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

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1. Introduction

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

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

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

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

which period co-existence of larvae in the intestinal wall and young worms in the intestinal content is seen (Ferdushy et al., 2013; Herd and McNaught, 1975; Katakam et al., 2010). Recently, Luna-Olivares et al. (2012) suggested that “mucosal phase” may be a more appropriate term than “histotrophic phase” (lamina propria invasive) as the larvae may not penetrate as deep into the intestinal tissue as originally thought. They reported that most larvae were observed in the lumen (but in close contact with the epithelium) (63%) followed by “within epithelium” (32%) and only few in the lamina propria (5%). However, only the very early time-point 3 days p.i. was investigated and it is uncertain what happened later in the histotrophic/mucosal phase. However, Katakam et al. (2010) was able to recover all larvae by an EDTA method, i.e. no additional larvae were recovered when applying additional pepsin digestion after EDTA incubation of intestinal samples taken 2 weeks p.i. indicating that lamina propria associated larvae are few also at this time point.

The chicken spleen works as a secondary lymphoid organ where innate and adaptive immune responses are efficiently mounted. It is hypothesized that the avian spleen plays an even more important immunological role than in mammals as avian lymphatic vessels and lymph nodes are poorly developed. The aim of this study was to investigate systemic immunological responses at different stages of an *A. galli* infection by comparing gene expression profiles in spleen tissue between infected and control chickens at week 2, 6 and 9 post infection (p.i.).

2. Materials and Methods

2.1. Animals

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

2.2. Experimental outline

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

2.3. A. galli-specific IgG ELISA

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

2.4. Faecal A. galli egg excretion

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

2.5. RNA extraction

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

2.6. cDNA synthesis and pre amplification of mRNA

cDNA synthesis and preamplification was performed as described previously (Skovgaard et al., 2013). Extracted total RNA was converted into cDNA by reverse transcription of 480 ng RNA using the QuantiTECT Reverse Transcription kit (Qiagen), cDNA was diluted 1:5 in low EDTA TE-buffer (VWR – Bie & Berntsen) prior to preamplification. Preamplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems) and a 200 nM pooled primer mix was prepared combining each primer used in the present study. TaqMan PreAmp Master Mix (5 µl) was mixed with 2.5 µl 200 nM pooled primer mix and 2.5 µl diluted cDNA, and incubated at 95°C for 10 min and 16 cycles of 95°C for 15 sec and 60°C for 4 min. 16 U of Exonuclease I (New England BioLabs) was added to the preamplified cDNA, thermal cycling conditions were set to 37°C for 30 min followed by 80°C for 15 min. Preamplified cDNA was diluted 1:10 in low EDTA TE-buffer (VWR – Bie & Berntsen) before qPCR. Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) as described in (Skovgaard et al., 2010), and purchased from Sigma-Aldrich. Primer sequences, efficiencies and amplicon length are shown in Table 1.

2.7. qPCR

Gene expression mRNA was analysed by quantitative real-time PCR (qPCR) performed in Dynamic Array Integrated Fluidic Circuits (Fluidigm) following the protocol described previously (Skovgaard et al., 2013). The following cycle parameter was used: 2 min at 50°C, 10 min at 95°C, followed by 35 cycles with denaturing for 15 sec at 95°C and annealing/ elongation for 1 min at 60°C. Melting curves were generated after each run to confirm a single PCR product (from 60°C to 95°C, increasing 1°C/ 3 sec). Reactions were performed in duplicates (cDNA replicates). Non template 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

