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Immune gene expression in the spleen of chickens experimentally infected with *Ascaridia galli*

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16 **1. Introduction**

17 *Ascaridia galli* is a gastrointestinal nematode infecting chickens (Permin et al., 1999; Permin et al.,
18 1997). Substitution of traditional cages with alternative rearing systems in modern poultry
19 production has led to an increase in the prevalence of *A. galli* and recent reports from Denmark
20 and neighbouring countries show that the majority of chickens kept in free-range systems are
21 indeed infected with *A. galli* (Jansson et al., 2010; Kaufmann et al., 2011; Permin et al., 1999).
22 Infection with *A. galli* may directly contribute to economic losses due to higher feed conversion
23 rates/reduced weight gain and decreased egg production (Permin and Ranvig, 2001; Skallerup et
24 al., 2005). In severe cases, *A. galli* infections are furthermore associated with increased mortality
25 (Das et al., 2010; Gauly et al., 2005; Kilpinen et al., 2005; Permin et al., 2006), increased
26 susceptibility to secondary infections (Dahl et al., 2002; Eigaard et al., 2006; Permin etal., 2006;
27 Saif et al., 2003), impaired vaccine responses (Pleidrup et al., 2014) and even migration of worms
28 into eggs of laying hens (Fioretti et al., 2005; Reid et al., 1973). Previously, *A. galli* control has been
29 based on synthetic anthelmintics, but concerns about parasite drug resistance and left-over
30 residues in food products call for alternative disease control strategies (Sangster, 1999). An
31 attractive alternative is vaccination, but no successful *A. galli* vaccines have yet been developed.

32 Natural acquired immunity is described for avian coccidiosis, another important parasitic disease.
33 Thus, trickle immunization may induce immunity against homologous *Eimeria* challenge (Brake et
34 al., 1997; Joyner and Norton, 1973). Extensive *Eimeria* studies have been performed in order to
35 understand host protective immune responses and aid vaccine development (Lillehoj et al., 2007).
36 Natural acquired immunity against *A. galli* is less well described, but reports exist on variability in
37 disease susceptibility. The outcome of infection may e.g. be influenced by age (Idi et al., 2004;

38 Tongson and McCraw, 1967) and host genetics (Herd and McNaught, 1975; Kaufmann et al., 2011;
39 Permin and Ranvig, 2001). Estimated heritabilities for resistance/susceptibility to *A. galli* infections
40 suggest that selective breeding for disease resistance may be possible (Gauly et al., 2002;
41 Kaufmann et al., 2011; Schou et al., 2003). In addition, several reports describe the presence of
42 very small larvae (with so called arrested development) in the late stages of an *A. galli* infection
43 and acquired immunity was suggested to be related to this phenomenon (Chamanza et al., 1999a;
44 Ferdushy et al., 2014; Herd and McNaught, 1975). Interestingly, Herd et al. (1975) reported that
45 the proportion of larvae with arrested development was very low in chickens treated with an
46 immunosuppressive agent. In general, it appears that development of anti-helminthic vaccines is
47 far more challenging than the development of vaccines directed against viral and bacterial
48 pathogens. This is in part due to their complex life cycles and the changing host-pathogen
49 interactions occurring during different stages of helminth infections. Thus, a detailed
50 understanding of anti-helminth immunity is essential for future disease control.

51 The life cycle of *A .galli* is direct, starting with embryonation of shedded eggs in litter or soil. After
52 10-20 days infective L3 stage larvae are found within the parasite eggs (Permin et al., 1997). When
53 ingested by chickens, the *A. galli* eggs hatch within the first 24 hours either in the proventiculus or
54 the duodenum of the host (Idi et al., 2004; Saif et al., 2003). After three to nine days the larvae
55 enter their histotrophic phase where they move deeper into the mucosal layers of the intestine
56 (Luna-Olivares et al., 2012; Saif et al., 2003; Tugwell and Ackert, 1952). Larvae recovery from the
57 intestinal wall during the first week of infection was highest in the anterior part of the jejunum,
58 but after day 7 post infection (p.i.) larvae was also found in the posterior part of the jejunum
59 (Ferdushy et al., 2013). A high infection dose of parasite eggs may lead to a prolonged histotrophic
60 phase, but usually young adult worms return to the intestinal lumen by day 17-30 of age during

