From the Clinic for Poultry and Fish Medicine

Department for Farm Animals and Veterinary Public Health

University of Veterinary Medicine Vienna, Austria

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Resolving local and systemic immune response in turkeys and chickens to *Histomonas meleagridis* vaccination and infection by gene expression profiles and quantification of immune cells

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Submitted by

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1. Abbreviations

18S rRNA: 18S ribosomal RNA
28S rRNA: 28S ribosomal RNA
ABL: abelson murine leukemia
ACTA1: actin alpha 1
ACTB: actin beta
ALB: albumin
ATP5B/ATP5G1: adenosine tri phosphate synthase complex 5B/G1
B2M: β_2 microglobulin
CC: control chickens
CT: control turkeys
DPI: day post inoculation
EEF1: eukaryotic elongation factor 1
FCM: flow cytometry
FCS: fetal calf serum
G6PDH: glucose-6-phosphate dehydrogenase
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HPRT: hypoxanthine-guanine phosphoribosyltransferase
GUSB: glucuronidase Beta
HMBS: hydroxymethylbilane synthase
IC infected chickens
IELs: intraepithelial lymphocytes
IT: infected turkeys
LDHA: lactate dehydrogenase-A
LS: lesion score
MRPS27: mitochondrial small ribosomal protein subunit 27
MT-CO2:mitochondrially encoded cytochrome C Oxidase II
PBMCs: peripheral blood mononuclear cells
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PGK: phosphoglycerate kinase
RNA: ribosomal nucleic acid
RPL13: ribosomal protein L13
RPL19: ribosomal protein L19
RPL30: ribosomal protein L30
RPL32: ribosomal protein L32
RPL4: ribosomal protein L4
RPL5: ribosomal protein L5
RPS30: ribosomal protein L30
RPS7: ribosomal protein L7
RT-qPCR: reverse transcriptase-quantitative polymerase chain reaction
SDHA: succinate Dehydrogenase A
TBP: TATA box binding protein
TFRC: transferrin receptor protein 1
TUBAT: testis-specific alpha-tubulin
TUBB: tubulin Beta
UB: ubiquitin
VC: vaccinated chickens
VIC: vaccinated and infected chickens
VIM: vimentin
VIT: vaccinated and infected turkeys
VT: vaccinated turkeys
YWHAZ: 14-3-3 protein zeta/delta

2. Publications included in the thesis

2.1. Main publications

- **The 60S ribosomal protein L13 is the most preferable reference gene to investigate gene expression in selected organs from turkeys and chickens, in context of different infection models.**

  Mitra T, Bilic I, Hess M, Liebhart D

  Veterinary Research, 2016 October 20; 47(1):105.

  Impact factor of the journal (2015): 2.928

  Authors´ contribution: DL, TM, IB, and MH conceived and designed the experiments. TM and DL performed the experiments. TM, IB and DL analyzed and interpreted the data. TM, DL, IB and MH drafted the manuscript. All authors read and approved the final manuscript.

- **Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens**

  Mitra T, Gerner W, Saalmüller A, Kidane FA, Wernsdorf P, Hess M, Liebhart D

  Vaccine, 2017 June 26; 35(33):4184-4196.

  Impact factor of the journal (2015): 3.413

  Authors´ contribution: DL, MH, AS, WG and TM conceived and designed the work. TM, DL, KFA and PW performed the animal trial and extraction of lymphocytes. TM performed data acquisition and analysis. TM, DL and WG interpreted the data. TM, DL and WG drafted the manuscript. MH and AS revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

2.2. Additional works

- ***In situ* hybridization to detect and localize signature cytokines of T-helper (Th) 1 and Th2 immune responses in chicken tissues.**

  Kidane FA, Bilic I, Mitra T, Wernsdorf P, Hess M, Liebhart D.

  Veterinary Immunology and Immunopathology, 2016 May 12; 175:51-56.

  Impact factor of the journal (2015): 1.664

  Authors´ contribution: DL, KFA and MH conceived and designed the experiments. KFA, TM and DL performed the experiments. KFA, DL, IB and TM, and analyzed and interpreted the data. KFA, DL, TM, IB and MH and drafted the manuscript. All authors read and approved the final manuscript.

- **Expression patterns of IFN-γ and IL-13 differ with the virulence of *Histomonas meleagridis* in turkeys and chickens**

  Mitra T, Kidane FA, Wernsdorf P, Hess M, Liebhart D.

  In the process of publication

  Authors´ contribution: DL, TM, KFA and MH and conceived and designed the experiments. TM, KFA, DL and PW performed animal trial. TM performed, analyzed and interpreted the data of RT-qPCR. TM, DL and MH drafted the manuscript.

2.3. Abstract publications

- **Different kinetics of B cells and T cell subsets ($CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$) are characteristic following vaccination of turkeys and chickens against histomonosis**

  Mitra T, Gerner W, Saalmüller A, Kidane FA, Wernsdorf P, Hess M, Liebhart D.

  WVPA XXth Congress 2017, 4th to 8th September 2017. Edinburgh, UK.

- **Virulent *Histomonas meleagridis* causes pronounced changes in T cell subsets and B cells of turkeys and chickens compared to attenuated parasites.**

  Mitra T, Gerner W, Saalmüller A, Kidane FA, Wernsdorf P, Hess M, Liebhart D.

  XIVth Avian Immunology Research Group (AIRG) Meeting, September-2016, Herrsching, Germany.

- **RPL13 is the most preferable reference gene for gene quantification studies considering selected tissues in turkeys and chickens for different infection models.**

  Mitra T, Bilic I, Hess M, Liebhart D.

  XIVth Avian Immunology Research Group (AIRG) Meeting, September-2016, Herrsching, Germany.

- **Expression patterns of interferon gamma and interleukin 13 differ with the virulence of *Histomonas meleagridis* used to infect turkeys and chickens.**

  Kidane FA, Wernsdorf P, Mitra T, Hess M, Liebhart D.

  XIVth Avian Immunology Research Group (AIRG) Meeting, September-2016, Herrsching, Germany.

- **Divergent cellular immune response of turkeys and chickens against *Histomonas meleagridis.***

  Mitra T, Gerner W, Saalmüller A, Kidane FA, Wernsdorf P, Hess M, Liebhart D.

  3rd International Symposium on Parasite Infections in Poultry, July-2016, Vienna, Austria.

- **Changes in T- and B-cell frequencies in different organs and blood of turkeys infected with virulent or attenuated *Histomonas meleagridis*.**

  Mitra T, Gerner W, Saalmüller A, Kidane FA, Wernsdorf P, Hess M, Liebhart D.

  5th European Veterinary Immunology Workshop, September-2015, Vienna, Austria.

- **Suitability of cross-reactive monoclonal antibodies to investigate turkey immune cells by flow cytometry**

  Mitra T, Gerner W, Hess M, Liebhart D.

  PaP Symposium, January-2015, University of Veterinary Medicine, Vienna, Austria.

- **Determination of suitable reference genes for turkey and chicken tissue samples to investigate gene expressions during *Histomonas meleagridis* infection**

  Mitra T, Bilic I, Hess M, Liebhart D.

  PaP Symposium, January-2016, University of Veterinary Medicine, Vienna, Austria.

3. Acknowledgements

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do this without the support and guidance that I received from many people.

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My special thanks go to Dr. Ivana Bilic, Clinic for Poultry and Fish Medicine and Ass. Prof. Dr. Wilhelm Gerner, Institute of Immunology for patience, immense knowledge and guidance on molecular and immunological works. Their valuable advices have helped me to overcome the obstacles in my laboratory works.

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My appreciation extends to all colleagues at Clinic for Poultry and Fish Medicine and to all of my friends outside of the clinic, who supported me during the PhD, and incented me to strive towards my goal. Without their support and help, this thesis would not have been possible.

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Taniya Mitra

Vienna, 2017

4. Declaration

I hereby declare that the work included in this thesis with the title 'Resolving local and systemic immune response in turkeys and chickens to *Histomonas meleagridis* infection and vaccination by gene expression profiles and quantification of immune cells' was performed during my PhD study at Clinic for Poultry and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria. I the author confirm that the rules of Good Scientific Practice have been followed in all aspects. In addition, I certify that neither whole nor part of this work have been submitted before to any other academic institutions for obtaining any sort of degrees or academic awards.

Taniya Mitra

Vienna, 2017

5. Summary

The protozoan parasite *Histomonas meleagridis* is the causative agent of histomonosis in gallinaceous birds. In turkey flocks, the disease can reach high mortalities due to severe pathological lesions characterized by necrotic inflammation of the caeca and the liver. In chickens, the disease is milder and pathological manifestations are generally less severe. It can be hypothesized that the extracellular location of the parasite activates the type 2 pathway of host birds and involvement of cellular immune responses. Experimental vaccination was shown to be effective to protect birds from histomonosis. However, data on the immune response against histomonads is limited, especially in a vaccination model. Therefore, the aim of the present study was to comparatively investigate the relevant key cytokines for Th pathways, T cells and B cells of turkeys and chickens, against attenuated and/or virulent histomonads. For that, different groups of birds of both species were experimentally inoculated with i) attenuated histomonads, ii) virulent histomonads, iii) attenuated and virulent histomonads or iv) were kept as negative control birds without infection. Three birds per group were killed on days 4, 7, 10, 14 and 21 post inoculation (dpi) and birds were monitored for clinical signs throughout the experiment. Following necropsy, lesion scores in caecum and liver were assessed and various organs together with blood were collected for different experiments.

During post-mortem investigations most severe lesions were observed in caecum and liver of non-vaccinated turkeys inoculated with virulent histomonads which succumbed to the disease. None of the vaccinated or vaccinated and challenged birds died and lesions were much less severe. In chickens lesion scores were generally lower but a similar graduation related to the used inoculum was observed.

In a first step, RT-qPCR was applied to investigate gene expression of key cytokines of the Th1 and Th2 pathways (IFN-γ respectively IL-13). For that, it was necessary to normalize the

experiment by reference genes for turkeys and chickens. Transcripts of eight candidate genes, such as HMBS, HPRT1, TBP, VIM, TFRC, RPLP0, RPL13 and RPS7 representing different metabolic processes affecting either heme production or purine synthesis, were quantified and investigated for their ability as suitable reference genes. In order to analyze a broad variation of physiological conditions spleen, liver, caecum and caecal tonsil of different aged SPF layer chickens together with commercial turkeys, with or without infection with the extracellular pathogen *H. meleagridis,* were included. Five different algorithms (GeNorm, NormFinder, BestKeeper, delta CT, RefFinder) were applied to survey the stability of each reference gene. According to these evaluations, in tissue samples of chickens RPL13 and TBP and were found to be highly stable reference genes using all five algorithms. Further testing of the selected reference genes in spleen, liver, caecum and caecal tonsil of uninfected and infected SPF broiler chickens with the intracellular pathogen fowl aviadenovirus confirmed their stability. In organs of turkeys infected with histomonads or collected from non-infected control birds, RPL13 and TFRC genes showed highest stabilities. The selected reference genes were further used for the gene expression profile of the cytokines.

For assessing cytokine expressions of the mentioned animal experiment by RT-qPCR, samples collected 10 and 21 dpi were selected to investigate caecum, liver and spleen of all groups of turkeys and chickens. At 10 dpi infected turkeys showed up-regulations of IL-13 and IFN-γ in every organ compared to samples of the control birds, whereas in vaccinated and challenged turkeys a higher expression of both cytokines was restricted to the caecum. Only vaccinated birds did not show distinct changes at 10 dpi. At 21 DPI, non-vaccinated turkeys did not survive the challenge, whereas in the other two groups, expression of IFN-γ and IL-13 was found to be up-regulated in caecum. Expression of IFN-γ was down-regulated in spleen. In chickens an up-regulation of IFN-γ was only measured in the caecum of infected birds at 10 dpi, when both cytokines increased in the liver of the same birds. Only vaccinated

chickens showed an up-regulation in the liver and a down regulation in the spleen. Later on, at 21 dpi, IFN-γ as well as IL-13 was found to be up-regulated in the caecum of all inoculated chickens. Hence, it can be concluded that there is a clear difference in the adaptive immune response between turkeys and chickens inoculated with *H. meleagridis* which is related to the different clinical outcome of the disease for both species. Furthermore, in both host species differences in the immune responses could be to be related to the virulence of the parasite.

For further characterization of the mentioned findings, analysis B cells and T cell subsets ($CD3^+CD4^+CD8\alpha^-$ and $CD3^+CD4^-CD8\alpha^+$) from mononuclear cells of caecum, liver, spleen and blood were performed by flow cytometry to elucidate the local and systemic cellular immune response. Thereby a decrease of B cells in the spleen of turkeys shortly after infection (4 dpi) with virulent histomonads was observed followed by a robust increase of the same cells in the liver and the caecum at the following sampling days. In the same birds $CD4^+$ T cells were reduced in the spleen at 4 dpi as well as on the upcoming sampling days in the caecum. $CD8^+$ T cells were not affected in the spleen of those birds but increased in the caecum after one week post inoculation. Compared to turkeys, chickens infected with virulent histomonads showed less changes in B cells and T-cell subpopulations both at the local and systemic level. In addition, in both bird species attenuated histomonads caused less pronounced changes in subpopulations of lymphocytes compared to virulent parasites. The challenge of vaccinated turkeys significantly increased all lymphocyte subpopulations in the blood already at 4 dpi, indicating a fast recall response of the previously primed immune system.

In conclusion, an earlier and more intense immune response for adaptive stage cytokines as well as for the different T cells and B cells was observed in organs of infected turkeys compared to infected chickens, which is in agreement with the different clinical and pathological outcome of the disease in both host species.

6. General introduction to histomonosis

Histomonosis (syn. Histomoniasis, blackhead, infectious enterohepatitis) is a fatal disease of gallinaceous birds resulting in severe typhlohepatitis, caused by the protozoan parasite *H. meleagridis* (Smith, 1895). The disease was firstly described by (Cushman, 1893). Several species of birds can be affected by this parasite but outbreaks in chicken and turkey flocks can cause severe morbidity and up to 100% mortality. *H. meleagridis* is a member of the family Dientamoebidae, order Tritrichomonadida, class Tritrichomonadea (Cepicka et al., 2010). Histomonosis can be treated with nitroimidazoles and prevented with nitrofurans or arsenicals. The ban of all available therapy and prophylaxis in the USA and Europe (CEC, 1995; CEC, 2002; FDA, 2015a; FDA, 2015b) caused a re-emerge of the disease and outbreaks of the disease lead to economic losses for the poultry industry (Liebhart et al., 2017). This situation demands for new strategies for prevention and therapy.

