented and not supplemented PHA stimulated PBMC

194 were estimated in a semi-quantified manner by densitometry (Gel Logic 200 Imaging System),
195 comparing the intensity of the band, relative to those of the GAPDH gene (housekeeping gene).
196 Results are presented as the relative intensity ratio of cytokine mRNA/GAPDH mRNA. Primers for
197 porcine cytokines detection have been previously described (Hernandez et al., 2001) and are shown
198 in Table 1.

199

200 2.8. Real time PCR for GATA3 and TBX21

201

202 PCR was performed with a Brilliant Quantitative PCR core reagent kit (Stratagene, La Jolla, Ca),
203 and a SmartCycler system (Cephe) as previously described (Flores-Mendoza et al., 2008). The
204 amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min; and 40 cycles of 95 °C
205 for 15 s and 60 °C for 1 min. Fluorescence signals measured during amplification were processed
206 post-amplification. For quantification of mRNA, differences in Ct values between supplemented
207 and non-supplemented PBMC were evaluated with the $2^{-\Delta\Delta C_t}$. The Ct values from supplemented and
208 non-supplemented cells were normalized against an endogenous control (Peptidylprolyl isomerase
209 A, PPIA), and results are reported as the fold change from non-supplemented and supplemented
210 PBMC. The primers and probes used are listed in Table 1.

211

212 2.9. Quantification of α -tocopherol from PBMC

213

214 Alpha-tocopherol was extracted from 5×10^6 cells supplemented and not supplemented as described
215 by Hess *et al.* (1991) with some modifications: 500 μ l ethanol (containing BHT 0.025%) was added
216 to the cell suspension and vortexed for 10 s. Then, 700 μ l of hexane (containing BHT 0.025%) was
217 added and shaken for 10 min, centrifuged at 14,000 rpm for 5 min, and 600 μ l of the hexane layer
218 was removed and dried at room temperature. The dried samples were dissolved in 400 μ l methanol
219 and vortexed for 10 min. This mixture was injected into the high performance liquid

220 chromatography (HPLC) column. A Varian Solvent Delivery module Pro-Star 220 and variable
221 wavelength UV-detector Varian 9050 were used and set at an excitation wavelength of 290 nm.
222 Separation was achieved using a Microsolv C-18 (R-0089200E3) C-18 (100 mm x 4.6 mm) column
223 (Varian, USA). The mobile phase was methanol: (98:2) water (98:2). The detection limit was 0.02
224 $\mu\text{g/ml}$.

225

226 *2.10. Statistical analysis*

227

228 Data were analyzed by one way analysis of variance (ANOVA) using NCSS 2000 to evaluate the
229 effect of vitamin E supplementation level on proliferation and cytokine production. Significant
230 differences among treatments were determined by Tukey test ($P < 0.05$). Kruskal-Wallis test was
231 used to assess the effect of vitamin E on TBX21 and GATA3 mRNA expression.

232

233 **3. Results**

234

235 *3.1. Uptake of α -tocopherol*

236

237 PBMC (n=8) were supplemented with 10, 50 and 100 μM of vitamin E for 24 h and the vitamin E
238 content was quantified by HPLC (Fig. 1). The basal content of vitamin E in 5×10^6 cells was $0.37 \pm$
239 $0.06 \mu\text{M}$ (mean \pm SEM), whereas cells supplemented with 10, 50 and 100 μM , had 2.83 ± 0.27 ,
240 4.32 ± 0.27 , and $5.05 \pm 0.47 \mu\text{M}$, respectively of vitamin E ($P < 0.05$), showing a significant
241 increase in a dose dependent manner.

242

243 *3.2. Effect of vitamin E on lymphocyte proliferation*

244

245 In order to evaluate the effect of vitamin E on lymphocyte proliferation, cells (n=8) were stained
246 with CFSE and cultured for 3 days in the presence of different levels of vitamin E. Our results
247 showed that vitamin E increased the percentage of proliferating cells ($P < 0.05$) when cells were
248 supplemented with any of the vitamin E levels, but there was not a further increase after the first
249 level of supplementation ($P > 0.05$). When cell cycles of proliferation were evaluated, vitamin E
250 induced multiple cycles of proliferation in comparison to cells without supplementation. Cells with
251 $10\mu\text{M}$ of vitamin E had 4 and 5 cycles of maximum proliferation (Fig. 2A), but at 50 and $100\mu\text{M}$
252 cells had 7 cycles of proliferation (Fig. 2B-C).