61 which period co-existence of larvae in the intestinal wall and young worms in the intestinal
62 content is seen (Ferdushy et al., 2013; Herd and McNaught, 1975; Katakam et al., 2010). Recently,
63 Luna-Olivares et al. (2012) suggested that “mucosal phase” may be a more appropriate term than
64 “histotrophic phase” (lamina propria invasive) as the larvae may not penetrate as deep into the
65 intestinal tissue as originally thought. They reported that most larvae were observed in the lumen
66 (but in close contact with the epithelium) (63%) followed by “within epithelium” (32%) and only
67 few in the lamina propria (5%). However, only the very early time-point 3 days p.i. was
68 investigated and it is uncertain what happened later in the histotrophic/mucosal phase. However,
69 Katakam et al. (2010) was able to recover all larvae by an EDTA method, i.e. no additional larvae
70 were recovered when applying additional pepsin digestion after EDTA incubation of intestinal
71 samples taken 2 weeks p.i. indicating that lamina propria associated larvae are few also at this
72 time point.
73 The chicken spleen works as a secondary lymphoid organ where innate and adaptive immune
74 responses are efficiently mounted. It is hypothesized that the avian spleen plays an even more
75 important immunological role than in mammals as avian lymphatic vessels and lymph nodes are
76 poorly developed. The aim of this study was to investigate systemic immunological responses at
77 different stages of an *A. galli* infection by comparing gene expression profiles in spleen tissue
78 between infected and control chickens at week 2, 6 and 9 post infection (p.i.).
79

80 **2. Materials and Methods**

81 *2.1. Animals*

82 In the experiment, chickens of mixed gender from the Aarhus University L133 were used. Line 133
83 is of White Leghorn origin and contains only birds with the major histocompatibility complex
84 (MHC) haplotype B13. Water and commercial chicken feed were supplied *ad libitum.* The lighting
85 period was 12 h daily, and the chickens were kept at a temperature of 21°C. All experimental
86 chickens were produced from MHC-characterized parents, and the MHC haplotypes of the
87 offspring were confirmed by genotyping the LEI0258 microsatellite locus (McConnell et al., 1999)
88 by PCR-based fragment analysis as earlier described (Dalgaard et al., 2005). Some birds in the
89 current experiment were shared with an already published experiment (Pleidrup et al., 2014).

90 *2.2. Experimental outline*

91 Experimental chickens were divided into two treatment groups; 1) negative control chickens and
92 2) chickens subjected to *A. galli* infection that were kept in separate rooms of the chicken facility.
93 At 4 weeks of age, chickens in group 2 were orally infected with 1750 embryonated *A. galli* eggs
94 recovered from female worm uteri obtained from naturally infected commercial hens and
95 embryonated in H2SO4 as described in Permin et al. (1997). Sixteen animals from each group were
96 used for weekly blood sampling and seven other animals from each group were sacrificed at week
97 2, 6 and 9 p.i. for spleen collection. At week 6 and 9 p.i. faecal samples were collected before
98 sacrificing the chickens. Licence to conduct the animal experiment was obtained from the Danish
99 Ministry of Justice, Animal Experimentation Inspectorate by Helle R. Juul-Madsen. The experiment
100 was conducted according to the ethical guidelines

101 *2.3. A. galli-specific IgG ELISA*

102 Blood samples from infected animals were taken at weeks 0, 6, 7, 8, 9 p.i. and from negative
103 controls at week 0, 6, 9 p.i. and serum was used for detection of *A. galli*-specific IgG antibodies as
104 earlier described (Norup et al., 2013).