6.1. Diagnosis and pathology

The first signs of histomonosis are reflected by clinical changes such as depression, drowsiness, droopy wings and roughed feathers. Some days later birds show yellowish diarrhoea and death (Hess et al., 2015). The pathogen primarily targets the caecum before it reaches the liver through the hepatic portal vein. Histomonosis can be a fatal disease resulting in typhlohepatitis with pathological lesions characterized by a severe inflammation of the caeca and focal necrosis in the livers (Tyzzer, 1920), frequently observed in infected turkeys. In chickens the clinical picture of histomonosis is usually less severe but can cause similar organ lesions and a drop in laying performance (Esquenet et al., 2003; Gerth et al., 1984; Liebhart et al., 2013).

6.2. Vaccination

The increasing interest concerning this disease, demands for improved control of histomonosis, in order to minimize the direct negative impact on animal welfare and economic losses (McDougald, 2005). Protection by vaccination would help to prevent histomonosis by triggering the bird's immune system to provide a prophylactic way of controlling it. Earlier, Tyzzer described a protective effect of *in vitro* attenuated histomonads in chickens and turkeys, but the immunizing properties decreased during an extended time of *in vitro* cultivation (Tyzzer, 1936). Later on, it was reported that virulent histomonads did not cause the disease when the birds were initially inoculated with a non-pathogenic strain specified as *Histomonas wenrichi* (Lund, 1963). At the same time, Clarkson found precipitating antibodies in turkeys and chickens recovering from the disease. However, it was not possible to protect naïve turkeys by transferring serum of infected birds (Clarkson, 1963). Recent studies on inactivated vaccines reported no effective protection of turkeys against histomonosis (Bleyen et al., 2009; Hess et al., 2008). In comparison, it could be demonstrated that vaccination using *in vitro* attenuated histomonads protects turkeys against a severe challenge (Hess et al., 2008). In that study a clonal culture of *H. meleagridis* was attenuated by long-term cultivation (up to 295 *in vitro* passages) and shown to protect turkeys from a severe challenge. The same *in vitro* attenuated vaccine candidate also prevented layers from a drop in egg production caused by histomonosis (Liebhart et al., 2013). Additionally further studies confirmed safety and stability of this vaccination in chickens and turkeys (Liebhart et al., 2011; Sulejmanovic et al., 2013). Most recently, it has been demonstrated that cross-protection is possible by the above mentioned attenuated clonal culture against genetically different isolates of *H. meleagridis* in turkeys (Sulejmanovic et al., 2016). However, the establishment of the vaccine raised questions regarding protective mechanisms and to resolve immunological traits in turkeys and chickens involved in protection.

6.3. Host immune interactions during histomonosis

So far, very little is known about the immune mechanisms in chickens and turkeys against *H. meleagridis*. Previously, it was demonstrated that in turkeys interaction with the parasite involves T cells at the sites of infiltration (Singh et al., 2008). Later on, expression profiles of cytokines and the involvement of different immune cells in histological samples were investigated during histomonosis in chickens and turkeys (Powell et al., 2009). The last mentioned study indicated a Th2 type pathway during infection with histomonads. Another study on *Heterakis gallinarum* mono-infection and co-infection with *H. meleagridis* in layer chickens measured the presence of immune cells, cytokine expression and electrophysiological responses in the intestine in Ussing chambers but the results could not exclusively be related to histomonosis due to the mixed infection (Schwarz et al., 2011). In this thesis, a detailed comparison of the immune reaction of turkeys with responses of chickens inoculated with attenuated and/or virulent *H. meleagridis* has been demonstrated, by investigating cytokine expressions together with changes in the major immune cell populations in caecum, liver, spleen and blood. This delivers insights in the local and systemic immunological responses of vaccinated and/or infected birds implicating the role of the host species, as explicated in this thesis.

7. General information on reference genes used for RT-qPCR normalisation

Assessing cytokines mRNA expression by RT-qPCR requires normalization of the results to exclude non-biological effects introduced during gene expression studies (Bustin, 2002; Pfaffl, 2004). There are several methods reported in literature (table 1). Mostly recognized is the determination of stable genes in the respective samples to be used as reference. The variability of a reference gene represents the cumulative error of the entire process and must

therefore be assessed in detail. So far, only a low number of studies reported valid genes in chickens or turkeys to be used as reference for gene expression which are listed in table 2. Especially, reference genes to be used in spleen, liver and intestine in context of a parasitic disease in birds like histomonosis have not been validated.

Table 1: Methods to normalize gene expression investigated by RT-qPCR

method	Specifications	reference	remarks
generic normalization against total mRNA	during reverse transcription this method would incorporates a long tailed sequence to each mRNA and after double stranded cDNA synthesis the tailed sequence can be quantified	Argyropoulos et al., 2006	mispriming of tailed oligo-dT may affect the quantification of mRNA
gene expression normalization strategy based on DNA	measures the DNA content of a total nucleic acids extract by PCR and uses this as normalization factor.	Talaat et al., 2002	extraction yields may vary between different samples, with the DNA yields being suboptimal.
	DNA content is determined spectroscopically and the sample is spiked with a cocktail of artificial RNA molecules that is proportional to the DNA content.	Kanno et al., 2006	
in situ calibration	based on knowledge about the biological system, selects a gene whose expression is correlated or anti-correlated with that of the gene of interest.	Ståhlberg et al., 2003	this approach does not really allow the comparison of expression levels of a given gene between samples, it is only effective when the expression ratio of two marker genes is significant for a disease
amplification of Arthrobacter luteus (Alu) restriction endonuclease	using a repetitive sequence in the human transcriptome as a measure for mRNA fraction	Marullo et al., 2010	well characterized only for human genes

Table 2: Survey of studies validating reference genes in chickens and turkeys.

experimentation model	target gene	validated gene/s	reference
lipopolysaccharide inflammation in chicken	GAPDH, HPRT, β-Actin,G6PDH,UB	GAPDH and UB	De Boever et al., 2008
chicken embryo fibroblasts cell culture infected with H5N1 avian influenza virus	ALB, B2M, HPRT1, RPL30, RPL4, SDHA, TBP, TUBB, YWHAZ, ACTB	ACTB and RPL4	Yue et al., 2010
influenza virus infected chicken lung derived cells	18S rRNA, ACTB, GAPDH, ATP5B/ATP5G1	18S rRNA	Kuchipudi et al., 2012
chicken embryo fibroblasts infected with avian leukosis virus subgroup J	B2M, ACTB, TUBB, GAPDH, ALB, TBP, YMHAZ, RPL4, HPRT1, RPL30, SDHA	RPL30 and SDHA	Yang et al., 2013
brain of domestic chicken, domestic turkey	18S, ABL, GAPDH, GUSB, HMBS, HPRT, PGK1, RPL13, RPL19, RPS7, SDHA, TFRC, VIM, YWHAZ	18S, PGK1, RPS7, TFRC, YWHAZ	Olias et al., 2014
pectoralis major, biceps femoris, liver and abdominal fat of chicken	B2M, RPL32, SDHA, TBP, YWHAZ	YWHAZ and TBP for biceps femoris and liver, YWHAZ and RPL32 for pectoralis major and RPL32 and B2M for abdominal fat	Bagés et al., 2015
pectoralis major muscle in different development periods and under different diets of chicken	ACTA1, ACTB, B2M, EEF1, GAPDH, HMBS, HPRT1, LDHA, MRPS27, RPS30, RPL5, TFRC, UBC, MT-CO2	HMBS and HPRT1	Nascimento et al., 2015
leukocytes from Bursa of Fabricius, spleen and thymus of chicken	ACTB, B2M, GAPDH, GUSB, TBP, TUBAT, 28S	TBP, GAPDH, r28S	Borowska et al., 2016
thymus, Bursa of Fabricius, spleen, caecal tonsil, heart, liver, kidney, lung, duodenum, ileum, colon, skin and cells of chick Embryo Fibroblasts infected with avian influenza virus	ACTB, B2M HMBS, HPRT1, PGK1, PLA2, PPIA, RPL13, RPL0, TBP	all reported stable depending on tissue type and experimental study	Staines et al., 2016

8. Expression analysis of key cytokines of Th1/Th2 pathway

More recently, the chicken was the first agricultural species for which a genome sequence map was published (Hillier et al., 2004). This information has allowed for an in depth analysis and identification of immunological genes in the chicken such as cytokines and chemokines (Kaiser et al., 2005). The Th1 and Th2 arms of the immune system are vital in the control of infectious disease. In 2004, the ability of a non-mammalian species, the chicken, was demonstrated to mount a Th1 and Th2 immune response through the quantification of signature cytokines during intracellular and extracellular infection (Avery et al., 2004; Degen et al., 2005). IFN-γ and IL-13 are regarded as key markers in the study of chicken Th1/Th2 pathways (Degen et al., 2005; Powell et al., 2009). The mentioned cytokines are secreted by a broad range of cell types that play pivotal roles in the regulation of inflammation and the immune response. Difference in the function of Th1 and Th2 pathway in regards to cytokines and cells, is given below in table 3:

Table 3: Features of Th1 and Th2 pathways

characteristics	Th1	Th2	
key cytokines involved in development of the pathways	IFN-γ, IL-12	IL-13, IL-4	
cytokine secretion profile	IFN-γ, IL-12, IL-2, IL18, TNF-β	IL-13, IL-1β, IL-4, IL-5, IL-6, IL-10	Abbas et al., 1996; Singh et al., 1999; Degan et al., 2005
effect on inflammatory response	pro-inflammatory	anti-inflammatory	
type of immune response	cell mediated immune response	humoral response	
cells primarily effected	NK cells, macrophages, $CD8^+$ T cells	B cells	

9. Cellular immune response of the host

The existence of functionally polarized responses by the $CD4^+$ T helper (Th) and the $CD8^+$ T cytotoxic (Tc) cell subsets plays a major role in host-pathogen interaction and outcome of diseases. So far very little is known on the host-pathogen interaction between turkey and chicken species with *H. meleagridis*. The parasite *H. meleagridis* primarily targets the caecum mucosa and submucosa and cause severe necrosis, which leads to inflammation and thickening of the caecal wall and formation of fibrin. Due to the destruction of intestinal cells, it reaches the liver via the hepatic portal vein and causes focal necrosis (Hess et al., 2013). In the final stage, the disease may become systemic when the parasite spreads to various organs of the host (Grabensteiner et al., 2006; Singh et al., 2008). Infiltration of leukocytes due to histomonosis has been reported earlier (Tyzzer, 1936). More recent studies demonstrated the involvement of T cells (Singh et al., 2008). $CD3^+CD4^+CD8^-$ T cells are predominantly of helper phenotype; act as coordinators of the immune response by producing a variety of cytokines, soluble molecules secreted to the extracellular space which affect other cells of the immune system. In contrast, the $CD3^+CD4^-CD8\alpha^+$ T cells are cytotoxic cells, promoting the cytolytic pathway. A protective immune response often relies on the ability of $CD4^+$ T cells to accumulate high numbers of effector cells to activate a response against an invading pathogen. They can promote a differentiated B cell immunity with antibody production or, on the opposite, directly modulate, respectively control, the activity of different types of T cells. Secreted cytokines can activate macrophages and other cells through cell to cell signal communication. In addition, invasion of extracellular pathogens causes infiltrations of heterophils which possess phagocytic activity in avian species. Therefore, the aim of the present work was to investigate changes in the kinetics

of leukocytes during inoculation of turkeys and chickens with attenuated and virulent *H. meleagridis*. $CD3^{+}CD4^{+}CD8\alpha^{-}$ and $CD3^{+}CD4^{-}CD8\alpha^{+}$ T cells together with B cells in different organs and blood were investigated by flow cytometry for the first time, to obtain insights into the local and systemic cellular immune response after vaccination and/or infection with *H. meleagridis*. In addition, data on the cell dynamics of B cells, total T cells, macrophages/monocytes together with heterophils in the whole blood of chickens were determined by FCM analysis in chickens.

10. Objectives of the thesis

The current thesis was performed to obtain an in-depth knowledge on the immunological scenario during the host and pathogen interaction of attenuated and virulent form of *H. meleagridis* in *Meleagris gallopavo* and *Gallus gallus.*

In order to achieve this, the following tasks were performed:

(i) establishing reference genes for gene expression in tissues of turkeys and chickens

(ii) determining cross reactivity of antibodies for immune cells of turkeys

(iii) comparing the local and systemic immune responses against virulent and attenuated *H. meleagridis* in turkeys and chickens

a. determine the expression difference of T and B cell populations in caecum, liver, spleen and blood of turkeys and chickens

b. measuring macrophages/monoytes, heterophils and TCR-γδ in the mentioned samples of chickens

c. analysing expression of IL-13 and IFN-γ the key cytokines of Th1/Th2 pathways in in caecum, liver and spleen of both species

11. Publications

In this thesis, two main publications are included, addressing the objectives mentioned above. The first manuscript describes the examination and evaluation of reference genes for turkeys and chickens in different infection models to precede accurate analysis of gene expression by RT-qPCR. The second publication outlined changes of the cellular immune response of turkeys and chickens during histomonosis and vaccination against the disease. The manuscripts are published in Veterinary Research and Vaccine, respectively.