253

254 3.3. Cytokine production of PBMC

255

256 Figure 3 shows the results of the effect of vitamin E on Th1 and Th2 cytokine production evaluated
257 at mRNA and protein level. Data are representative of eight pigs. The mRNA for IL-2 increased in
258 cells stimulated with PHA (relative intensity, $\text{RI} = 232 \pm 84$), compared to control unstimulated cells
259 (100 ± 82 ; $P < 0.05$). PHA-stimulated cells supplemented with $100\mu\text{M}$ vitamin E showed a higher
260 ($P < 0.05$) IL-2 mRNA ($\text{RI} = 265 \pm 88$) than non supplemented-PHA-stimulated cells ($\text{RI} = 336 \pm$
261 65). In contrast, $50\mu\text{M}$ of vitamin E decreased the expression of IL-2 ($P < 0.05$) compared to other
262 treatments (0 , 10 and $100\mu\text{M}$ of vitamin E) and did not show differences regarding to PHA-
263 unstimulated cells (control). Supplementation with $10\mu\text{M}$ vitamin E was not significantly different
264 to that of $0\mu\text{M}$ vitamin E. The percentage of cells producing IL-2 was higher in PHA-stimulated
265 than unstimulated cells (9 ± 1 and $24 \pm 4\%$, respectively; $P < 0.05$), and $10\mu\text{M}$ of vitamin E was
266 sufficient to increase the proportion ($28 \pm 7\%$; $P < 0.05$). In the presence of 50 or $100\mu\text{M}$, the
267 proportion of cells producing IL-2 was 22 ± 5 and $25 \pm 4\%$, respectively ($P > 0.05$). The mRNA
268 expression of IFN- γ was did not show a regular pattern of response. Cells stimulated with PHA
269 expressed higher levels of mRNA for IFN- γ ($\text{RI} = 134 \pm 107$) than unstimulated cells ($\text{RI} = 62 \pm 46$;
270 $P < 0.05$), but no differences were observed due to supplementation of 10 , 50 or $100\mu\text{M}$ of vitamin

271 E (RI= 216 ± 191 , 115 ± 70 , and 274 ± 117 ; respectively; $P > 0.05$). Similar results were observed
272 in the percentage of cells producing IFN- γ . A significant increment was observed when comparing
273 PHA-stimulated (15 ± 2 %) vs. unstimulated cells (RI= 4 ± 1 %; $P < 0.05$), but no further changes
274 were caused by 10 (17 ± 3 %), 50 (12 ± 3 %) or 100 μM (13 ± 4 %) of vitamin E ($P > 0.05$). The
275 mRNA expression for IL-4 was low in unstimulated cells (RI= 8 ± 13) and increased after PHA
276 stimulation (218 ± 109 ; $P < 0.05$). PHA stimulated cells incubated with 10 μM or 50 μM vitamin E
277 showed a trend to decrease the expression of IL4 mRNA (RIs 101 ± 103 , 134 ± 108 respectively; P
278 > 0.05), which was less evident in presence of 100 μM of vitamin E (RI= 187 ± 81). The percentage
279 of cells producing IL-4 was greater in stimulated (18 ± 2 %) than unstimulated cells (6 ± 1 %; $P <$
280 0.05). In the presence of 10 μM of vitamin E a significant reduction in the percentage of cells
281 producing IL-4 was noticed (11 ± 3 %; $P < 0.05$) compared to those with 50 μM (14 ± 5 %) or 100
282 μM (13 ± 4 %; $P > 0.05$). The mRNA expression of IL-10 increased with stimulation of cells (from
283 RI= 166 ± 118 to 318 ± 111 for unstimulated and stimulated cells; $P < 0.05$), and vitamin E
284 significantly reduced its expression (RI= 123 ± 47 , and 118 ± 36 , for 10 μM and 50 μM
285 respectively; $P < 0.05$) the expression of IL-10. No changes were observed when cells were
286 supplemented with 100 μM (RI= 185 ± 85 ; $P > 0.05$). Stimulation with PHA increased the
287 percentage of IL-10 cells (6 ± 2 to 18 ± 3 %; $P < 0.05$). The addition of vitamin E (10, 50 or 100
288 μM), reduced the number of cells producing IL-10 (9 ± 1 , 12 ± 2 , 9 ± 2 %, respectively; $P < 0.05$).