105 *2.4. Faecal A. galli egg excretion*

106 Faecal samples were obtained from *A. galli*-infected chickens before sacrificing them for spleen
107 sampling at weeks 6 and 9 p.i. Faeces was not sampled from chickens sacrificed 2 weeks p.i. as
108 adult egg secreting worms are not developed until week 5-8 p.i. (Permin and Hansen, 1998). The
109 faecal samples were examined for the presence of *A. galli* eggs using a modified McMaster
110 counting technique (Henriksen and Aagaard, 1976; Permin et al., 1997) with a detection limit of 20
111 eggs per gram faeces (EPG).

112

113 *2.5. RNA extraction*

114 After collection, spleens were sectioned (triangular cross-sectional slice from upper part) and
115 identical samples from each chicken were immediately placed in RNAlater (Ambion/Life
116 Technologies), kept overnight at 4°C and then at -20°C until further processing. Amounts of 7 to 15
117 mg tissue were homogenised on a TissueLyzer LT (Qiagen), and RNA isolation and DNA digestion
118 was done using the NucleoSpin 96 RNA kit (Macherey-Nagel) according to the manufacturer's
119 instructions. RNA quality was controlled on a 1 % agarose gel and the RNA concentration and
120 purity were determined using a NanoDrop spectrophotometer (Saveen and Werner AB).

121

122 *2.6. cDNA synthesis and pre amplification of mRNA*

123 cDNA synthesis and preamplification was performed as described previously (Skovgaard et al.,
124 2013). Extracted total RNA was converted into cDNA by reverse transcription of 480 ng RNA using
125 the QuantiTECT Reverse Transcription kit (Qiagen), cDNA was diluted 1:5 in low EDTA TE-buffer
126 (VWR – Bie & Berntsen) prior to preamplification. Preamplification was performed using TaqMan
127 PreAmp Master Mix (Applied Biosystems) and a 200 nM pooled primer mix was prepared
128 combining each primer used in the present study. TaqMan PreAmp Master Mix (5 µl) was mixed
129 with 2.5 µl 200 nM pooled primer mix and 2.5 µl diluted cDNA, and incubated at 95°C for 10 min
130 and 16 cycles of 95°C for 15 sec and 60°C for 4 min. 16 U of Exonuclease I (New England BioLabs)
131 was added to the preamplified cDNA, thermal cycling conditions were set to 37°C for 30 min
132 followed by 80°C for 15 min. Preamplified cDNA was diluted 1:10 in low EDTA TE-buffer (VWR –
133 Bie & Berntsen) before qPCR. Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-
134 0.4.0/) as described in (Skovgaard et al., 2010), and purchased from Sigma-Aldrich. Primer
135 sequences, efficiencies and amplicon length are shown in Table 1.
136
137 *2.7. qPCR*
138 Gene expression mRNA was analysed by quantitative real-time PCR (qPCR) performed in Dynamic
139 Array Integrated Fluidic Circuits (Fluidigm) following the protocol described previously (Skovgaard
140 et al., 2013). The following cycle parameter was used: 2 min at 50°C, 10 min at 95°C, followed by
141 35 cycles with denaturing for 15 sec at 95°C and annealing/ elongation for 1 min at 60°C. Melting
142 curves were generated after each run to confirm a single PCR product (from 60°C to 95°C,
143 increasing 1°C/ 3 sec). Reactions were performed in duplicates (cDNA replicates). Non template
144 controls (NTC) were included to indicate potential problems with non-specific amplification or