11.1. The 60S ribosomal protein L13 is the most preferable reference gene to investigate gene expression in selected organs from turkeys and chickens, in context of different infection models

Mitra *et al. Vet Res (2016) 47:105*
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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

The 60S ribosomal protein L13 is the most preferable reference gene to investigate gene expression in selected organs from turkeys and chickens, in context of different infection models

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Abstract

Evaluation of reference genes for expression studies in chickens and turkeys is very much limited and unavailable for various infectious models. In this study, eight candidate reference genes HMBS, HPRT1, TBP, VIM, TFRC, RPLP0, RPL13 and RPS7 were evaluated by five different algorithms (GeNorm, NormFinder, BestKeeper©, delta CT, RefFinder) to assess their stability. In order to analyze a broad variation of tissues, spleen, liver, caecum and caecal tonsil of different aged specific pathogen free (SPF) layer chickens and commercial turkeys, uninfected or infected with the extracellular pathogen *Histomonas meleagridis*, were included. For tissue samples from SPF chickens RPL13 and TBP were found to be the most stable reference genes. Further testing of RPL13 and TBP in the same organs of uninfected and infected SPF broiler chickens with the intracellular pathogen fowl aviadenovirus confirmed this finding. In tissue samples from turkeys, a stable expression of RPL13 and TFRC genes was noticed. Overall, the determined reference genes should be considered whenever gene expression studies in spleen, liver, caecum and caecal tonsil of chickens and turkeys are performed.

Introduction

Gene expression analysis provides insights into complex biological regulatory processes and has become an essential part in various molecular biology studies. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is, in many studies, the method of choice for the detection and quantification of mRNA [1]. However, this method can be affected by technical variations in template quantity, quality, reverse transcription process and data analysis which impede correct measurements of true biological deviations [2, 3]. It is therefore essential to normalize these variations. There are several methods to eliminate technically induced variations from the true biological diversity such as in situ calibration, generic normalization against total mRNA, measuring DNA content of total nucleic acid or normalization with validated reference genes [4]. According to minimum information for publication of quantitative real-time pcr experiments (MIQE) guidelines, a reliable method of normalization uses reference genes which should be validated for every species and also on the basis of different experimental treatments [5]. The use of a single reference gene is considered to be an improper approach for gene expression studies, and the application of several genes for normalization is highly recommended to avoid erroneous results introduced by technical manipulation of samples [4, 6]. In recent years, reference genes were established for different animal species, such as cattle, pig, sheep, goat, horse and fish [7–12]. Also in chickens

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(*Gallus gallus*), a number of studies validated reference genes under different physiological conditions [13–20]. However, the assessment of genes used for normalization of gene expression during infection with an extracellular pathogen is completely lacking. Furthermore, only a single study evaluated reference genes for brain tissue in turkeys (*Meleagris gallopavo*) [18]. This prompted us to validate reference genes in spleen, liver, caecum and caecal tonsils from healthy and infected SPF layer chickens and turkeys with the extracellular pathogen *Histomonas meleagridis* at different ages. In SPF broiler chickens, preselected reference genes were further evaluated using samples from birds infected with the intracellular pathogen fowl aviadenovirus (FAdV).

Materials and methods

Sample selection

A total of 252 different tissue samples from 27 SPF layer-type chickens (VALO, BioMedia, GmBH, Osterholz-Scharmbeck, Germany), 12 SPF broiler chickens (Animal Health Service, Deventer, Netherlands) and 24 commercial turkeys (B.U.T.6™; Aviagen Turkeys Ltd, Tattenhall, UK) were included in the analysis (Table 1). Samples from spleen, liver, caecum and caecal tonsil were collected from three non-infected SPF layer-type chickens and three turkeys at six time points between their first and their 49th day of life, to cover age related changes in gene expression. In addition, the effect of infectious pathogens on the expression of reference genes in host birds was investigated by collecting samples during the course of disease. For that, the same organs were sampled on three different time points from SPF layer-type chickens and on two time points from turkeys following infection with the extracellular pathogen *H. meleagridis* between the 35th and 49th day of life. Infected birds showed inflammation of the caeca and the livers with the exception of chickens sampled on the day 49, when two out of three birds had no lesions. Healthy SPF broiler chickens were sacrificed at the age of 4, 7 and 21 days of life to collect spleen, liver, caecum, and caecal tonsils of three birds each time point. The same organs of three additional SPF broiler chickens that were infected with the intracellular pathogen fowl aviadenovirus were sampled at day 7 of life. Swollen marble-like livers with a colour ranging from yellow to brown were observed in infected birds [21].

All samples were collected separately during post mortem investigations directly after euthanization of birds and stored in RNA*later* RNA stabilization reagent (Qiagen, Hilden, Germany) at −80 °C.

Gene selection

Based on findings from previous gene expression studies on different bird tissues [13, 14, 17, 18], eight genes—HMBS, HPRT1, TBP, VIM, TFRC, RPLP0, RPL13 and RPS7, known to be involved in different basic metabolic and structural pathways were selected to be tested for their suitability as reference genes (Table 2). Analyses of these genes were performed at the time points mentioned above in spleen, liver, caecum and caecal tonsil samples from SPF layer chickens and commercial turkeys. Samples from healthy birds together with those from birds infected with *H. meleagridis* were processed. Further analyses of RPL13 and TBP were performed using the same organs from healthy and fowl aviadenovirus infected SPF broiler chickens.

Total RNA extraction and analysis for purity and integrity

Total RNA was prepared from RNA*later*® (Qiagen) stabilized liver, spleen, caecum and caecal tonsil tissues. Tissue samples were homogenised separately using QIAshredders (Qiagen) and total RNA was extracted by RNeasy® mini kit (Qiagen) according to manufacturer's instructions and RNA was stored at −80 °C before further use. Every sample was assessed according to following criteria: the nucleic acid purity was analyzed with A260/280 and additionally A260/230 ratio by NanoDrop

Table 1 Organ samples used in this study

Animal type/species	Day of life								
	1	4	7	14	21	28	35	38	49
Healthy SPF layer chickens	x	n.d.	n.d.	x	n.d.	x	x	x	x
H. meleagridis-infected SPF layer chickens	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	x	x	x
Healthy turkeys	x	n.d.	n.d.	x	n.d.	x	x	x	x
H. meleagridis-infected turkeys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	x	x	n.d.
Healthy SPF broiler chickens	n.d.	x	x	n.d.	x	n.d.	n.d.	n.d.	n.d.
Fowl aviadenovirus-infected SPF broiler chickens	n.d.	n.d.	x	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

From different groups, spleen, liver, caecum and caecal tonsil were sampled in between 1st and 49th day of life.

x: sampling of three birds.

n.d.: not done.

Table 2 Details of selected potential reference genes

Gene/symbol	Protein	Physiological functions	Accession number for chicken	Accession number for turkey	Primer and probe sequences (F-forward primer; R-reverse primer; P-probe)
TFRC	Transferrin receptor protein	Cellular uptake of iron	NM_205256.2	XM_003209136.2	F:AGCTGTGGGTGCTACTGAA R:GGCAGAAATCTTGACATGG P:HEX-CTCTGCCATGCTGCAT GCCA-BHQ1
TBP	Binding protein TATA box	Transcription factor	NM_205103.1	XM_010707033.1	F:CTGGGATAGTGCCACAGCTA R:GCACGAAGTGCAATGGTTT P:ROX-TGCAACCAAGATTCAC CGTGGA-BHQ2
HPRT1	Hypoxanthine–guanine phosphoribosyltransferase I	Enzyme in the purine pathway	NM_204848.1	XM_010715191.1	F:GCTCATCATGGACAGGACAG R:CACAGAGAGCTACAATGTGGTG P:CY5-TGCCCTTCATAATTTCACGT GCCA-BHQ3
VIM	Vimentin	Cytoskeletal component responsible for maintaining cell integrity	NM_001048076.1	XM_010712706.1	F-TGAGTCCCTGCAAGAAGAAA R:CAGGGCAGCAGTAAGATCAG P:ROX-TCCGGGAACTGCAGGCT CAA-BHQ2
RPS7	40S acidic ribosomal protein S7	Small ribosomal subunit	XM_004940516.1	NM_001285787.1	F-TGGTATATCCCAGGCTCTCC R-TCAGCTGAGGAACTGGTACG P:FAM-TCAACTCCCGCAGCT GAGCC-TAM
RPL13	60S ribosomal protein L13	Large ribosomal subunit	NM_204999.1	XM_010718177.1	F:GGAGGAGAAGAACTTCAAGGC R:CCAAAGAGACGAGCGTTTG P:HEX-CTTTGCCAGCCTGCG CATG-BHQ1
HMBS	Hydroxymethyl-bilane synthase	Production of heme	XM_417846.5	XM_010723546.1	F:CCTGCCAACTTCTCTTCCTC R:CAACAGCATCAAGTGGGTTC P:FAM-TGCAAATAGCACCAATGG TAAAGCCA-TAM
RPLP0	60S acidic ribosomal protein P0	Large ribosomal subunit	NM_204987.2	XM_003211079.2	F-CAATGGCAGCATTTACAACC R:CAGGAAACGCTTGTGCAG P:CY5-TCTCCTCAGTGATGTCCAG CACTTCA-BHQ3

2000 (ThermoFisher scientific, Vienna, Austria) to ascertain that the RNA was free from contaminates like guanidine, glycogen and EDTA. The RNA quality and quantity of every sample was further surveyed by chip-based capillary electrophoresis Bioanalyzer 2100 (Agilent technologies, Waldbronn, Germany). In this process, the RNA concentration and the integrity of total RNA together with the presence or absence of degradation products were estimated by measuring the entire electrophoretic trace of each sample, given by the RNA integrity number (RIN) [22].

RT-qPCR

An identical set of primer and probe sequences were designed for both poultry species, chickens and turkeys, to target highly conserved regions of reference genes. Primers and probes were selected by using the respective sequence information from the NCBI database (Table 2) and GenScript real-time PCR (TaqMan) primer design software with default settings. One step real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) used TaqMan chemistry and Brilliant III Ultra-Fast QRT-PCR master mix kit (Agilent technologies). Amplification and quantification of reference genes mRNA were performed using AriaMx real-time PCR system (Agilent Technologies) together with the Agilent AriaMx1.0 software (Agilent Technologies). Thermal cycle profile for RT-qPCR was adjusted as follows: 1 cycle of reverse transcription at 50 °C for 10 min followed by 95 °C for 3 min to hot start, 40 cycles of amplification at 95 °C for 5 s and 60 °C for 10 s. Concentrations of 200–900 nM for primers and 100 nM for probes were ascertained by tenfold serial dilutions of RNA (100, 10, 1, 0.1 ng) to determine the optimal primer concentration and the highest efficiency of RT-qPCR reactions. Further details on the selected primer concentrations and the efficiency values are given in the Additional file 1. All samples were analysed in duplicate and different types of controls such as NRT (non reverse transcriptase) and NTC (non template control) were run with every plate to determine possible genomic DNA contamination and overall PCR contamination. The mean CT value of each

duplicate was further used for the gene expression analysis. Overall, the RT-qPCR investigation complies with the MIQE guidelines [5].

Gene expression analysis

Organ samples of healthy and infected SPF layer chickens and turkeys were analyzed separately. The stability of gene expression was determined by calculations using different software algorithms: GeNorm, NormFinder, BestKeeper© and delta CT. In addition, RefFinder was used to rank the genes on the basis of stability from the most to the least stable reference genes.

GeNorm

The GeNorm algorithm was used to calculate the average of pairwise variation of one gene with all the other potential reference genes and to identify their average expression stability (M). The gene with lowest M value was assumed to be the highest stable gene [23]. Genes with a threshold of 1.5 were considered stable whereas a threshold of 1.0 and below characterized the most stable genes [17].

NormFinder

NormFinder software generates a stability measure (Sv) which indicates an increased stability in gene expression by a low value. NormFinder software allows direct estimation of expression variation between different organ groups and ranked genes according to the similarity of their expression profiles by using a model-based approach. The chance to introduce systemic errors in genes with a low Sv value were found to be marginal, as previously described [24].

BestKeeper©

BestKeeper© calculated the standard deviation (SD) based on raw crossing point (CP) including the inter-gene relationship with the help of Pearson correlation coefficient matrix. Highly correlated genes were combined into an index which is called BestKeeper© index. The comparison of the correlation of each gene with BestKeeper© index gave a correlation coefficient value (r) with the probability value (p), as explained earlier [25]. BestKeeper© calculated the most stable gene by the lowest coefficient of variance (r) and standard deviation (SD).

Delta CT

Delta CT method was used to compare CT values of all possible gene combinations. An increased or decreased level of deviation in delta CT pattern is formed by comparing the possible gene combinations. Least amount of deviation means least amount variability of gene expression within the samples [26].

RefFinder

RefFinder is a web-based tool that integrates the current major computational programs, including GeNorm, Normfinder, BestKeeper©, and the delta CT method, to compare and rank the stability of the investigated candidate reference genes. Based on the rankings from each program, RefFinder assigns an appropriate value to an individual gene and calculate the geometric mean of their weights for the overall final ranking [27]. Ranking is from most stable gene to least stable gene in an ascending order.

Results

RNA purity and integrity

All RNA samples included in the present work were within the range of 1.5 and 2.3 ratio of 260/280 value and secondary measures of nucleic acid purity with 260/230 value was equal or above 2 by NanoDrop 2000 (ThermoFisher Scientific). The integrity of each RNA sample considered for RT-qPCR analysis was ensured by reaching a RIN value of 6.5 to 10 (see Additional file 2).

Expression stability of candidate reference genes

The results obtained by each single algorithm or as comparisons following calculation using the CT values (Additional file 3) with RefFinder are summarized below and listed in detail in Tables 3 and 4.

GeNorm

According to GeNorm stability criteria in healthy chicken samples, RPL13 (1.302), TFRC (1.302) and TBP (1.364) were within the threshold range of M value $\leq$ 1.5 indicating a reliable stability and in case of infected chickens RPL13 (0.753), TBP (0.753), TFRC (0.848) showed even more stable threshold with M values $\leq$1.0 (Table 3). In turkey samples, RPL13 and TFRC genes demonstrated stability similar to chicken samples with the M values of 1.333 for healthy and 0.868 in infected birds (Table 4).