289

290 3.4. Modulation of TBX21 and GATA3 by vitamin E

291

292 Figure 4 shows the relative changes in TBX21 and GATA3 on PBMC stimulated with PHA and
293 supplemented with vitamin E (n=4). TBX21 and GATA3 mRNA levels were not significantly
294 different by vitamin E supplementation ($P > 0.05$). However, the analysis of the proportion of
295 TBX21 mRNA levels related to GATA3 mRNA levels obtained, revealed that only the lowest

296 vitamin E concentration used (10 μ M) induced higher levels of TBX21 mRNA over GATA3
297 mRNA (1.5 fold difference; $P = 0.08$).

298

299 **4. Discussion**

300

301 The aim of this work was to evaluate the ability of vitamin E to modulate the production of Th1 and
302 Th2 cytokines. Our results showed that the addition of vitamin E to PBMC cultures increased their
303 proliferation irrespective of the dose of vitamin E used. However, IL-2 production increases only at
304 the highest concentration of vitamin E (100 μ M) used. No changes were observed in the production
305 of IFN- γ , but a reduction in the production of IL-4 and IL-10 was noticed with the lowest
306 concentration of vitamin E (10 μ M). These changes were associated with a discrete increased
307 mRNA expression ($P = 0.08$) of TBX-21 over the expression of GATA3 with the lowest
308 concentration of vitamin E.

309

310 After 24 h of supplementation, we observed that vitamin E was incorporated into the membrane of
311 PBMC in proportion to the amount supplemented in the media culture. When proliferation was
312 evaluated, higher proliferation was observed in presence of vitamin E. These results are in
313 agreement with previous reports in pigs (Larsen and Tollersrud, 1981; Pinelli-Saavedra, 2003) and
314 other species (Adolfsson, 2001; Han et al., 2006), which described that PBMC of mice
315 supplemented with vitamin E induced the cells to enter more proliferation cycles, by increasing IL-
316 2 expression and its high affinity receptor CD25 (Adolfsson, 2001). In mice, vitamin E increases the
317 expression of cell-related proteins cyclin B, Cdc2, and Cdc6, which are important in the regulation
318 of the cell cycle (Han et al., 2006). IL-2 is the main cytokine involved in the proliferation of T cells.
319 In our experiment, the change in IL-2 relative to a housekeeping gene (GAPDH) and the number of
320 cytokine-producing cells was evaluated. Although the intracellular cytokine staining method gives
321 information on cytokine production at the single-cell level, these data are relatively qualitative in

322 nature (Zhang et al., 2005). Therefore, we decided not to compare the cytokine level on per cell
323 basis. We observed that IL-2 mRNA expression level responded to vitamin E level differently than
324 the proportion of cells producing IL-2. At the mRNA level, we only observed significant changes
325 when 100 μ M of vitamin E was added, but the proportion of cells producing IL-2 was increased with
326 10 μ M. Our results suggest that vitamin E increases both the proportion of cells producing IL-2 and
327 the total amount of message, and these results could explain why proliferation was consistently
328 increased following addition of different concentrations of vitamin E.

329

330 A previous report has described the effects of vitamin E on cells in Peyer's patches and mesenteric
331 lymph nodes of weaned pigs. Vitamin E can increase the number of IgA⁺ B-lymphocytes compared
332 with control animals, but no effects were observed on the numbers of CD4⁺ or CD8⁺ T-
333 lymphocytes (Fragou et al., 2006). To our knowledge, data regarding the effects of vitamin E on
334 Th1 or Th1 cytokine response in pigs are not available. In mice and to some extent in humans
335 (Malmberg et al., 2002), vitamin E has the ability to up-regulate Th1 cytokines, especially IFN- γ .
336 According to our results, vitamin E did not up-regulate the expression of IFN- γ mRNA or the
337 number of cells producing IFN- γ , though we did observe a non-significant trend for 10 and 100 μ M
338 of vitamin E to increase expression. Some functions of vitamin E may be specie-dependent.