3.1. (Pro-)Inflammatory cytokines

Only few studies have been published concerning innate immune responses towards *A. galli* in chickens and focus has been on local responses in the small intestine. Thus, a single study reports increased numbers of mast cells in the chicken jejunum 2 weeks post *A. galli* infection (Darmawi et al., 2013). Another study reports increased numbers of presumably heterophils in the jejunum 3 days after an *A. galli* infection (Luna-Olivares et al., 2012). Interestingly, a genetic association study indicated that chicken IFN-γ gene variants may influence *A. galli* susceptibility (Luhken et al., 2011). In the present study, we analysed the expression of inflammatory cytokines in the spleen (Table 2). Surprisingly, the expression of IFN-α, IL-1β, IL-12β and Il-18 was up regulated at week 6 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 week 6 p.i. in *A. galli*-infected chickens. Despite structural differences most avian cytokines display conserved functions compared to their mammalian counterparts (Staeheli et al., 2001), and roles in the chicken inflammatory response have been described for IL-8, IL-1β, IL-18, IL-12β (Balu and Kaiser, 2003; Barker et al., 1993; Laurent et al., 2001; Schneider et al., 2000; Weining et al., 1998; Withanage et al., 2004). Also chicken IFN-α (ChIFN-I) was identified to have a similar function to the mammalian counterpart as a potent antiviral agent (Schultz et al., 1995; Sick et al., 1996). It is now accepted that IFN-α in mammals beside its antiviral properties shows additional immunomodulating effects. Although little is known of IFN-α's role in parasite infections, treatment of helminth disease in mice has been attempted with recombinant IFN-α (Godot et al., 2003).

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

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

3.4. Th signature cytokines

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

3.5. Anti-inflammatory cytokines

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

3.6. Conclusion

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

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

Conflict of interest statement

The authors declare to have no conflicts of interest.

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References

Balu, S., Kaiser, P., 2003. Avian interleukin-12beta (p40): cloning and characterization of the cDNA and gene. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research 23, 699-707.

Barker, K.A., Hampe, A., Stoeckle, M.Y., Hanafusa, H., 1993. Transformation-associated cytokine 9E3/CEF4 is chemotactic for chicken peripheral blood mononuclear cells. Journal of virology 67, 3528-3533.

Brake, D.A., Fedor, C.H., Werner, B.W., Miller, T.J., Taylor, R.L., Jr., Clare, R.A., 1997. Characterization of immune response to Eimeria tenella antigens in a natural immunity model with hosts which differ serologically at the B locus of the major histocompatibility complex. Infection and immunity 65, 1204-1210.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55, 611-622.

Chamanza, R., van Veenm, L., Tivapasi, M.T., Toussaint, M.J.M., 1999. Acute phase proteins in the domestic fowl. World's Poultry Science Journal 55, 61-71.

Dahl, C., Permin, A., Christensen, J.P., Bisgaard, M., Muhairwa, A.P., Petersen, K.M., Poulsen, J.S., Jensen, A.L., 2002. The effect of concurrent infections with Pasteurella multocida and Ascaridia galli on free range chickens. Veterinary microbiology 86, 313-324.

Dalgaard, T.S., Vitved, L., Skjodt, K., Thomsen, B., Labouriau, R., Jensen, K.H., Juul-Madsen, H.R., 2005. Molecular characterization of major histocompatibility complex class I (B-F) mRNA variants from chickens differing in resistance to Marek's disease. Scandinavian journal of immunology 62, 259-270.

Darmawi, D., Balqis, U., Hambal, M., Tiuria, R., Frengki, F., Priosoeryanto, B.P., 2013. Mucosal Mast Cells Response in the Jejunum of Ascaridia galli-Infected Laying Hens. Media Peternakan 36, 113-119.

316 Das, G., Kaufmann, F., Abel, H., Gauly, M., 2010. Effect of extra dietary lysine in Ascaridia galli-infected
317 grower layers. Veterinary parasitology 170, 238-243.

318 Degen, W.G., Daal, N., Rothwell, L., Kaiser, P., Schijns, V.E., 2005. Th1/Th2 polarization by viral and helminth
319 infection in birds. Veterinary microbiology 105, 163-167.

320 Eigaard, N.M., Schou, T.W., Permin, A., Christensen, J.P., Ekstrom, C.T., Ambrosini, F., Cianci, D., Bisgaard,
321 M., 2006. Infection and excretion of Salmonella Enteritidis in two different chicken lines with concurrent
322 Ascaridia galli infection. Avian pathology : journal of the W.V.P.A 35, 487-493.