145 sample contaminations. Non-reverse transcriptase controls were included to assess potential DNA
146 contamination.
147
148 Expression data (Cq values) were acquired using the Fluidigm Real-Time PCR Analysis software
149 3.0.2 (Fluidigm) and exported to GenEx (MultiD) for data pre-processing including interplate
150 correction, correction for PCR efficiency for each primer assay individually, normalising to six
151 highly stable reference genes, and averaging of cDNA technical repeats. Using GeNorm (17) and
152 NormFinder (18), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2 microglobulin (B2M),
153 peptidylprolyl isomerase A (PPIA), hypoxanthine phosphoribisyl transferase I (HRPT1), TATA-box
154 binding protein (TBP), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation
155 protein, zeta polypeptide (YWHAE) were identified as the most stably expressed reference genes
156 out of eight candidates. For each primer assay, the mean relative expression level of the control
157 group was scaled to one during data transformation log2 (Cq) to linear scale. Gene expression data
158 were $\log_2$-transformed before testing for normal distribution, Student t test was used to analyse
159 normally distributed data, while the non-parametric test (Wilcoxon–Mann–Whitney test) was
160 used when data was non-normal distributed. Gene expression was considered significantly
161 different if the P value was less than 0.05 and the relative expression was greater than 2.0.
162 Experimental practice and reporting have been performed according to the Minimum Information
163 for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).
164

165 **3. Results and Discussion**

166 According to earlier studies, week 2 p.i. represents the mucosal phase of the *A. galli* larvae
167 whereas at weeks 6 and 9 adult worms are present in the intestinal lumen. In the present

168 experiment only 43 % of the animals (data not shown) shedded *A. galli* eggs in faeces at week 6

169 p.i., and we hypothesise that the *A. galli* worms are young and have just recently started

170 producing eggs. Presumably some larvae are also still present in the mucosa at this time point as

171 earlier reported by Ferdushi et al. (2013). In contrast, 73 % of the chickens (data not shown)

172 shedded *A. galli* eggs in faeces at week 9 p.i. and with a higher mean EPG per animal than at week

173 6 (Figure 1a). Thus, this time point may represent more mature adult worms. None of the chickens

174 in the *A. galli*-free group tested EPG positive at any time-point during the experiment (data not

175 shown). Additional chickens allocated to blood sampling were sero-negative at the day of infection

176 (data not shown). Chickens in the blood sampled *A. galli*-inoculated group had seroconverted by

177 week 6 p.i. and showed positive titres of *A. galli-specific* serum IgG throughout the rest of the

178 experiment. Chickens from the blood sampled negative control group were tested at weeks 6 and

179 9 p.i. and were found to be sero-negative at both time-points (Fig. 1.b). A systemic humoral

180 immune response is reported by others as early as 2 weeks p.i., but serum titres do not appear to

181 correlate with egg excretion or worm burden (Marcos-Atxutegi et al., 2009; Norup et al., 2013;

182 Schwarz et al., 2011).

183

184 In order to understand systemic molecular response mechanisms in different stages of an *A. galli*

185 infection we studied gene expression profiles in spleen sampled 2, 6 and 9 weeks after the

186 experimental infection. Twelve genes (representing inflammatory cytokines, antimicrobial

187 peptides, acute phase proteins, soluble pattern recognition receptors and T cell signature

188 cytokines) were differentially expressed ($P < 0.05$) at at least one of the three analysed time points

189 after the *A. galli* infection compared to the control group.