339

340 Vitamin E activates protein kinase C (Tasinato et al., 1995) and NF- κ B (Li-Weber et al., 2002), an
341 important transcription factor in the synthesis of many cytokines including IFN- γ . However, one of
342 the main transcription factors responsible for IFN- γ expression is TBX21 (Szabo et al., 2003).
343 TBX21 initiates Th1 cell differentiation by activating Th1 genetic programs and repressing Th2
344 phenotypes. In order to explain the involvement of vitamin E in the regulation of TBX21 and
345 GATA3 genes, the mRNA expression of these genes was quantified by qPCR. Our results showed
346 that only the lowest concentration of vitamin E (10 μ M) up-regulated the expression of TBX21 over
347 GATA3. Interesting, similar results were observed when the IFN- γ was evaluated. These results

348 provide preliminary evidence that vitamin E could be involved in regulation of TBX21 and GATA3
349 in pigs. The regulation of T-bet (TBX21) by vitamin E has been evaluated previously in aged mice
350 (Jones et al., 2003). In that report, vitamin E was able to regulate the expression of TBX21 and
351 PPAR- α , and in consequence to control the dysregulated IL-2 and IFN- γ production of aged mice.

352

353 Regarding to Th2 cytokines, IL-4 was down-regulated at the protein level and no significant
354 changes were observed at the transcriptional level, although there was a trend towards low mRNA
355 expression with 10 μ M of vitamin E. Supplementation of 50 or 100 μ M of vitamin E did not modify
356 the IL-4 mRNA or protein. In humans, it has been shown that low *in vitro* supplements of vitamin E
357 reduced the transcription of IL-4 and this was associated with the inhibition of AP-1 transcription
358 factor (Li-Weber et al., 2002). In our results, we observed that mRNA levels of GATA-3 were low
359 with 10 μ M of vitamin E, which suggests that in addition to AP-1, inhibition of GATA-3 could be
360 implicated in the down-regulation of IL-4. However, this hypothesis needs to be tested.

361

362 The major effects of vitamin E were observed for IL-10 production. According to our results,
363 vitamin E reduces the expression of IL-10 mRNA and the proportion of IL-10 producing cells.
364 These data suggest that vitamin E provided *in vitro* decreases the development of Th2 cytokine
365 response and/or Treg cells. Modulation of Treg by vitamin E has not been evaluated and needs to be
366 analyzed in future works. Some studies have reported that vitamin E and C treated-human dendritic
367 cells reduce the allogeneic T cell response and increase the expression of Th2 cytokines (IL-4 and
368 IL-10) (Tan et al., 2005). This discrepancy may be attributed to the different cell type used in both
369 experiments or by the combined effect of vitamin E with vitamin C. In the present work, the low
370 level of IL-10 observed should favor Th1 responses, increasing T-bet expression and greater
371 inflammation; however no changes on IFN- γ production were observed. The effect of vitamin E on
372 IL-10 production has been contradictory, while some authors reported a decreased production
373 (Sabat et al., 2001; Venkatraman and Chu, 1999; Wang et al., 1995) others reported no changes

374 (Hsieh and Lin, 2005). The mechanisms proposed to explain this decrease in IL-10 include the
375 ability of vitamin E to inhibit PKC (Tasinato et al., 1995), since it has been shown that expression
376 and synthesis of IL-10 requires the early activation of PKC (Meisel et al., 1996). Another possible
377 mechanism is the decreased expression of the transcription factor GATA3, which can modulate the
378 expression of IL-10 (Chang et al., 2007). Further experiments are needed to test these hypotheses.

379

380 In conclusion, vitamin E was incorporated into lymphocytes in a dose dependent manner. The
381 lowest level of vitamin E (10 μ M) was sufficient to maximally increase lymphocyte proliferation,
382 enhance the proportion of cells producing IL-2, and to diminish IL-4 and IL-10 levels. Vitamin E
383 clearly has immunomodulatory effects and further work *in vivo* is necessary to determine the
384 physiological nature of these effects.

385

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387

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389

390 **References**

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513

514 **Figure legends**

515

516 **Figure 1.** PBMC content of vitamin E. PBMC were supplemented with different concentrations of
517 vitamin E (α -tocopherol) for 24 h and the content was quantified by HPLC. Results represent the
518 Mean \pm SEM of 8 pigs. Statistical analyses were performed using one-way ANOVA followed by
519 Tukey's multiple comparison test. Different letters denote significant differences ($P \leq 0.05$).