NormFinder

Analysis with NormFinder resulted with the same three genes as the most stable ones: TBP, RPL13 and TFRC. The ranking in selected organs of healthy layer chickens was RPL13 (0.745), TBP (0.978) and TFRC (1.192), whereas it was slightly perturbed in tissues from infected chickens, with TBP (0.377) as the most stable followed by RPL13 (0.509) and TFRC (0.719) (Table 3). In turkeys, the ranking was as follows: for samples from healthy

Table 3 Results of four different algorithms for SPF layer-type chicken tissue samples of healthy birds or those infected with *Histomonas meleagridis*

Healthy SPF chickens						Infected with *H. meleagridis*				
Genes	**Delta CT**	**BestKeeper©**	**NormFinder**	**GeNorm**	**RefFinder**	**Delta CT**	**BestKeeper©**	**NormFinder**	**GeNorm**	**RefFinder**
RPL13	*2.49*	*0.73*	*0.745*	*1.302*	*1*	*1.58*	*0.7*	*0.509*	*0.753*	*2*
TFRC	*2.61*	*1.24*	*1.192*	*1.302*	*2*	*1.64*	*0.88*	*0.719*	*0.848*	*3*
TBP	*2.52*	*1.32*	*0.978*	*1.364*	*3*	*1.52*	*0.82*	*0.377*	*0.753*	*1*
VIM	2.86	1.3	1.406	1.963	4	2.21	1.37	1.733	1.402	5
RPS7	3.14	2.12	2.377	1.626	5	1.87	1.04	1.132	1.112	4
HMBS	3.6	2.27	2.862	2.31	6	2.41	1.43	1.984	1.837	6
HPRT1	3.73	2.52	2.944	2.666	7	2.4	1.92	2	1.634	7
RPLP0	4.96	2.88	4.588	3.239	8	2.61	1.79	2.261	2.03	8

Delta CT compares CT value from two reference genes; BestKeeper© calculates standard deviations (SD) based on raw crossing point; NormFinder gives stability value (Sv) by comparing inter and intra group variations; GeNorm value represents the average expression stability (M); RefFinder compares all other algorithms and gives an overall ranking on the basis of geometric mean.

Table 4 Results of four different algorithms for commercial turkey tissue samples of healthy birds or those infected with *Histomonas meleagridis*

Healthy turkeys						Infected with *H. meleagridis*				
Genes	**Delta CT**	**BestKeeper©**	**NormFinder**	**GeNorm**	**RefFinder**	**Delta CT**	**BestKeeper©**	**NormFinder**	**GeNorm**	**RefFinder**
TFRC	*2.39*	*1.22*	*0.908*	*1.333*	*1*	*1.9*	*0.59*	*1.188*	*0.868*	*2*
RPL13	*2.41*	*0.91*	*0.923*	*1.333*	*2*	*1.7*	*0.64*	*0.707*	*0.868*	*1*
RPS7	2.41	1.24	0.918	1.45	3	2.2	1.77	1.623	2.033	6
VIM	3.19	1.36	2.466	1.998	4	2.19	1.19	1.653	1.393	3
HMBS	2.99	2.13	2.148	2.466	5	2.23	1.47	1.687	1.872	7
TBP	3.21	2.06	2.488	2.252	6	2.31	1.8	1.826	2.103	8
RPLP0	3.33	3.21	2.711	2.664	7	2.17	1.43	1.635	1.683	4
HPRT1	3.95	2.78	3.45	2.984	8	2.12	1.67	1.501	1.97	5

Delta CT compares CT value from two reference genes; BestKeeper© calculates standard deviations (SD) based on raw crossing point; NormFinder gives stability value (Sv) by comparing inter and intra group variations; GeNorm value represents the average expression stability (M); RefFinder compares all other algorithms and gives an overall ranking on the basis of geometric mean.

birds RPL13 (0.923), TFRC (0.908) and for infected birds RPL13 (0.707) and TFRC (1.188) (Table 4).

BestKeeper©

BestKeeper© found RPL13 as the most stable gene for both healthy (0.73) and infected layer chickens (0.7) (Table 3). Similar to that, RPL13 (0.91) showed highest stability in healthy turkey tissues also, however, RPL13 (0.64) was ranked behind TFRC (0.59) in samples from turkeys infected with histomonads (Table 4).

Delta CT

The delta CT results supported GeNorm, NormFinder, BestKeeper© findings. RPL13 (healthy 1; infected 1.58), TFRC (healthy 2.61; infected 1.64), TBP (healthy 2.52; infected 1.52) genes showed the most constant CT values in chicken samples (Table 3). The same trend was found for turkey samples, for which RPL13 (healthy 2.41; infected 1.7) and TFRC (healthy 2.39; infected 1.9) showed lowest variation in CT values (Table 4).

RefFinder

Conclusive calculations using RefFinder included all above mentioned algorithms: RPL13 (healthy 1; infected 2), TBP (healthy 3; infected 1) and TFRC (healthy 2; infected 3) were most stable in contrast to RPLP0, HPRT1, HMBS which were found to be the least stable genes for healthy and infected layer chickens (Table 3). For turkeys, TFRC (healthy 1; infected 2) and RPL13 (healthy 2; infected 1) showed a consistent stability in both the conditions unlike the remaining reference gene candidates (Table 4).

Verification of stability for validated reference genes

Additionally, the variation in gene expression of the two most stable genes in layer-type chickens, RPL13 and TBP, was investigated in SPF broiler chickens. The same organs sampled from SPF layer chickens were also investigated from healthy and fowl aviadenovirus-infected SPF broiler chickens (Additional file 3). A stable expression of RPL13 with CT values between 17.8 and 20.39 and TBP with values of 23.43 and 26.05 was noticed in all tissue samples of differently aged healthy SPF broilers (Figure 1A). Following fowl aviadenovirus infection, the CT value was even less diverse, and ranged between 17.24–18.22 for RPL13 and 23.59–25.75 for TBP (Figure 1B).

Discussion

Recent studies focused on the validation of reference genes applied on different avian tissues [13, 17, 18]. However, so far validation of genes for their stable expression pattern in spleen, liver, caecum, and caecal tonsils of chickens and turkeys in context of an infection with an extracellular pathogen was not performed. Therefore, a wide range of possible physiological conditions (e.g. infections status, age and genetics), which potentially influences the gene expression, were investigated in the present study to identify suitable reference genes.

In a first step candidate reference genes, namely TBP, HPRT1 and HMBS, were selected in the present work according to their previously described stability in gene expression studies of various avian tissues or cells, such as muscular tissues, liver and leukocytes isolated from spleen, thymus and Bursa of Fabricius of chickens [13, 14, 17]. Expression of RPS7 together with TFRC was described in chicken and turkey brain tissue [18]. In addition to the above mentioned genes, VIM, RPL13 and RPLP0 were also validated in the present analysis. VIM and RPL13 were not found to be stable in a study evaluating pan-avian reference genes on brain samples [18] and RPLP0 was only analyzed in organs of mammals [28]. Anyhow, they were included in the current study to broaden the spectrum of metabolic and structural pathways. Other previously reported reference genes like 18S and 28S rRNA or GAPDH were not validated in

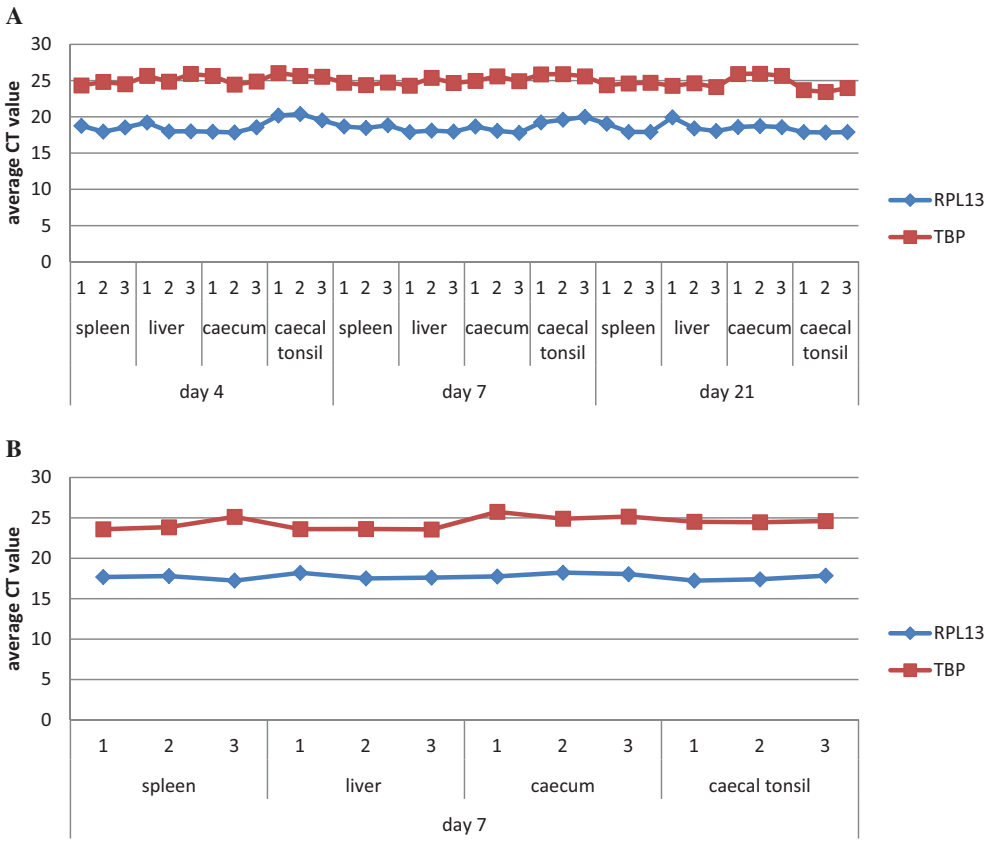

Figure 1 CT value fluctuations in organs of (A) healthy SPF broiler chickens and (B) birds infected with fowl aviadenovirus. Graphs were plotted against average raw CT values acquired by RT-qPCR experiment on different tissues of obtained from three SPF broilers (denoted as 1, 2 and 3 in the graph) at different ages. The fluctuation of the CT value was <2.59 for RPL13 and <2.62 for TBP in organs of healthy SPF broilers and <0.98 for RPL13 and <2.1 for TBP in tissues of infected birds.

the present work due to certain aspects that makes them unlikely to be used for normalization: the expression of 18S and 28S rRNA is regulated by the RNA polymerase I enzyme, whereas the synthesis of mRNAs to be measured is processed by the RNA polymerase II [6, 29]. Furthermore, both genes do not harbour any introns, which implicates that the amplification of genomic DNA, if not removed properly, is possible. In agreement with that, Olias et al. [18], who performed a multi avian species study, also recommended to avoid 18S rRNA as a reference gene, despite of the proven stability. GAPDH, on the other hand, is involved in the glycolytic pathway and its expression depends on the respective tissue and different experimental conditions such as glucose deprivation or stress. Therefore, also GAPDH was described to be unsuitable for normalization of RT-qPCR experiments [6, 29].

According to the MIQE guidelines reference genes can vary between different species and have to be validated even between closely related species [5]. Consequently, the expression of all reference candidates of this study was investigated separately in chickens and turkeys. Furthermore, genetic variations as well as previously demonstrated immunological differences between SPF layer- and SPF broiler chickens [30], which potentially affects the gene expression, were considered in the present work. Previous studies also described different expression patterns of reference gens according to the type of organ [13, 17, 18]. This prompted us to include the immune organs, spleen and caecal tonsils, together with the liver and the caecum, organs that are fundamental in the metabolism of birds. Age dependent changes in gene expression were covered by using SPF layer chickens and turkeys from day-old to 49th day of life, and SPF broiler chickens from day-old to 21st day of life. Finally, the impact of different kind of pathogens on the gene expression in organs of host birds was investigated. For that, chickens and turkeys were infected with the extracellular pathogen *H. meleagridis* following a well-defined infection model [31]. Hence, reference gene candidates in inflamed and immune organs of birds at different time points following infection were included. Accordingly, the most suitable reference gene candidates were used in organ samples from SPF broiler chickens infected with fowl aviadenovirus to validate the stability following infection with an intracellular pathogen.

The expression of each reference gene for every sample was analyzed with different algorithms: GeNorm, NormFinder, BestKeeper© and delta CT. The rankings of assayed genes were not always identical due to variations in statistical calculations by different algorithms, a phenomenon recently reported [17]. Therefore, it was crucial to get a consensus in the outcome of the applied algorithms, for which purpose the RefFinder software was applied. Overall, it was found that RPL13 gene was the most stable expressed gene in the examined tissues of SPF layer chickens regardless of an infection with *H. meleagridis*. TBP and TFRC were both shown to be stable as well; however, there were slightly higher variations in the expression levels of TFRC in tissues of infected SPF layer chickens. Depending on the experimental setup TFRC also can be used as reference gene along with RPL13 and TBP for chickens. All other reference gene candidates calculated with RefFinder were not considered as stable for SPF layer chickens due to severe variations in their expression profiles. Hence, RPL13 and TBP were further investigated in tissues of non-infected and infected SPF broiler chickens with fowl aviadenovirus. These additional investigations confirmed the stable expression of both genes in SPF broiler chicken spleen, liver, caecum and caecal tonsil samples. Our findings demonstrated that neither the genetic background of chickens nor the nature of an infectious agent caused remarkable variations of RPL13 and TBP expression in spleen, liver, caecum and caecal tonsil, suggesting them as optimal candidates for normalization of RT-qPCR experiments. Similar to the results found in chicken tissues, RPL13 was also determined to be the most stable gene, followed by TFRC in spleen, liver, caecum and caecal tonsil samples from healthy and infected turkeys with the extracellular pathogen. In contrast to data from chickens, presented here and elsewhere [13, 14], TBP of turkeys ranked on the eight position using RefFinder, which indicates the heterogeneity in the expression of certain candidate reference genes between gallinaceous species. Thus, our findings also show the possibility of a variation of reference genes expression between different bird species, even if they are closely related. On the other hand, TFRC was stable in both poultry species which is in the agreement with previous findings investigating brain tissue [18].

According to the MIQE guidelines, a reference gene needs to be validated and established for every species and for different physiological conditions. The guidelines furthermore specify verification of a used reference gene for every experimental settings, otherwise the variation of reference genes expression may adversely affect the results and with it the biological conclusion. The RPL13 and the TBP genes of chickens and the RPL13 and the TFRC genes of turkeys were shown to be highly stable in the present experimental setting and are therefore recommended to be first priority candidates for gene expression studies in case spleen, liver, caecum and caecal tonsil tissues are targeted.

Additional files

Additional file 1. Candidate reference genes efficiency. Optimized concentrations of all candidate reference genes primers and probes are given with efficiency value for chicken and turkey species.