190

191 *3.1. (Pro-)Inflammatory cytokines*

192 Only few studies have been published concerning innate immune responses towards *A. galli* in
193 chickens and focus has been on local responses in the small intestine. Thus, a single study reports
194 increased numbers of mast cells in the chicken jejunum 2 weeks post *A. galli* infection (Darmawi et
195 al., 2013). Another study reports increased numbers of presumably heterophils in the jejunum 3
196 days after an *A. galli* infection (Luna-Olivares et al., 2012). Interestingly, a genetic association
197 study indicated that chicken IFN-γ gene variants may influence *A. galli* susceptibility (Luhken et al.,
198 2011). In the present study, we analysed the expression of inflammatory cytokines in the spleen
199 (Table 2). Surprisingly, the expression of IFN-α, IL-1β, IL-12β and Il-18 was up regulated at week 6
200 p.i., but not at week 2 p.i. or week 9 p.i. The IL-8 expression was up regulated at week 2 as well as
201 week 6 p.i. in *A. galli*-infected chickens. Despite structural differences most avian cytokines display
202 conserved functions compared to their mammalian counterparts (Staeheli et al., 2001), and roles
203 in the chicken inflammatory response have been described for IL-8, IL-1β, IL-18, IL-12β (Balu and
204 Kaiser, 2003; Barker et al., 1993; Laurent et al., 2001; Schneider et al., 2000; Weining et al., 1998;
205 Withanage et al., 2004). Also chicken IFN-α (ChIFN-I) was identified to have a similar function to
206 the mammalian counterpart as a potent antiviral agent (Schultz et al., 1995; Sick et al., 1996). It is
207 now accepted that IFN-α in mammals beside its antiviral properties shows additional
208 immunomodulating effects. Although little is known of IFN-α's role in parasite infections,
209 treatment of helminth disease in mice has been attempted with recombinant IFN-α (Godot et al.,
210 2003).

211 *3.2. Antimicrobial peptides*

212 The expression of DEFβ1 was significantly reduced at week 2 p.i. and significantly increased at
213 weeks 6 and 8 p.i. in spleen tissue of *A. galli*-infected chickens (Table 2). Antimicrobial peptides
214 like defensins play an important role in innate immunity, and activity directed against bacteria,
215 fungi and viruses has been reported (Ganz, 2003). Interestingly, defensins may influence adaptive
216 immune responses as they can affect the maturation of dendritic cells as well as effector T cell
217 recruitment (Yang et al., 2002). In the chicken genome, 14 beta-defensin/gallinacin genes exist and
218 the nomenclature AvBD1-14 was suggested (Lynn et al., 2007). Local expression of several of the
219 AvBD genes and their antimicrobial activity against avian enteric pathogens have been described
220 (Evans and Harmon, 1995; Harmon, 1998; Hong et al., 2012; Sugiarto and Yu, 2004). However, the
221 role of AvBD in innate immunity towards helminth infections is not clear. In humans, some beta-
222 defensins are up regulated by pro inflammatory cytokines (McDermott et al., 2006; Scott and
223 Hancock, 2000). In the present study an increased expression of DEFβ1/AvBD1 coincided with an
224 increase in the expression of pro-inflammatory cytokine genes at week 6 p.i., but not at week 9 p.i.

225 *3.3. Acute phase proteins*

226 Mannose binding protein (MBL) and C-reactive protein (CRP) are soluble pattern recognition
227 receptors. Few reports exist on chicken CRP, but it appears that infections with Eimeria spp. and
228 Histomonas meleagridis induce high levels of CRP (Chamanza et al., 1999). In mammals, MBL binds
229 to microbial surface carbohydrates and mediates opsonophagocytosis directly or through
230 activation of the lectin complement pathway. A conserved function of MBL in the chicken was
231 suggested as cMBL in a heterologous in vitro assay was shown to enhance human complement
232 factor 4 (C4) deposition in a calcium dependent way (Norup and Juul-Madsen, 2007). As in
233 mammals, reduced levels of serum MBL in chickens may lead to increased disease susceptibility to

234 viral and bacterial infections (Juul-Madsen et al., 2007; Schou et al., 2010). Chicken MBL is mainly
235 produced in the liver, but constitutive and inducible local expression of the gene has also been
236 reported (Hogenkamp et al., 2006; Laursen et al., 1998; Nielsen et al., 1998). In this study, MBL
237 expression was significantly increased in spleen tissue of *A. galli*-infected chickens 6 weeks p.i.
238 (Table 2). An *in vivo* function of MBL in intestinal helminth infections has not yet been determined,
239 but preliminary results suggest that faecal shedding of *A. galli* eggs is reduced in infected inbred
240 chickens with high MBL serum levels (unpublished, Norup).