520

521 **Figure 2.** Effect of vitamin E on PBMC proliferation after 3 days of culture. (Top) A representative
522 experiment showing the analysis on gated blast cells in a proliferating culture of PHA-stimulated
523 CFSE-stained PBMC show a sequential halving of fluorescence intensity (1-7) that corresponds to
524 cell divisions (continuous lines). The number 0 represent the population without cell division;
525 proliferating cells without vitamin E (filled histogram) or with vitamin E (open histogram) at
526 concentration of 10 μ M (A), 50 μ M (B) and 100 μ M (C). (Bottom) Blast cell percentages of PHA-
527 stimulated PBMC and supplemented (10, 50 and 100 μ M) or not with vitamin E. Data represent the
528 mean and the individual data of 8 pigs.

529

530 **Figure 3.** Expression of cytokines on vitamin E supplemented-PBMC. Total RNA was extracted
531 and the mRNA relative expression (cytokine/GADPH) of cytokines was evaluated by conventional
532 RT-PCR. The percentage of positive cells was evaluated on 24 h PHA-stimulated PBMC by using
533 intracellular two-stain label as is described in the Material and Methods section. Results are
534 expressed as Mean \pm SEM of 8 pigs. Data were analyzed by one-way ANOVA followed by Tukey
535 test. Different letters represent significant differences ($P < 0.05$).

536

537 **Figure 4.** TBX21 and GATA3 mRNA expression. Total RNA was extracted and the mRNA
538 expression of cytokines was quantified by real time PCR. For quantification, differences in Ct
539 values between supplemented and non-supplemented PBMC were evaluated with the delta-delta

540 equation. The Ct values were normalized against the PPIA gene, as endogenous control, and results
541 (Mean \pm SEM of 4 pigs) are reported as the relative fold change from non-supplemented vs. TBX21
542 (black bars), and GATA3 (clear bars). Statistical analyses were performed using Kruskal-Wallis
543 test.
544

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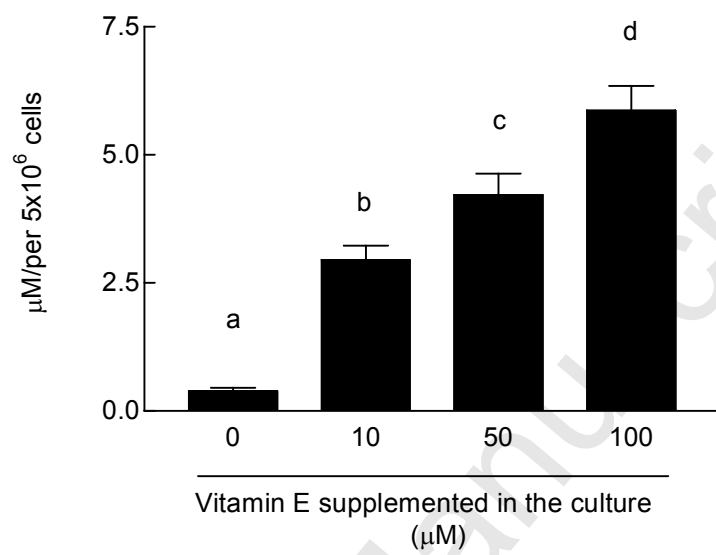
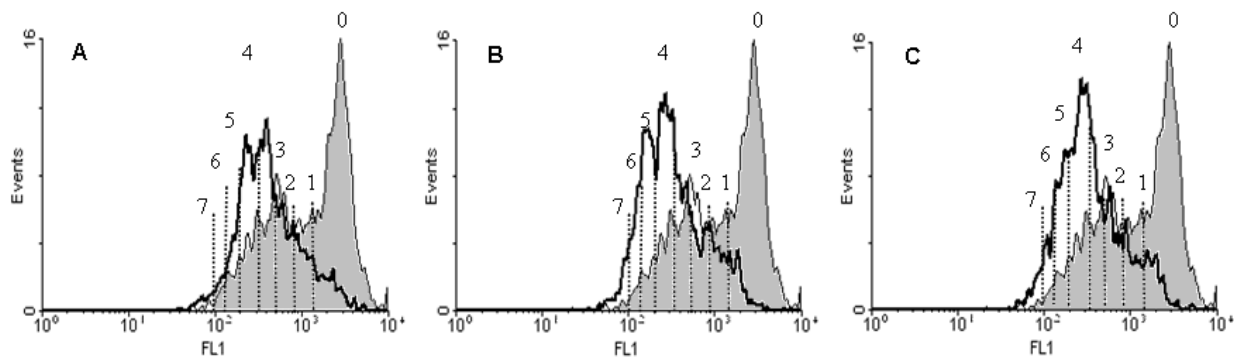
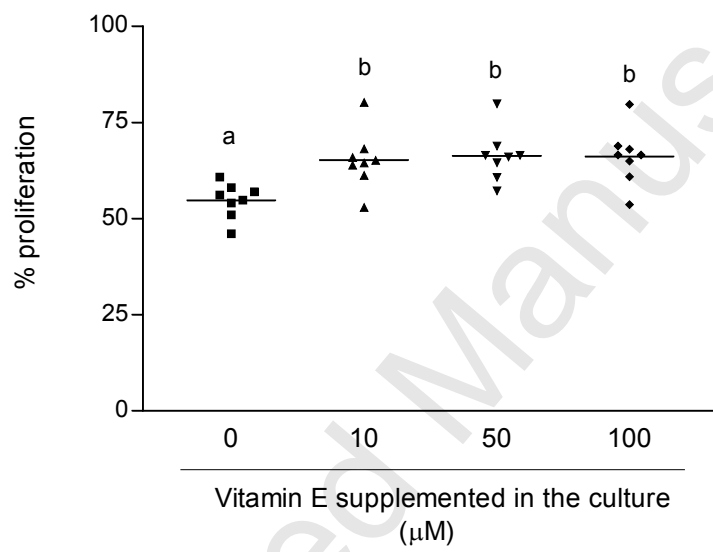