323 Evans, E.W., Harmon, B.G., 1995. A review of antimicrobial peptides: defensins and related cationic
324 peptides. Veterinary clinical pathology / American Society for Veterinary Clinical Pathology 24, 109-116.

325 Ferdushy, T., Luna-Olivares, L.A., Nejsum, P., Roepstorff, A.K., Thamsborg, S.M., Kyvsgaard, N.C., 2013.
326 Population dynamics of Ascaridia galli following single infection in young chickens. Parasitology 140, 1078-
327 1084.

328 Ferdushy, T., Schou, T.W., Norup, L.R., Dalgaard, T.S., Thamsborg, S.M., Nejsum, P., Permin, A., Juul-
329 Madsen, H.R., Kyvsgaard, N.C., 2014. Acquisition of resistance after continuous infection with Ascaridia galli
330 in chickens. Parasitology, 1-8.

331 Fioretti, D.P., Veronesi, F., Diaferia, M., Franciosini, M.P., Proietti, P.C., 2005. Ascaridia galli: a report of
332 erratic migration. Ital J Anim Sci 4, 310-312.

333 Ganz, T., 2003. Defensins: antimicrobial peptides of innate immunity. Nature reviews. Immunology 3, 710-
334 720.

335 Gauly, M., Bauer, C., Preisinger, R., Erhardt, G., 2002. Genetic differences of Ascaridia galli egg output in
336 laying hens following a single dose infection. Veterinary parasitology 103, 99-107.

337 Gauly, M., Homann, T., Erhardt, G., 2005. Age-related differences of Ascaridia galli egg output and worm
338 burden in chickens following a single dose infection. Veterinary parasitology 128, 141-148.

339 Godot, V., Harraga, S., Podoprigora, G., Liance, M., Bardonnet, K., Vuitton, D.A., 2003. IFN alpha-2a protects
340 mice against a helminth infection of the liver and modulates immune responses. Gastroenterology 124,
341 1441-1450.

342 Harmon, B.G., 1998. Avian heterophils in inflammation and disease resistance. Poultry science 77, 972-977.

343 Henriksen, S.A., Aagaard, K., 1976. [A simple flotation and McMaster method (author's transl)]. Nordisk
344 veterinaermedicin 28, 392-397.

345 Herd, R.P., McNaught, D.J., 1975. Arrested development and the histotropic phase of Ascaridia galli in the
346 chicken. International journal for parasitology 5, 401-406.

347 Hogenkamp, A., van Eijk, M., van Dijk, A., van Asten, A.J., Veldhuizen, E.J., Haagsman, H.P., 2006.
348 Characterization and expression sites of newly identified chicken collectins. Molecular immunology 43,
349 1604-1616.

350 Hong, Y.H., Song, W., Lee, S.H., Lillehoj, H.S., 2012. Differential gene expression profiles of beta-defensins in
351 the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines.
352 Poultry science 91, 1081-1088.

353 Idi, A., Permin, A., Murrell, K.D., 2004. Host age only partially affects resistance to primary and secondary
354 infections with Ascaridia galli (Schrank, 1788) in chickens. Veterinary parasitology 122, 221-231.

355 Jakowlew, S.B., Mathias, A., Lillehoj, H.S., 1997. Transforming growth factor-beta isoforms in the developing
356 chicken intestine and spleen: increase in transforming growth factor-beta 4 with coccidia infection.
357 Veterinary immunology and immunopathology 55, 321-339.

358 Jansson, D.S., Nyman, A., Vagsholm, I., Christensson, D., Goransson, M., Fossum, O., Hoglund, J., 2010.
359 Ascarid infections in laying hens kept in different housing systems. Avian pathology : journal of the W.V.P.A
360 39, 525-532.

361 Joyner, L.P., Norton, C.C., 1973. The immunity arising from continuous low-level infection with Eimeria
362 tenella. Parasitology 67, 333-340.