Additional file 2. RNA integrity number measured with Bioanalyzer2100. The RIN values of all the samples used in the experiment are given.

Additional file 3. Raw CT values obtained in the present work. The raw CT values of all RT-qPCR experiments were used for calculations with different algorithms to assess the stability of each reference gene candidate.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conceived and designed the experiments: TM, IB, MH and DL. Performed the experiments: TM and DL. Analysed and interpreted the data: TM, IB and DL. Drafted the manuscript: TM, IB, MH and DL. All authors read and approved the final manuscript.

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Ethics approval
All organ samples used in the present work were obtained from animal trials approved by institutional ethics committee and the national authority according to §26 of law for the animal experiments, Tierversuchsgesetz 2012—TVG 2012 (license number: GZ 68.205/0147-II/3b/2013 and GZ 68.205/0041-WF/II/3b/2014).

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Additional file 1. Candidate reference genes efficiency. Optimized concentrations of all candidate reference genes primers and probes are given with efficiency value for chicken and turkey species.

gene[1]	primer concentrations for chicken (nM)	efficiency for chicken (%)	primer concentrations for turkey (nM)	efficiency for turkey (%)
TFRC	400	98	400	100.9
TBP	600	100.4	600	99.2
HPRT1	700	97.3	700	98.2
VIM	400	96.4	500	97.8
RPS7	400	97.3	400	95.2
RPL13	500	95.1	300	96
HMBS	400	100.3	400	98.7
RPLP0	600	96.4	600	96.8

[1] For every candidate gene, 100nM of probe concentration was used for both the species. Concentrations of primers were different for chickens and turkeys.

Additional file 2. RNA integrity number measured with Bioanalyzer2100. The RIN values of all the samples used in the experiment are given.

	sample name	RIN					
		day of life					
		35	38	49			
layer chickens with *Histomonas meleagridis* infection	spleen 1	8.3	9.9	8.4			
	liver 1	6.9	8.9	10			
	caecum 1	10	6.6	8.8			
	caecal tonsil 1	10	10	10			
	spleen 2	6.5	9.5	8			
	liver 2	9.9	6.8	9.1			
	caecum 2	9.6	7.8	9			
	caecal tonsil 2	10	9.8	9.4			
	spleen 3	7.5	9.9	9.7			
	liver 3	10	8	7.8			
	caecum 3	7.6	7.5	7.7			
	caecal tonsil 3	9.8	9.5	9.7			
		day of life					
		1	14	28	35	38	49
healthy layer chickens	spleen 1	10	7.3	7	8.4	9.7	6.7
	liver 1	10	10	8.1	8.9	8.9	9.9
	caecum 1	8.5	8.2	9.5	7	9.1	9.7
	caecal tonsil 1	9.7	7.3	9.8	9.9	10	9.7
	spleen 2	10	7.6	6.5	8.9	8.6	8.4
	liver 2	10	7.2	10	8.6	7.4	7.1
	caecum 2	8.3	6.7	8.7	8.2	8.2	9.1
	caecal tonsil 2	10	6.6	6.7	9.8	10	10
	spleen 3	10	7	7.3	7.7	7.1	7.7
	liver 3	10	10	10	8	7.6	7.8
	caecum 3	10	7.9	7.2	7.5	9.4	9.1
	caecal tonsil 3	10	8.9	7.8	10	9.8	10
		day of life					
		35	38				
turkeys with *Histomonas meleagridis* infection	spleen 1	7.5	7.6				
	liver 1	7.2	6.9				
	caecum 1	6.7	7				
	caecal tonsil 1	6.7	8.1				
	spleen 2	9.8	6.5				
	liver 2	8.8	6.8				
	caecum 2	6.6	7.3				
	caecal tonsil 2	7.2	8.7				
	spleen 3	8.1	8.6				
	liver 3	8.3	6.6				
	caecum 3	7.8	6.9				
	caecal tonsil 3	8.5	7.1				

		day of life					
		1	**14**	**28**	**35**	**38**	**49**
	spleen 1	8.7	6.9	7.7	7.9	6.8	7.5
	liver 1	10	6.5	7.3	9.9	7.3	8.5
	caecum 1	7.3	7.9	7.9	8.6	8.8	6.6
	caecal tonsil 1	7.6	9.4	9	9.9	9.2	10
	spleen 2	8.1	7	9.2	8.6	7.6	7.2
	liver 2	9.6	7.4	6.7	8.5	7.4	6.9
	caecum 2	10	8.3	6.9	9.3	8.5	6.7
	caecal tonsil 2	7.2	8.2	7.1	9.8	10	8.6
	spleen 3	7.1	7.3	6.7	8	9	8.8
	liver 3	8.2	6.7	7.5	6.8	9.4	9.4
	caecum 3	9.3	6.8	6.8	6.6	9.3	10
healthy turkeys	caecal tonsil 3	10	6.9	8.9	9.1	10	8.7
		day of life					
		7					
	spleen 1	8.3					
	liver 1	9.2					
	caecum 1	7.5					
	caecal tonsil 1	8.5					
	spleen 2	6.9					
	liver 2	8.5					
	caecum 2	7.3					
	caecal tonsil 2	6.6					
broiler chickens	spleen 3	6.7					
with fowl	liver 3	8.5					
aviadenovirus	caecum 3	6.9					
infection	caecal tonsil 3	7.6					
		day of life					
		4	**7**	**21**			
healthy broiler	spleen 1	8	6.7	6.6			
chickens	liver 1	9.3	7.5	6.7			
	caecum 1	6.5	9	9.3			
	caecal tonsil 1	7.9	8.4	9.3			
	spleen 2	9.7	8.2	7.2			
	liver 2	6.8	7.2	7.5			
	caecum 2	8.6	7.2	6.9			
	caecal tonsil 2	7.1	8.1	7.7			
	spleen 3	7	6.7	7.5			
	liver 3	7.7	6.9	7.6			
	caecum 3	7.2	6.8	7.7			
	caecal tonsil 3	7.2	7.6	8.5			

Additional file 3. Raw CT values obtained in the present work. The raw CT values of all RT-qPCR experiments were used for calculations with different algorithms to assess the stability of each reference gene candidate.

control layer chickens	**average CT value**								
age of the birds	**TBP**	**RPLP0**	**HMBS**	**HPRT**	**TFRC**	**RPL13**	**RPS7**	**VIM**	**organ**
day 1	26.55	18.7	27.54	27.26	20.76	21.06	34.8	21.91	spleen
day 1	25.88	17.86	28.35	25.62	23.12	20.84	33.66	22.79	
day 1	25.65	18.37	27.26	25.42	21.11	21.48	35.13	21.68	
day 15	25.94	21.54	23.99	27.16	24.92	21.42	34.24	21.75	
day 15	25.95	21.64	24	28.76	25.72	21.67	34.19	22.15	
day 15	25.84	22.64	23.92	29.76	24.99	21.58	35	21.8	
day 28	25.14	19.98	26.11	27.16	23.56	20.81	33.22	20.12	
day 28	24.31	20.4	25.46	28.76	23.44	20.39	35.05	20.5	
day 28	23.74	20.12	25.8	25.62	23.7	20.94	34.12	20.8	
day 35	26.07	22.11	27.55	29.81	22.93	20.61	33.49	22.44	
day 35	26.32	21.96	29.41	32.89	22.97	21.68	33.48	24.66	
day 35	25.54	21.46	26.1	35	23.15	21.35	32.84	22.46	
day 38	26.67	22.04	29.97	26.02	23.14	22.62	33.79	22.04	
day 38	26.55	21.15	29.27	28.82	23.06	22.51	34.32	22.19	
day 38	26.47	23.54	30.73	25.34	23.35	22.3	34.28	22.14	
day 49	27.04	23.25	34.3	31.63	25.76	21.05	34.12	24.62	
day 49	27.97	18.91	34.24	34.11	24.83	20.92	34.54	23.31	
day 49	27.1	23.55	34.85	24.23	24.32	20.35	34.9	23.22	
day 1	24.05	16.24	25.11	29.95	22.58	19.38	32.3	22.65	liver
day 1	24.16	18.97	25.48	26.03	22.43	20.47	32.24	22.85	
day 1	24.55	18.31	25.02	27.85	22.25	20.34	31.5	22.6	
day 15	23.44	19.25	22.68	27.16	23.08	21.07	33.34	21.19	
day 15	23.41	18.91	21.78	28.76	23.29	21.58	33.83	21.3	
day 15	23.28	18.36	20.9	29.76	23.33	21.59	31.51	21.75	
day 28	23.54	19.26	24.92	27.16	22.91	20.69	33.31	21.57	
day 28	23.61	18.91	24.57	28.76	23.63	20.91	34.5	22.15	
day 28	24.21	18.27	24.8	25.62	22.36	20.29	32.1	21.84	
day 35	23.69	23.25	23.97	29.9	25.02	20.55	30.88	24.49	
day 35	23.99	23.97	24.17	31.15	24.07	22.2	30.88	24.09	
day 35	25.28	27.4	23.9	29.13	23.12	20.61	29.28	25.18	
day 38	24.45	22.04	26.4	26.55	21.81	22.3	30.98	22.56	
day 38	24.13	21.15	26.61	25.35	21.78	22.48	31.92	22.45	
day 38	23.41	23.54	26.32	25.76	23.12	22.62	30.86	20.89	
day 49	25.67	23.25	33.6	22.84	23.93	21.49	34.8	24.56	

day 49	24.55	18.91	34.63	22.59	24.8	21.77	34.4	24.42	
day 49	23.4	23.55	33.04	23.21	23.09	21.82	35.13	25.76	
day 1	21.87	18.92	25.39	22.54	21.72	19.62	31.82	20.7	caecum
day 1	22.57	18.09	27.89	21.69	22.02	19.69	29.65	20.91	
day 1	22.24	20.22	24.68	21.44	23.69	19.26	31.46	21.84	
day 15	25.57	20.41	23	24.37	23.54	20.58	34.8	20.3	
day 15	25.52	20.19	22.9	26.76	24.43	20.95	33.5	20.57	
day 15	26.32	21.14	23.68	25.6	23.89	21.32	34.54	20.53	
day 28	23.84	18.64	24.98	25.16	22.15	20.52	33.64	22.02	
day 28	24.06	19.5	24.86	28.76	22.71	20.05	34.81	22.31	
day 28	23.84	20.35	24.92	27.62	22.53	19.88	34.67	21.84	
day 35	22.94	22.54	23.65	25.49	20.62	19.81	29.68	24.03	
day 35	22.73	21.3	23.57	25.2	19.88	19.98	30.54	23.86	
day 35	22.87	20.12	25.9	26.95	18.8	20.05	30.52	24.09	
day 38	22.9	22.04	26.24	26.48	20.99	19.85	29.75	23.53	
day 38	23.19	21.15	26.39	26.61	20.86	20	29.84	24.81	
day 38	23.2	23.54	26.19	26.26	21.98	20.48	29.8	23.85	
day 49	23.16	25.05	25.38	25.87	20.33	19.42	30.11	22.64	
day 49	23.16	19.84	24.83	25.96	19.8	19.85	29.74	24.66	
day 49	23.18	23.55	24.81	25.74	20.54	19.87	30.16	23.38	
day 1	22.34	17.68	24.73	23.24	20.73	19.65	31.3	18.45	caecal tonsil
day 1	21.79	18.55	24.57	22.72	21.14	20.15	30.68	18.4	
day 1	22	17.94	24.66	22.81	22.17	20.98	29.48	18.97	
day 15	24.95	18.99	22.78	24.14	23.98	20.91	35	19.23	
day 15	24.52	19.2	21.95	25.56	24.02	21.35	34.7	19.19	
day 15	22.89	18.98	22.44	25.94	24.16	21.06	34.9	20.02	
day 28	23.9	19.84	25.31	25.16	24.02	20.57	34.51	21.82	
day 28	22.24	18.48	25.88	28.76	23.11	20.86	33.64	21.03	
day 28	22.76	19.29	25.58	27.62	23.05	20.46	33.49	21.97	
day 35	22.07	19.84	22.66	23.08	20.92	19.44	28.2	22.7	
day 35	22.2	20.9	22.49	23.54	20.84	19.8	28.63	21.22	
day 35	22.86	19.17	22.2	23.96	21.41	19.8	27.78	23.69	
day 38	21.8	25.15	23.81	22.39	20.69	19.58	27.68	22.25	
day 38	21.35	35.66	23.88	22.29	21.97	19.77	27.79	24.12	
day 38	23.13	37.02	23.97	22.66	21.08	19.41	27.52	22.85	
day 49	21.13	35.42	23.18	33.84	22.08	20.48	28.01	24.8	
day 49	21.62	34.84	23.1	34.9	20.15	20.51	27.82	24.99	
day 49	22.96	34.91	22.97	34.1	21.57	19.07	27.92	22.09	