Figure 1.



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4 **Figure 2.**

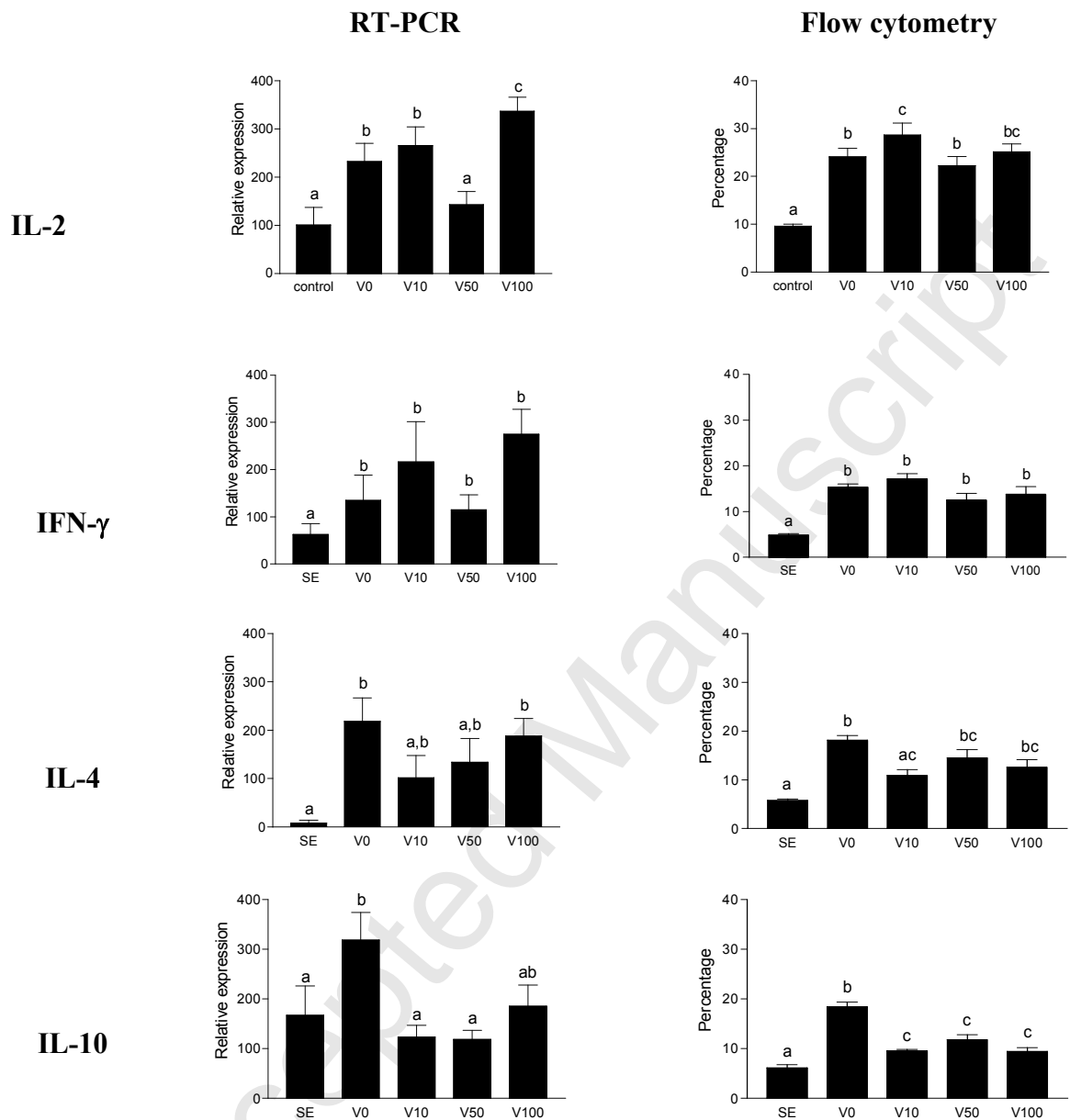
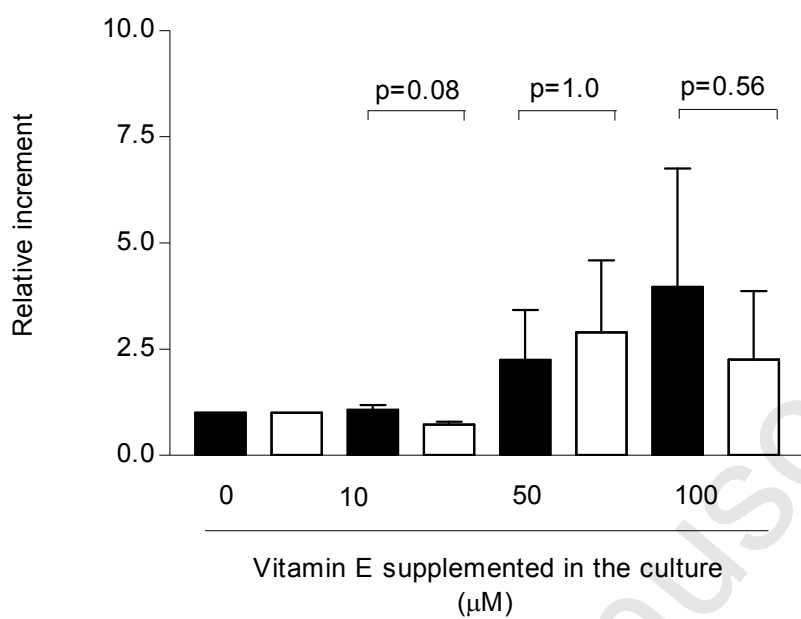