241 *3.4. Th signature cytokines*

242 In mammals, Th2 polarised cells drive responses to helminth infections. Also in the chicken a Th2
243 polarised cytokine response was reported in the jejunum and spleen of *A. galli*-infected chickens 2
244 weeks p.i. (Degen et al., 2005; Kaiser, 2007; Pleidrup et al., 2014; Schwarz et al., 2011). In
245 agreement with former studies, we observed an increased expression of the Th2 signature
246 cytokine IL-13 at 2 weeks p.i. in the spleen of *A. galli*-infected chickens, but not at later stages of
247 the infection (Table 2). This time-point corresponds to the mucosal phase of the infection which
248 co-incides with influx of both αβ (including CD4+ve cells) and γδ T cells in the jejunal mucosa as
249 reported by others (Schwarz et al., 2011). In the present study we observed a slightly decreased
250 expression of the Th1 signature cytokine IFN-γ at week 9 p.i. in spleen tissue of *A. galli*-infected
251 chickens. This observation is in contrast to earlier findings by Degen et al. (2005) who reported
252 decreased relative cytokine mRNA ratios (infected/non-infected) for IFN-γ as early as 2 weeks p.i.
253 Earlier reports do suggest that onset and length of the larvae mucosal phase depend on infection
254 dose which differed between the two experiments.

255 *3.5. Anti-inflammatory cytokines*

256 In human and murine infections the survival strategy of helminth parasites is largely based on
257 immunoregulation by excretory-secretory (ES) products through mechanisms involving regulatory
258 T cells (Taylor et al., 2012). No Foxp3 orthologue has been identified in the chicken, but thymic
259 CD4+CD25+ T cells were characterised as counterparts of mammalian natural Tregs by production
260 of IL-10 and TGF-β (Shanmugasundaram and Selvaraj, 2011). In the present study, an increased
261 expression of TGF-β4 was observed 6 weeks p.i. in spleen tissue of *A. galli*- infected chickens
262 (Table 2). The chicken TGF-β gene-family includes: TGF-β2, TGF-β3 and TGF-β4, of which the latter
263 is the chicken orthologue of mammalian TGF-β1 acting as an anti-inflammatory cytokine
264 (Jakowlew et al., 1997; Pan and Halper, 2003). IL-10 has a conserved function in the chicken acting
265 as an anti-inflammatory cytokine (Rothwell et al., 2004). No increased expression of IL-10 was
266 observed in the present study; instead the expression was lower in the spleen tissue of *A. galli*-
267 infected chickens 6 and 9 weeks p.i. than in controls where expression increased by age (data not
268 shown). We have no explanation for this and further studies in other inbred chicken lines as well
269 as outbred lines need to be conducted in order to elucidate if this is a general response in *A. galli*
270 infections. Further studies of the expression of anti-inflammatory cytokines may also help us to
271 understand why *A. galli* infected chickens appear to have impaired vaccine responses towards
272 third party antigens (Pleidrup et al., 2014).

273 *3.6. Conclusion*

274 In summary, we have investigated the avian systemic immune response to *A. galli* infection by
275 expression analyses of immune genes in spleen. Interestingly, we observed only few differentially
276 expressed genes at week 2 p.i. which corresponds to the larvae mucosal phase. In contrast, by
277 week 6 p.i. where the larvae expectedly have matured and migrated back into the intestinal

278 lumen, we observed increased expression of pro-inflammatory cytokines and acute phase
279 proteins. It is yet to be determined if the observed pro-inflammatory response is caused by *A. galli*
280 specific pathogen-associated molecular pattern molecules (PAMPs), host specific damage-
281 associated molecular pattern molecules (DAMPs) released by tissue damage, DAMP homologues
282 in parasite secretions of even by opportunistic secondary infections.

283

284 **Conflict of interest statement**

285 The authors declare to have no conflicts of interest.

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293

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