infected layer chickens	average CT value								
age of the birds	RPS7	RPLP0	HMBS	HPRT	TFRC	RPL13	TBP	VIM	organ
day 35	23.75	16.39	22.45	27.38	24.64	19.79	28.09	21.39	spleen
day 35	22.93	14.36	22.6	24.18	25	19.89	28.18	21.67	
day 35	23.5	16.78	22.86	27.24	25.89	19.92	28.02	22.44	
day 38	22.22	16.79	23.94	21.77	22.15	19.22	25.65	20.43	
day 38	22.19	16.42	23.98	23.26	22.51	19.26	25.71	20.88	
day 38	21.68	15.44	23.94	23.9	22.22	19.21	26.16	22.36	
day 49	22.51	14.5	25.74	22.01	22.02	18.37	26.62	20.7	
day 49	22.8	13.25	25.87	22.44	22.96	17.85	26.1	20.77	
day 49	22.78	15.7	26.12	20.62	22.82	18.49	26.05	20.68	
day 35	24.67	18.27	22.7	26.65	24.98	21.5	28.11	24.47	liver
day 35	24.55	13.59	22.08	28.22	24.8	21.19	28.33	24.81	
day 35	23.82	17.4	23.91	27.69	24.4	20.95	28.16	25.29	
day 38	24.08	16.79	23.99	26.91	22.93	19.55	26.66	17.67	
day 38	24.09	16.42	24.44	28.01	23.33	19.9	26.86	17.78	
day 38	24.17	15.44	24.43	27.51	25.18	20.1	26.29	18.98	
day 49	21.62	15.45	26.05	28.46	23.97	19.79	27.69	23.6	
day 49	21.57	19.45	26.01	27	23.19	19.86	27.73	23.19	
day 49	21.69	17	27.09	25.65	25.48	20.82	27.55	23.69	
day 35	21.93	22.34	23.08	24.37	23.54	20.96	27.8	20.47	caecum
day 35	21.94	21.96	22.47	24.33	24.58	20.67	28	20.1	
day 35	22.12	17.11	21.19	24.23	23.27	21.12	27.83	20.45	
day 38	25.55	20.45	25.72	23.7	23.51	19.02	27.63	19.13	
day 38	25.69	22.05	25.79	25.44	23.86	18.95	27.96	19.41	
day 38	25.77	19.78	25.83	25.4	24.41	19.71	27.8	19.44	
day 49	24.09	18.61	26.03	22.98	23.05	19.74	26.83	21.11	
day 49	23.86	19.84	26.34	22.39	23.14	19.2	27.44	22.27	
day 49	23.79	17.16	26.68	22.52	23.88	19.77	27.82	22.18	
day 35	21.87	18.16	21.66	23.5	24.03	20.3	27.48	21.24	caecal tonsil
day 35	24.99	19.05	22.3	23.45	24.86	20.6	26.95	20.78	
day 35	22.02	18.74	21.75	23.88	24.3	20.16	26.74	21.69	
day 38	22.83	16.76	23.81	22.43	24.24	19.22	25.56	19.44	
day 38	22.35	16.89	23.84	22.09	22.81	18.69	25.97	19.43	
day 38	22.15	15.33	23.81	22.38	24.64	19.03	25.24	21.4	
day 49	22.84	15.45	25.93	20.96	22.84	18.85	26.38	20.44	
day 49	23.12	19.45	25.48	23.44	22.5	18.87	26.27	20.48	
day 49	22.97	17	25.57	21.64	22.75	19.02	26.21	20.48	

control turkeys	average CT value								
age of the birds	HMBS	RPL13	RPS7	HPRT	VIM	RPLO	TBP	TFRC	organ
day 1	22.91	20.14	23.99	25.95	24.64	20.64	28.47	19.68	spleen
day 1	22.3	20.17	24	24.42	25.54	20.64	28.62	20.83	
day 1	22.48	21.33	23.92	26.97	25.45	20.59	28.68	20.65	
day 15	25.94	20.98	23.94	27.16	24.92	21.42	34.24	19.75	
day 15	25.95	20.4	24.94	28.76	25.72	21.67	34.19	20.15	
day 15	25.84	20.12	25.9	29.76	24.99	21.58	35	20.8	
day 28	25.14	21.54	26.11	27.16	23.56	20.81	33.22	20.12	
day 28	24.31	21.64	25.46	28.76	23.44	20.39	35.05	20.5	
day 28	23.74	22.64	25.8	25.62	23.7	19.94	34.12	20.8	
day 35	31.87	22.63	25.72	24.33	23.02	26.87	31.7	22.54	
day 35	32.58	20.78	25.6	22.55	23.78	27.82	33.6	21.89	
day 35	32.55	22.89	26.1	24.17	26.03	25.92	34.09	22.43	
day 38	26.8	20.51	27.25	27.41	21.52	27.11	31.43	22.46	
day 38	27.55	21.56	25.96	26.43	20.18	26.4	30.74	21.71	
day 38	29.67	21.04	27.87	28.55	25.82	27.64	34.66	23.59	
day 49	25.82	23.89	28.14	38.78	21.46	25.45	30.36	22.84	
day 49	29	22.99	26.84	36.84	20.68	26.71	29.96	22.39	
day 49	27.7	22.45	27.25	36.72	22.44	26.53	31.86	23.39	
day 1	24.1	20.67	22.68	28.14	23.29	20.45	27.81	21.19	liver
day 1	24.22	20.99	21.78	27.62	23.28	20.15	27.73	21.3	
day 1	24.31	20.7	20.9	28.48	25.82	20.95	27.76	21.75	
day 15	23.44	20.25	24.92	27.16	23.08	21.07	33.34	21.57	
day 15	23.41	21.91	24.57	28.76	23.29	21.58	33.83	22.15	
day 15	23.28	20.36	24.8	29.76	23.33	21.59	31.51	21.84	
day 28	23.54	21.26	25.43	27.16	22.91	20.69	33.31	23.6	
day 28	23.61	21.91	24.73	28.76	23.63	20.91	34.5	24.06	
day 28	24.21	22.27	25.11	25.62	22.36	20.29	32.1	23.98	
day 35	35.66	23.86	26.16	27.64	23.01	28.93	32.57	25.62	
day 35	28.67	21.82	24.79	27.89	21.94	28.47	31.68	24.4	
day 35	29.41	22.25	25.89	26.44	23.06	30.94	31.39	24.47	
day 38	27.59	23.36	26.58	28.41	23.24	29.45	31.74	23.58	
day 38	26.92	22.97	25.3	29.69	22.13	27.41	31.17	23.14	
day 38	26.4	23.34	23.63	29.39	20.38	26.37	30.2	24.97	
day 49	28.38	23.45	27.75	35.05	23.84	27.68	32.59	24.23	
day 49	26.95	24.4	25.6	32.33	20.25	26.39	30.63	24.12	
day 49	25.1	24.12	26.17	34.59	21.73	27.58	30.64	22.21	
day 1	22.91	20.59	23	22.88	25.14	20.98	29.37	20.3	caecum
day 1	22.3	20.17	22.9	23.78	26.32	20.15	28.69	20.57	

day 1	22.48	20.84	23.68	25.21	24.98	20.92	29.17	20.53	
day 15	25.57	20.41	24.98	24.37	23.54	20.58	35.8	20.53	
day 15	25.52	20.19	24.86	26.76	24.43	20.95	35.5	21.62	
day 15	26.32	21.14	24.92	25.6	23.89	21.32	35.54	21.14	
day 28	23.84	21.64	24.19	25.16	22.15	20.52	33.64	22.02	
day 28	24.06	20.5	26.38	28.76	22.71	20.05	34.81	22.31	
day 28	23.84	20.35	25.31	27.62	22.53	19.88	34.67	21.84	
day 35	26.65	21.96	26.32	28.5	20.53	26.05	30.66	21.5	
day 35	27.17	20.7	27.13	30.96	21.27	27.23	32.63	24.41	
day 35	27.67	20.43	26.83	30.57	26.06	26.23	31.84	24.92	
day 38	27.21	21.33	26.45	24.36	21.3	26.47	30.74	22.48	
day 38	25.81	20.19	25.57	24.59	22.6	25.94	30.95	22.32	
day 38	24.17	21.79	27.36	25.14	24.38	27.64	36.51	24.29	
day 49	25.85	20.16	28.01	25.05	20.13	24.68	30.36	21.83	
day 49	26.53	20.12	25.71	25.12	20.13	26.21	30.08	22.67	
day 49	32.37	20.87	27.26	25.19	21.23	26.26	33.13	22.29	
day 1	24.02	20.66	22.78	22.27	21.95	20.7	26.18	19.23	caecal tonsil
day 1	23.74	20.98	21.95	22.36	22.48	20.59	26.95	19.19	
day 1	23.87	20.81	22.44	22.89	22.1	20.12	26.5	20.02	
day 15	24.95	20.99	24.46	24.14	23.98	20.91	35	21.82	
day 15	24.52	21.2	24.23	25.56	24.02	21.35	34.7	21.03	
day 15	22.89	21.98	24.34	25.94	24.16	21.06	34.9	21.97	
day 28	23.9	20.84	25.31	25.16	24.02	20.57	34.51	22.69	
day 28	22.24	21.48	25.88	28.76	23.11	20.86	33.64	22.66	
day 28	22.76	21.29	25.58	27.62	23.05	20.46	33.49	22.78	
day 35	27.99	22.41	25.91	35.21	23.1	27.81	34.72	22.98	
day 35	27.68	22.32	25.53	30.29	29.71	27.41	31.45	23.03	
day 35	29.72	21.41	25.05	32.77	22.86	29.15	31.08	21.45	
day 38	28.46	21.32	26.72	23.71	22.14	28.35	31.95	23.95	
day 38	26.37	22.51	26.11	22.6	21.59	27.21	31.66	22.02	
day 38	25.66	21.76	24.91	22.41	19.86	25.95	27.96	22.25	
day 49	27.21	22.53	28.32	21.78	23.15	27.65	32.36	24.28	
day 49	26.73	22.55	27.72	23.68	23.72	27.02	29.31	24.51	
day 49	26.43	22.26	28.19	24.14	20.55	27.57	30.15	24.69	

infected turkeys	**average CT value**								
age of the birds	**TFRC**	**RPLP0**	**HMBS**	**HPRT**	**RPS7**	**RPL13**	**TBP**	**VIM**	**organ**
day 35	23.72	16.8	23.04	23.73	23.65	17.9	27.2	20.34	spleen
day 35	22.5	19.69	22.54	23.9	24.14	17.95	27.31	20.69	
day 35	23.41	17.39	22.93	25.64	27.1	17.13	27.33	22.81	
day 38	21.13	17.2	25.39	24.73	22.13	17.48	27.55	20.51	
day 38	22.5	17.5	25.35	24.83	22.89	17.58	26.77	20.72	
day 38	20.83	19.35	25.72	24.93	22.55	17.48	27.19	20.12	
day 35	22.81	19.04	24.25	29.51	24.17	19.5	34.18	24.42	liver
day 35	22.74	18.95	24.19	29.8	25.15	19.52	33.25	23.57	
day 35	22.94	15.97	22.84	28.42	24.67	19.43	33.39	24.31	
day 38	23.23	20.6	27.08	29.56	26.43	18.31	29.58	21.29	
day 38	22.97	21.76	26.73	27.49	26.92	18.27	28.78	21.23	
day 38	23.17	21.49	27.16	27.69	27.73	18.22	28.7	21.93	
day 35	24.36	21.98	23.98	24.89	23.95	19.35	29	19.39	caecum
day 35	24.04	20.9	24.87	25.15	26.22	19.32	29.41	18.9	
day 35	24.21	20.74	24.69	25.24	26.32	19.71	29.32	19.26	
day 38	22.86	20.11	28.36	24.48	27.69	18.77	30.72	21.03	
day 38	22.99	21.24	28.2	27.37	27.6	18.79	31.35	21.26	
day 38	22.98	20.76	28.5	27.57	28.97	18.95	31.24	21.04	
day 35	23.01	20.97	23.69	23.37	24.18	19.77	28.39	23.46	caecal tonsil
day 35	23.01	20.68	23.37	23.52	23.73	18.29	28.35	22.05	
day 35	22.91	20.36	23.66	23.99	24.19	18.88	28.28	21.99	
day 38	23.81	18.65	26.48	26.05	21.7	18.44	26.19	21.35	
day 38	23.98	17.67	24.97	25.17	23.45	18.2	25.99	22.65	
day 38	23.98	19.78	25.07	25.35	21.68	18.63	26.08	22.39	

control broiler chickens	average CT value		
age of the birds	**RPL13**	**TBP**	**organs**
day 4	18.76	24.33	spleen
day 4	17.96	24.82	
day 4	18.54	24.51	
day 4	19.21	25.65	liver
day 4	17.98	24.85	
day 4	18	25.92	
day 4	17.94	25.63	caecum
day 4	17.84	24.46	
day 4	18.56	24.88	
day 4	20.18	26.05	caecal tonsil
day 4	20.39	25.64	
day 4	19.52	25.53	
day 7	18.67	24.71	spleen
day 7	18.47	24.38	
day 7	18.84	24.74	
day 7	17.9	24.3	liver
day 7	18.1	25.39	
day 7	17.96	24.67	
day 7	18.67	24.96	caecum
day 7	18.07	25.59	
day 7	17.8	24.92	
day 7	19.23	25.86	caecal tonsil
day 7	19.6	25.89	
day 7	19.99	25.59	
day 21	19.04	24.37	spleen
day 21	17.92	24.61	
day 21	17.91	24.71	
day 21	19.93	24.29	liver
day 21	18.41	24.63	
day 21	18.04	24.11	
day 21	18.59	25.91	caecum
day 21	18.72	25.95	
day 21	18.58	25.66	
day 21	17.89	23.69	caecal tonsil
day 21	17.84	23.43	
day 21	17.89	23.97	

infected broiler chickens	**average CT value**		
age of the birds	**RPL13**	**TBP**	**organs**
day 7	17.68	23.59	spleen
day 7	17.8	23.84	
day 7	17.24	25.13	
day 7	18.2	23.61	liver
day 7	17.51	23.62	
day 7	17.6	23.57	
day 7	17.75	25.75	caecum
day 7	18.22	24.9	
day 7	18.04	25.16	
day 7	17.24	24.52	caecal tonsil
day 7	17.4	24.46	
day 7	17.85	24.61	

11.2. Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens

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Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens

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ABSTRACT

The protozoan parasite *Histomonas meleagridis* is the causative agent of histomonosis in gallinaceous birds. In turkeys, the disease can result in high mortality due to severe inflammation and necrosis in caecum and liver, whereas in chickens the disease is less severe. Recently, experimental vaccination was shown to protect chickens and turkeys against histomonosis but dynamics in the cellular immune response are not yet demonstrated. In the present work, different groups of birds of both species were vaccinated with attenuated, and/or infected with virulent histomonads. Flow cytometry was applied at different days post inoculation to analyse the absolute number of T-cell subsets and B cells in caecum, liver, spleen and blood, in order to monitor changes in these major lymphocyte subsets. In addition, in chicken samples total white blood cells were investigated.

Infected turkeys showed a significant decrease of T cells in the caecum within one week post infection compared to control birds, whereas vaccination showed delayed changes. The challenge of vaccinated turkeys led to a significant increase of all investigated lymphocytes in the blood already at 4 DPI, indicating an effective and fast recall response of the primed immune system.

In the caecum of chickens, changes of B cells, $CD4^+$ and $CD8\alpha^+$ T cells were much less pronounced than in turkeys, however, mostly caused by virulent histomonads. Analyses of whole blood in non-vaccinated but infected chickens revealed increasing numbers of monocytes/macrophages on all sampling days, whereas a decrease of heterophils was observed directly after challenge, suggesting recruitment of this cell population to the local site of infection.