363 Juul-Madsen, H.R., Norup, L.R., Handberg, K.J., Jorgensen, P.H., 2007. Mannan-binding lectin (MBL) serum
364 concentration in relation to propagation of infectious bronchitis virus (IBV) in chickens. Viral immunology
365 20, 562-570.

366 Kaiser, P., 2007. The avian immune genome--a glass half-full or half-empty? Cytogenetic and genome
367 research 117, 221-230.

368 Katakam, K.K., Nejsum, P., Kyvsgaard, N.C., Jorgensen, C.B., Thamsborg, S.M., 2010. Molecular and
369 parasitological tools for the study of Ascaridia galli population dynamics in chickens. Avian pathology :
370 journal of the W.V.P.A 39, 81-85.

371 Kaufmann, F., Das, G., Preisinger, R., Schmutz, M., Konig, S., Gauly, M., 2011. Genetic resistance to natural
372 helminth infections in two chicken layer lines. Veterinary parasitology 176, 250-257.

373 Kilpinen, O., Roepstorff, A., Permin, A., Norgaard-Nielsen, G., Lawson, L.G., Simonsen, H.B., 2005. Influence
374 of Dermanyssus gallinae and Ascaridia galli infections on behaviour and health of laying hens (Gallus gallus
375 domesticus). British poultry science 46, 26-34.

376 Laurent, F., Mancassola, R., Lacroix, S., Menezes, R., Naciri, M., 2001. Analysis of chicken mucosal immune
377 response to Eimeria tenella and Eimeria maxima infection by quantitative reverse transcription-PCR.
378 Infection and immunity 69, 2527-2534.

379 Laursen, S.B., Hedemand, J.E., Nielsen, O.L., Thiel, S., Koch, C., Jensenius, J.C., 1998. Serum levels, ontogeny
380 and heritability of chicken mannan-binding lectin (MBL). Immunology 94, 587-593.

381 Lillehoj, H.S., Kim, C.H., Keeler, C.L., Jr., Zhang, S., 2007. Immunogenomic approaches to study host
382 immunity to enteric pathogens. Poultry science 86, 1491-1500.

383 Luhken, G., Gauly, M., Kaufmann, F., Erhardt, G., 2011. Association study in naturally infected helminth
384 layers shows evidence for influence of interferon-gamma gene variants on Ascaridia galli worm burden.
385 Veterinary research 42, 84.

386 Luna-Olivares, L.A., Ferdushy, T., Kyvsgaard, N.C., Nejsum, P., Thamsborg, S.M., Roepstorff, A., Iburg, T.M.,
387 2012. Localization of Ascaridia galli larvae in the jejunum of chickens 3 days post infection. Veterinary
388 parasitology 185, 186-193.

389 Lynn, D.J., Higgs, R., Lloyd, A.T., O'Farrelly, C., Herve-Grepinet, V., Nys, Y., Brinkman, F.S., Yu, P.L., Soulier,
390 A., Kaiser, P., Zhang, G., Lehrer, R.I., 2007. Avian beta-defensin nomenclature: a community proposed
391 update. Immunology letters 110, 86-89.

392 Marcos-Atxutegi, C., Gandolfi, B., Aranguena, T., Sepulveda, R., Arevalo, M., Simon, F., 2009. Antibody and
393 inflammatory responses in laying hens with experimental primary infections of Ascaridia galli. Veterinary
394 parasitology 161, 69-75.

395 McConnell, S.K., Dawson, D.A., Wardle, A., Burke, T., 1999. The isolation and mapping of 19 tetranucleotide
396 microsatellite markers in the chicken. Animal genetics 30, 183-189.

397 McDermott, A.M., Rich, D., Cullor, J., Mannis, M.J., Smith, W., Reid, T., Murphy, C.J., 2006. The in vitro
398 activity of selected defensins against an isolate of Pseudomonas in the presence of human tears. The British
399 journal of ophthalmology 90, 609-611.