Figure 3

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3 **Figure 4.**

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TABLE 1

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Primer and probe sequences

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Probe sequence (5' to 3')
TBX21 ^a	TGGACCCAAGTCAATTGC T	ACGGCTGGGAACGGGATA	TET ^b - ACCACTACTCTCCTCTCCTCCCAAC CAGT -BHQ1 ^c
GATA3	TCTAGCAAATCCAAAAAGTG CAAA	GGGTTGAACGAGCTGCTCTT	TET- TCCTCCAGCGTGTCTGTCACCT -BHQ1
PPIA ^d	GCCATGGAGCGCTTTGG	TTATTAGATTTGTCCACAGTCAGCAAT	TET- TGATCTTCTGCTGGTCTTGCCATTCT- BHQ1
IL-2	GATTACAGTTGCTTTTGAA	GTTGAGTAGATGCTTTGACA	
IL-4	TACCAGCAACTTCGTCCAC	ATCGTCTTTAGCCTTTCCAA	
IL-10	GCATCCACTTCCCAACCA	CTTCCTCATCTTCATCGTCAT	
IFN- γ	GTTTTTCTGGCTCTTACTGC	CTTCCGCTTTCTTAGGTAG	
GADPH	GTCTTCACCACCATGGAG	CCTAAAGTTGTCATGGATGACC	

^a Sequences for cytokines and PPIA from Porcine Immunology and Nutrition database

(<http://www.ars.usda.gov/Services/docs.htm?docid=6065>)

^b TET (6,carboxy-2',4,7,7'-tetrachlorofluorescein)

^c BHQ1 (black hole quencher)

^d PPIA (Peptidylprolyl isomerase A)