Our results showed that virulent histomonads caused more severe changes in the distribution of lymphocyte subsets in turkeys compared to chickens. Moreover, vaccination with attenuated histomonads resulted in less pronounced alterations in both species, even after challenge.

1. Introduction

The flagellated protozoan parasite *Histomonas meleagridis* is the aetiological agent of histomonosis (synonyms: enterohepatitis or blackhead disease) of poultry [1]. The pathogenesis can vary between species of gallinaceous birds: in turkeys (*Meleagris gallopavo*) the disease can cause high mortality whereas in chickens (*Gallus gallus*) histomonosis is generally less fatal. The pathogen primarily targets the caecum before it reaches the liver through the hepatic portal vein. The lesions are characterized by severe fibrinous inflammation of the caecum and multifocal areas of inflammation and necrosis in the liver [2]. Effective prophylactic and therapeutic options are not available for food producing birds in most industrial countries due to consumer safety regulations resulting in re-emergence of the disease and economic losses in the poultry industry [3,4].

Previous investigations on vaccination to prevent histomonosis showed that the transfer of antibodies or the use of inactivated

Abbreviations: DPI, day post inoculation; FCM, flow cytometry; VT, vaccinated turkeys; VC, vaccinated chickens; IT, infected turkeys; IC, infected chickens; VIT, vaccinated and infected turkeys; VIC, vaccinated and infected chickens; CT, control turkeys; CC, control chickens; LS, lesion score; PBS, phosphate buffered saline; FCS, fetal calf serum; PBMCs, peripheral blood mononuclear cells; IELs, intraepithelial lymphocytes; aa, amino acid; mAb, monoclonal antibody.

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H. meleagridis was not effective to protect birds from the disease [5–7]. In contrast, the application of attenuated histomonads to prevent histomonosis was earlier demonstrated [2] and recently performed experimental studies showed that clonal *in vitro* attenuated *H. meleagridis* are effective and safe in protecting turkeys and chickens [7–10]. However, data on the immune response against histomonads are limited. Varying cytokine expression profiles in caecum and liver between chickens and turkeys indicated an innate immune response of chickens against histomonosis [11]. In the same work, the occurrence of different populations of lymphocytes in liver and spleen by immunohistochemistry was demonstrated. Moreover, co-infection of *Heterakis gallinarum* and *H. meleagridis* of chickens showed the involvement of T cells in the caecum with induction of Th1 and Th2 type cytokines [12]. The activation of the local humoral immune response was demonstrated by detecting specific antibodies in different parts of the intestine of chickens infected with histomonads [13]. Anyhow, there are no data available about detailed changes in lymphocyte distribution following *H. meleagridis* infection or vaccination.

Therefore, the aim of the present work was to investigate changes in the kinetics of lymphocytes during inoculation of turkeys and chickens with attenuated and virulent *H. meleagridis*. $CD4^+$ and $CD8\alpha^+$ T cells together with B cells in different organs and blood were investigated by flow cytometry (FCM) for the first time to obtain insights into the local and systemic cellular immune response after vaccination and/or infection. In addition, data on the cell dynamics of B cells, total T cells, macrophages/monocytes together with heterophils in whole blood of chickens were generated by FCM analysis following inoculation with *H. meleagridis* in chickens.

2. Materials and methods

2.1. Birds

A total of sixty turkeys (B.U.T. 6™; Aviagen Turkeys Ltd, Tattenhall, UK) and the same number of specific pathogen free (SPF) layer type chickens (VALO, BioMedia, GmBH, Osterholz-Scharmbeck, Germany) were included in the present study. At the first day of life every bird was marked with subcutaneously fixed tags for identification.

2.2. Preparations of parasites for inoculation

The clonal culture *H. meleagridis*/Turkey/Austria/2922-C6/04 [14] was co-cultivated with intestinal flora of the host bird before used for vaccination and infection of the birds: attenuated histomonads, established by long-term cultivation for 295 passages, were used for vaccination whereas for infection the virulent cultured histomonads (21 passages) were administered as previously described [7]. Both cultures were stored at −150 °C prior to inoculation. 6×10^5 cells of *H. meleagridis* in 600 μl culture medium consisting of Medium 199 with Earle's salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco™ Invitrogen, Lofer, Austria), 15% foetal calf serum (FCS) (Gibco™ Invitrogen) and 0.66 mg rice starch (Sigma-Aldrich, Vienna, Austria) were administered per bird, split between the oral and cloacal route using a syringe together with a crop tube, respectively a pipette. Birds of the control groups were sham infected with the equal volume of pure culture medium.

2.3. Setup of the in vivo trial

Water and feed (unmedicated turkey, respectively chicken starter feed) were provided *ad libitum*, except for 5 h of feed restriction after inoculation. The different groups consisted of 15 birds of each species and were kept separated in four rooms depending on the inoculation scheme: vaccinated turkeys (VT), vaccinated chickens (VC), infected turkeys (IT), infected chickens (IC), vaccinated and infected turkeys (VIT), vaccinated and infected chickens (VIC), control turkeys (CT) and control chickens (CC) (Table 1). Vaccination of groups VIT and VIC was applied on the first day of life. The challenge infection of the same birds was performed 28 days later together with the inoculation of the IT and IC groups. On the same day (28th day of life) birds from the only vaccinated group were inoculated with the attenuated strain and control birds were inoculated with culture medium only. From this day onwards, 3 previously determined birds (ascending order of tag numbers) per group were sacrificed 4, 7, 10, 14 and 21 days post inoculation (DPI).

2.4. Clinical examination, post-mortem and sampling

Behaviour, plumage, faeces, feed and water intake together with body weight were examined throughout the experiment for any clinical signs indicative for histomonosis. Re-isolation of viable parasites from cloacal swabs of every bird was performed in intervals of 2–3 days following vaccination and/or infection to confirm the successful inoculation. For that, after sampling each swab was placed into a 2 ml Eppendorf tube containing culture medium as described above and incubated at 40 °C. Following a propagation period of 2–3 days the re-isolations were microscopically examined. Blood samples of every bird were collected directly before the birds were killed. Birds that were killed or had to be euthanized were anaesthetized by intravenous application of thiopental (Sandoz, Kundl, Austria) before bleeding to death. Dead birds were necropsied and pathological changes in caecum and liver were evaluated using a previously established lesion score (LS) system: LS 0 was applied for normal organs whereas LS 1–4 classified mild to severe changes [13]. Caecum, liver and spleen were weighed and collected in cold phosphate buffered saline (PBS) (Gibco™ Invitrogen) containing 2% FCS (Gibco™ Invitrogen) (PBS + FCS) as soon as possible after death and further processed as described below.

2.5. Cell isolation

Single cell suspensions were prepared according to standard procedures from caecum, liver, spleen and blood PBMCs together with total white blood cells. Brief descriptions are given below for each type of sample.

2.5.1. Caecum

Intraepithelial lymphocytes (IELs) were isolated from both caeca of each bird as described previously [15] with some modification. In detail, faeces were removed and the organ samples were rinsed with cold PBS + FCS. After cleaning, the caeca were cut longitudinally in pieces of approximately 1 cm before isolation was performed in a solution of 50 ml PBS (Gibco™ Invitrogen) containing 500 μl of 1 M DTT (Sigma-Aldrich) and 10 μl of 0.5 M EDTA at 37 °C for 30 min during continuous stirring. The sediment consisting of sloughed tissue was left behind and the supernatant was transferred into two 50 ml tubes in equal amounts. The tubes were filled up with cold PBS + FCS and centrifuged at 4 °C, 220*g* for 10 min. The supernatant was collected passed through 40 μm nylon cell strainer (BD Falcon©), whereas the pellet was resuspended in 50 ml cold PBS + FCS to obtain more single cells by further centrifugation steps at 4 °C, 350*g* for 10 min. By that, single cells of the supernatants from the centrifugation steps were obtained before passed through the cell strainer. Following centrifugation the pellet was resuspended in 10 ml PBS + FCS.

The prepared suspension was then slowly layered above a double volume of Histopaque®-1077 (Sigma-Aldrich, Vienna, Austria) for density gradient centrifugation. The cells from interphase layer

were collected and washed. Finally, the pellet was dissolved in 1 ml of the same solution.

2.5.2. Liver and spleen

Single cells from liver and spleen were obtained by mechanical dissection. Isolation of lymphocytes was performed by crushing the liver (plunger of a syringe) and by tearing apart the spleen (two forceps) in petri dishes containing up to 30 ml cold PBS + FCS. The cells were then separated from the remaining tissue through a 40 μm nylon cell strainer (BD Falcon©) in a 50 ml tube. Following sedimentation of bigger tissue pieces for 10 min the supernatant was collected and centrifuged at room temperature, 350*g* for 10 min. The pellet was resuspended in 5 ml cold PBS + FCS and separated by density gradient as described for IELs. Mononuclear cells were finally resuspended in 5 ml cold PBS + FCS.

2.5.3. Blood

For the separation of peripheral blood mononuclear cells (PBMCs) approximately 3 ml of blood was collected from the wing vein of each bird and immediately transferred into blood collection tubes containing EDTA (EDTA KE/1.3, Sarstedt, Nümbrecht, Germany). The blood was mixed with an equal volume of cold PBS + FCS and subjected to density gradient separation under the conditions mentioned above after which, the obtained PBMCs were diluted in 1 ml cold PBS + FCS.

For total white blood cells, between 0.75 and 1 ml of blood per bird was collected in EDTA tubes (EDTA KE/1.3, Sarstedt). The samples were further treated with TransFix® solution (Cytomark, Buckingham, UK) according to manufacturer's protocol for conservation of the cells. Agitation was performed using a Stuart general rotator, STR4 (Bibby Scientific Limited, Staffordshire, UK) in circular rotation with 45° angle of the tubes with 15 rpm at room temperature, to ensure a proper diffusion.

2.6. Flow cytometry

2.6.1. FCM staining protocol

Lymphocytes of blood and tissues were microscopically examined for their viability using Trypan Blue and counted by a Neubauer hemocytometer (Sigma-Aldrich, Vienna, Austria). A concentration of 2×10^7 cells/ml PBS + FCS was adjusted before the cells were stained. Different combinations of monoclonal antibodies (mAbs) were used for immunophenotyping of the isolated cells. Detailed information on antibody combinations for the two species

Table 1
Experimental vaccination and/or infection of chickens and turkeys. Post mortem examinations and sampling of organs together with blood from 3 birds per group for flow cytometry experiments were performed on 4, 7, 10, 14 and 21 days post inoculation.

Group	Day of life/day post inoculation						
	1	28	32/4	35/7	38/10	42/14	49/21
Vaccinated turkeys (VT)		Vaccination	x[a]	x	x	x	x
Vaccinated chickens (VC)			x	x	x	x	x
Infected turkeys (IT)		Infection	x	x	x	n.a.[b]	n.a.
Infected chickens (IC)			x	x	x	x	x
Vaccinated and infected turkeys (VIT)	Vaccination	Infection	x	x	x	x	x
Vaccinated and infected chickens (VIC)			x	x	x	x	x
Control turkeys (CT)			x	x	x	x	x
Control chickens (CC)			x	x	x	x	x

[a] Necropsy and sampling of three birds.
[b] Not applicable due to fatal histomonosis at earlier time points.

Table 2
Antibody panels. List of antibodies and antibody combinations used in this study.

Purpose	Species specificity	Antigen	Clone	Isotype	Fluoro-chrome	Labeling strategy	Source of primary mAb
Cross-reactivity of CD3-12	Human	CD3ε	CD3-12	Rat IgG1	AlexaFluor 647	Directly conjugated	AbD Serotec
	Chicken	CD3	CT3	Mouse IgG1	BV421	Biotin-streptavidin[a]	Southern-Biotech
H. meleagridis vaccination/ infection study: Turkey panel 1	Human	CD3ε	CD3-12	Rat IgG1	AlexaFluor 647	Directly conjugated	AbD Serotec
	Chicken	MHC-II	2G11	Mouse IgG1	BV421	Secondary antibody[b]	LMU Munich[d]
Turkey panel 2	Human	CD3ε	CD3-12	Rat IgG1	AlexaFluor 647	Directly conjugated	AbD Serotec
	Chicken	CD4	CT4	Mouse IgG1	BV421	Biotin-streptavidin[a]	Southern-Biotech
	Chicken	CD8α	3-298	Mouse IgG2b	R-PE	Directly conjugated	Southern-Biotech
Chicken panel 1	Human	CD3ε	CD3-12	Rat IgG1	AlexaFluor 647	Directly conjugated	AbD Serotec
	Chicken	Bu-1	AV20	Mouse IgG1	BV421	Biotin-streptavidin[a]	Southern-Biotech
Chicken panel 2	Human	CD3ε	CD3-12	Rat IgG1	AlexaFluor 647	Directly conjugated	AbD Serotec
	Chicken	CD4	CT4	Mouse IgG1	BV421	Biotin-streptavidin[a]	Southern-Biotech
	Chicken	CD8α	3-298	Mouse IgG2b	R-PE	Directly conjugated	Southern-Biotech
Chicken whole blood panel	Chicken	CD45	16-4	Mouse IgG2a	PerCP	Conjugation kit[c]	Southern-Biotech
	Chicken	Bu-1	AV20	Mouse IgG1	APC	Conjugation kit[c]	Southern-Biotech
	Chicken	Macrophags/ monocytes	Kul-01	Mouse IgG1	R-PE	Conjugation kit[c]	Southern-Biotech
	Chicken	CD4	CT4	Mouse IgG1	FITC	Directly conjugated	Southern-Biotech
	Chicken	CD8α	3-298	Mouse IgG2b	FITC	Directly conjugated	Southern-Biotech
	Chicken	TCR-γδ	TCR1	Mouse IgG1	FITC	Directly conjugated	Southern-Biotech

[a] Brilliant Violet 421™ Streptavidin, BioLegend.
[b] Rat anti-mouse IgG1 Brilliant Violet 421™, clone RMG1-1, BioLegend.
[c] LYNX Rapid Conjugation kits®, AbD serotec.
[d] Kindly provided by Prof. Dr. Thomas Göbel; Institute of Animal Physiology, Ludwig-Maximilian University, Munich, Germany.