400 Nielsen, O.L., Jorgensen, P.H., Hedemand, J., Jensenius, J.C., Koch, C., Laursen, S.B., 1998.
401 Immunohistochemical investigation of the tissue distribution of mannan-binding lectin in non-infected and
402 virus-infected chickens. Immunology 94, 122-128.

403 Norup, L.R., Dalgaard, T.S., Pleidrup, J., Permin, A., Schou, T.W., Jungersen, G., Fink, D.R., Juul-Madsen, H.R.,
404 2013. Comparison of parasite-specific immunoglobulin levels in two chicken lines during sustained infection
405 with Ascaridia galli. Veterinary parasitology 191, 187-190.

406 Norup, L.R., Juul-Madsen, H.R., 2007. An assay for measuring the mannan-binding lectin pathway of
407 complement activation in chickens. Poultry science 86, 2322-2326.

408 Pan, H., Halper, J., 2003. Cloning, expression, and characterization of chicken transforming growth factor
409 beta 4. Biochemical and biophysical research communications 303, 24-30.

410 Permin, A., Bisgaard, M., Frandsen, F., Pearman, M., Kold, J., Nansen, P., 1999. Prevalence of
411 gastrointestinal helminths in different poultry production systems. British poultry science 40, 439-443.

412 Permin, A., Bojesen, M., Nansen, P., Bisgaard, M., Frandsen, F., Pearman, M., 1997. Ascaridia galli
413 populations in chickens following single infections with different dose levels. Parasitology research 83, 614-
414 617.

415 Permin, A., Christensen, J.P., Bisgaard, M., 2006. Consequences of concurrent Ascaridia galli and Escherichia
416 coli infections in chickens. Acta veterinaria Scandinavica 47, 43-54.

417 Permin, A., Ranvig, H., 2001. Genetic resistance to Ascaridia galli infections in chickens. Veterinary
418 parasitology 102, 101-111.

419 Pleidrup, J., Dalgaard, T.S., Norup, L.R., Permin, A., Schou, T.W., Skovgaard, K., Vadekaer, D.F., Jungersen,
420 G., Sorensen, P., Juul-Madsen, H.R., 2014. Ascaridia galli infection influences the development of both
421 humoral and cell-mediated immunity after Newcastle Disease vaccination in chickens. Vaccine 32, 383-392.

422 Reid, W.M., Mabon, J.L., Harshbarger, W.C., 1973. Detection of worm parasites in chicken eggs by candling.
423 Poultry science 52, 2316-2324.

424 Rothwell, L., Young, J.R., Zoorob, R., Whittaker, C.A., Hesketh, P., Archer, A., Smith, A.L., Kaiser, P., 2004.
425 Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima.
426 Journal of immunology 173, 2675-2682.

427 Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E., 2003. Diseases of Poultry.
428 Wiley.

429 Sangster, N.C., 1999. Anthelmintic resistance: past, present and future. International journal for
430 parasitology 29, 115-124; discussion 137-118.

431 Schneider, K., Puehler, F., Baeuerle, D., Elvers, S., Staeheli, P., Kaspers, B., Weining, K.C., 2000. cDNA cloning
432 of biologically active chicken interleukin-18. Journal of interferon & cytokine research : the official journal
433 of the International Society for Interferon and Cytokine Research 20, 879-883.

434 Schou, T., Permin, A., Roepstorff, A., Sorensen, P., Kjaer, J., 2003. Comparative genetic resistance to
435 Ascaridia galli infections of 4 different commercial layer-lines. British poultry science 44, 182-185.

436 Schou, T.W., Permin, A., Christensen, J.P., Cu, H.P., Juul-Madsen, H.R., 2010. Mannan-binding lectin (MBL)
437 in two chicken breeds and the correlation with experimental Pasteurella multocida infection. Comparative
438 immunology, microbiology and infectious diseases 33, 183-195.

439 Schultz, U., Kaspers, B., Rinderle, C., Sekellick, M.J., Marcus, P.I., Staeheli, P., 1995. Recombinant chicken
440 interferon: a potent antiviral agent that lacks intrinsic macrophage activating factor activity. European
441 journal of immunology 25, 847-851.

442 Schwarz, A., Gauly, M., Abel, H., Das, G., Humburg, J., Rohn, K., Breves, G., Rautenschlein, S., 2011.
443 Immunopathogenesis of Ascaridia galli infection in layer chicken. Developmental and comparative
444 immunology 35, 774-784.

445 Scott, M.G., Hancock, R.E.W., 2000. Cationic Antimicrobial Peptides and Their Multifunctional Role in the
446 Immune System. 20, 24.

447 Shanmugasundaram, R., Selvaraj, R.K., 2011. Regulatory T cell properties of chicken CD4+CD25+ cells.
448 Journal of immunology 186, 1997-2002.

449 Sick, C., Schultz, U., Staeheli, P., 1996. A family of genes coding for two serologically distinct chicken
450 interferons. The Journal of biological chemistry 271, 7635-7639.

451 Skallerup, P., Luna, L.A., Johansen, M.V., Kyvsgaard, N.C., 2005. The impact of natural helminth infections
452 and supplementary protein on growth performance of free-range chickens on smallholder farms in El
453 Sauce, Nicaragua. Preventive veterinary medicine 69, 229-244.

454 Skovgaard, K., Cirera, S., Vasby, D., Podolska, A., Breum, S.O., Durrwald, R., Schlegel, M., Heegaard, P.M.,
455 2013. Expression of innate immune genes, proteins and microRNAs in lung tissue of pigs infected
456 experimentally with influenza virus (H1N2). Innate immunity 19, 531-544.

457 Skovgaard, K., Mortensen, S., Boye, M., Hedegaard, J., Heegaard, P.M., 2010. Hepatic gene expression
458 changes in pigs experimentally infected with the lung pathogen Actinobacillus pleuropneumoniae as
459 analysed with an innate immunity focused microarray. Innate immunity 16, 343-353.

460 Staeheli, P., Puehler, F., Schneider, K., Gobel, T.W., Kaspers, B., 2001. Cytokines of birds: conserved
461 functions--a largely different look. Journal of interferon & cytokine research : the official journal of the
462 International Society for Interferon and Cytokine Research 21, 993-1010.

463 Sugiarto, H., Yu, P.L., 2004. Avian antimicrobial peptides: the defense role of beta-defensins. Biochemical
464 and biophysical research communications 323, 721-727.

465 Taylor, M.D., van der Werf, N., Maizels, R.M., 2012. T cells in helminth infection: the regulators and the
466 regulated. Trends in immunology 33, 181-189.

467 Tongson, M.S., McCraw, B.M., 1967. Experimental ascaridiasis: influence of chicken age and infective egg
468 dose on structure of Ascaridia galli populations. Experimental parasitology 21, 160-172.

469 Tugwell, R.L., Ackert, J.E., 1952. On the tissue phase of the life cycle of the fowl nematode Ascaridia galli
470 (Schrank). The Journal of parasitology 38, 277-288.

471 Weining, K.C., Sick, C., Kaspers, B., Staeheli, P., 1998. A chicken homolog of mammalian interleukin-1 beta:
472 cDNA cloning and purification of active recombinant protein. European journal of biochemistry / FEBS 258,
473 994-1000.

474 Withanage, G.S., Kaiser, P., Wigley, P., Powers, C., Mastroeni, P., Brooks, H., Barrow, P., Smith, A., Maskell,
475 D., McConnell, I., 2004. Rapid expression of chemokines and proinflammatory cytokines in newly hatched
476 chickens infected with Salmonella enterica serovar typhimurium. Infection and immunity 72, 2152-2159.

477 Yang, D., Biragyn, A., Kwak, L.W., Oppenheim, J.J., 2002. Mammalian defensins in immunity: more than just
478 microbicidal. Trends in immunology 23, 291-296.

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480