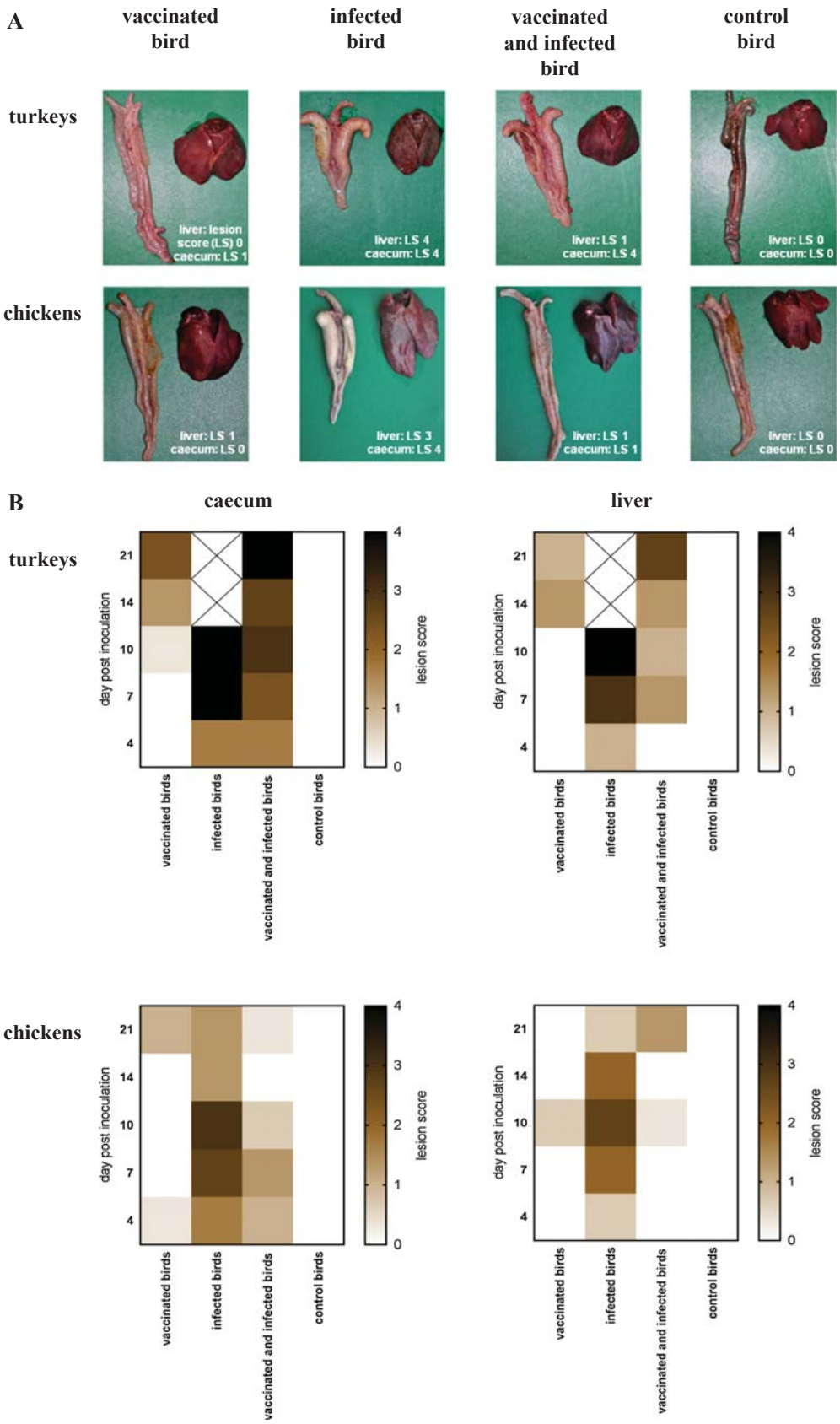

Fig. 1. Pathological changes in caecum and liver following vaccination and/or infection of chickens and turkeys. (A) Differences in the severity of lesions using defined lesion scores (ls 0–4) between the different groups are shown exemplarily at 10 day post inoculation in caecum and liver of 1 bird of every group. (B) Heat map comparatively indicating the mean lesion score of 3 birds of every group on the different days post inoculation (Software: Graph Pad PRISM® 7.02).

and, where applicable, their fluorescence labelling by second-step reagents is given in Table 2. The final concentration of every antibody was determined by titration and the respective isotype controls were included.

For staining of mononuclear cells isolated from blood and organs, 20 μl of the adjusted cell suspension was transferred into wells of 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) together with the respective primary antibodies, except anti-human CD3ε, for incubation for 30 min at 4 °C. Afterwards, cell pellets obtained by centrifugation at 4 °C, 250*g* for 4 min were washed two times with cold PBS + FCS. For biotinylated antibodies the secondary reagent Brilliant Violet 421™ Streptavidin (BioLegend, San Diego, CA, USA) was applied. Following another incubation step for 30 min at 4 °C further washing was performed. The cells were fixed with BD fixation buffer (BD Biosciences, San jose, CA, USA) according to manufacturer's protocol. Intracellular staining with the anti-human CD3ε mAb CD3-12 was performed after fixation and permeabilization. To achieve this, the BD Cytofix/Cytoperm™ fixation/permeabilization kit (BD Biosciences) was employed according to manufacturer's instructions. Afterwards the cells were incubated with CD3-12 antibody for 30 min followed by two washing steps. Finally, the pellets were resuspended in 200 μl cold PBS + FCS and kept at 4 °C until FCM analysis.

Total white blood cells were analysed according to a previously established protocol [16] with slight modifications. Briefly, blood samples were processed in BD Trucount Tubes® (BD Biosciences, Austria) and incubated with mouse anti-chicken CD45-PerCp (AbD Serotec), mouse anti-chicken Bu-1-APC (SouthernBiotech), mouse anti-chicken TCR-γδ-FITC, mouse anti-chicken CD8α-FITC, mouse anti-chicken CD4-FITC and mouse anti-chicken KUL-01-RPE (SouthernBiotech) (see Table 2 for details on antibodies) before FCM was performed.

2.6.2. FCM analysis

FCM of stained cells was performed on a FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer equipped with three lasers (405, 488 and 633 nm) and a high throughput sampler (HTS). At least 40,000 lymphocytes per sample were recorded. Analysis of FCM raw data was performed by FACSDiva Software version 6.1.3 (BD Biosciences).

2.6.3. Determination of absolute cell counts

For calculation of absolute cell numbers, percent values obtained by lymphocyte-subgating were multiplied by the total lymphocyte counts obtained by hemocytometer. After that, every cell population was calculated per gram or per ml according to the weight or volume of the tissue or blood, respectively, taken for the extraction procedure.

For total white blood cell counts at least 10,000 Trucount® beads were recorded in each sample and absolute numbers of individual cell populations were calculated using following formula: absolute cell count/μl blood = (cells counted/beads counted) × (total content of beads per tube/blood volume per tube) [16].

2.7. Statistical analysis

For statistical calculation of numbers of the different cell populations, the mean values of cell populations from three birds per group were used for each time point. Samples obtained from vaccinated and/or infected groups were individually compared to the equivalent cells isolated from birds of the negative control group. Significant differences were calculated using student's T-test, with a p-value ≤ 0.05.

3. Results

3.1. Clinical signs and post mortem

Turkeys infected with virulent histomonads (group IT), showed first clinical signs, such as depression, diarrhoea and ruffled feathers, on 7 DPI. The severity of histomonosis increased in birds of this group and therefore the remaining birds had to be euthanized before the pre-last sampling day, i.e. 14 DPI. In contrast, no clinical signs were noticed in any other group. Re-isolations of histomonads from cloacal swabs confirmed the presence of the parasite in every group with the exception of control birds (data not shown).

Different grades of pathological changes (Fig. 1A) and the mean LS on the respective sampling day of every group determined during the post mortem procedure are summarized in Fig. 1B. In the IT group first signs of inflammation were observed in caecum and liver at 4 DPI which later on increased until 10 DPI with the maximum LS 4 in both organs. In group VIT lesion scores in the caecum and liver were milder on the respective sampling days and increased until 21 DPI in the caecum to LS 4 and in the liver to LS 2.5. First lesions in only vaccinated turkeys (VT group) were observed at 10 DPI in the caecum and 4 days later in the liver. The obtained scores were rather low, between LS 1 and LS 3. The severity of lesions in chickens was graduated from LS of 3 in the caecum and 2.5 in the liver of IC at 10 DPI to lower values in group VC. Chickens of group VIC and VC entirely displayed LS below 2. None of the control birds in group CT or CC showed lesions at any sampling day.

3.2. Flow cytometry analysis

3.2.1. Cross-reactivity of mAb CD3-12 with turkey T cells

As no turkey-specific anti-CD3 antibody was available, cross-reactivity of the rat anti-human antibody CD3ε (clone CD3-12) was tested for lymphocytes of chicken and turkey in initial

Table 3
Amino acid sequence alignment of the epitope recognized by the monoclonal antibody CD3ε (clone CD3-12). Comparison of the relevant amino acid sequence of human T-cell surface glycoprotein CD3ε chain with the respective CD3ε molecule of chicken and turkey using protein BLAST®. The difference in the amino acid sequence is denoted in red colour.

Description	Sequence	Identity	Accession No.
CD3 epsilon chain precursor (*Homo sapiens*)	ERPPPVPNPDYEPI	100%	NP 000724.1
CD3 epsilon chain isoform X1(*Gallus gallus*)	QRPPPVPNPDYEPI	92%	XP 015153448.1
CD3 epsilon chain (*Gallus gallus*)	QRPPPVPNPDYEPI	92%	ACF04800.1
CD3 epsilon chain precursor (*Gallus gallus*)	QRPPPVPNPDYEPI	92%	NP 996787.1
CD3 epsilon chain (*Meleagris gallopavo*)	QRPPPVPNPDYEPI	92%	XP 003212775.1

experiments. The mAb CD3-12 recognizes an epitope of 14 amino acid residues within the cytoplasmic domain of the CD3ε molecule. By using Basic Local Alignment Search Tool (BLAST®) the amino acid sequences comprising this epitope of the following species were compared: *Meleagris gallopavo*; *Gallus gallus* and *Homo sapiens* (Table 3). The BLAST® showed that within the 14 amino acids only one amino acid, situated at N-terminus, is different for turkeys and chickens compared to the human sequence. FCM with lymphocytes isolated from chicken blood, spleen, liver, caecum and stained with rat anti-human CD3ε (clone CD3-12) versus mouse anti-chicken CD3 (clone CT3) revealed that both antibodies target the same cell population (Fig. 2, top panel). In parallel, lymphocytes derived from the same organs of turkeys were labelled also with rat anti-human CD3ε (clone CD3-12) and co-stained with a mouse anti-chicken CD4 mAb (with a documented cross-reactivity against turkey CD4 [17]). Results revealed that all $CD4^+$ cells were co-stained by the CD3-12 antibody in all investigated organs, indicating that these cells represent $CD3^+CD4^+$ T cells (Fig. 2, bottom panel). Moreover, a separate population of $CD3\text{-}12^+$ but $CD4^-$ population of cells was obtained, representing $CD3\varepsilon^+$ $CD8\alpha^+$ and γδ T cells (see also Fig. 3A, bottom panel).

3.2.2. Gating strategy for identification of B cells, $CD4^+$ and $CD8\alpha^+$ T cells in chickens and turkeys

FCM analyses were performed with lymphocytes isolated from caecum, liver, spleen, and blood of *H. meleagridis* infected/vaccinated turkeys and chickens. A uniform gating hierarchy was used throughout all sampling days and for both species (Fig. 3A + B). After gating on putative lymphocytes by light scatter properties, doublet cells were excluded in two consecutive gates based on uniform forward scatter and side scatter width versus height properties. For both species, total T cells were identified in a gate (illustrated in red) comprising by $CD3\varepsilon^+CD4^+$ T cells and $CD3\varepsilon^+CD4^-$ T cells and further sub-gated for analysis of CD4 and CD8α expression. Two subsets were identified in this way: $CD3\varepsilon^+CD4^+CD8\alpha^-$ (orange window) and $CD3^+CD4^-CD8\alpha^+$ T cells (blue window). For the identification of B cells different marker combinations were used for turkeys and chicken. Due to the lack of a B-cell specific antibody in turkeys, we decided to identify putative B cells in this species by a $CD3\varepsilon^-$MHC-II^+ phenotype (Fig. 3A, bottom panel, left, purple gate) since it has been shown that turkey B cells express MHC-II (clone 2G11) [18]. Chicken B cells were identified by the commercially available Bu1 (clone AV20) mAb in parallel to CD3ε staining. In this way $CD3\varepsilon^-Bu1^+$ cells were identified (Fig. 3B, bottom panel, left, purple gate) and designated as B cells for all chicken samples.

Following the identification of these lymphocyte subsets by the illustrated gates, the mean of absolute numbers of $CD4^+$ ($CD3\varepsilon^+CD4^+CD8\alpha^-$), $CD8\alpha^+$ ($CD3\varepsilon^+CD4^-CD8\alpha^+$) and B ($CD3\varepsilon^-$MHC-$II^+$ or $CD3\varepsilon^-Bu1^+$) cells in caecum, liver, spleen, and blood of turkeys and chickens were calculated and are comparatively given between 4 and 21 DPI.

3.2.3. Caecum

In the caecum, at 4 DPI, both investigated T-cell subsets ($CD3\varepsilon^+CD4^+CD8\alpha^-$, $CD3\varepsilon^+CD4^-CD8\alpha^+$) and B cells significantly decreased in birds of group VIT in comparison to controls (Fig. 4). Three days later, a decrease of the T-cell subsets was observed in birds of group IT whereas in all inoculated chicken groups IC, VIC, VC only $CD4^+$ T cells were found decreased. On the same day, B cells of chicken were only altered in group VIC, when higher numbers were counted. At 10 DPI, only turkeys of group IT were affected by a significant decrease of $CD4^+$ T cells but increased numbers of $CD8\alpha^+$ T cells and B cells. On the last two sampling days, the groups VIT and VT showed a reduction of all lymphocyte subpopulations but not all values were statistically relevant. The

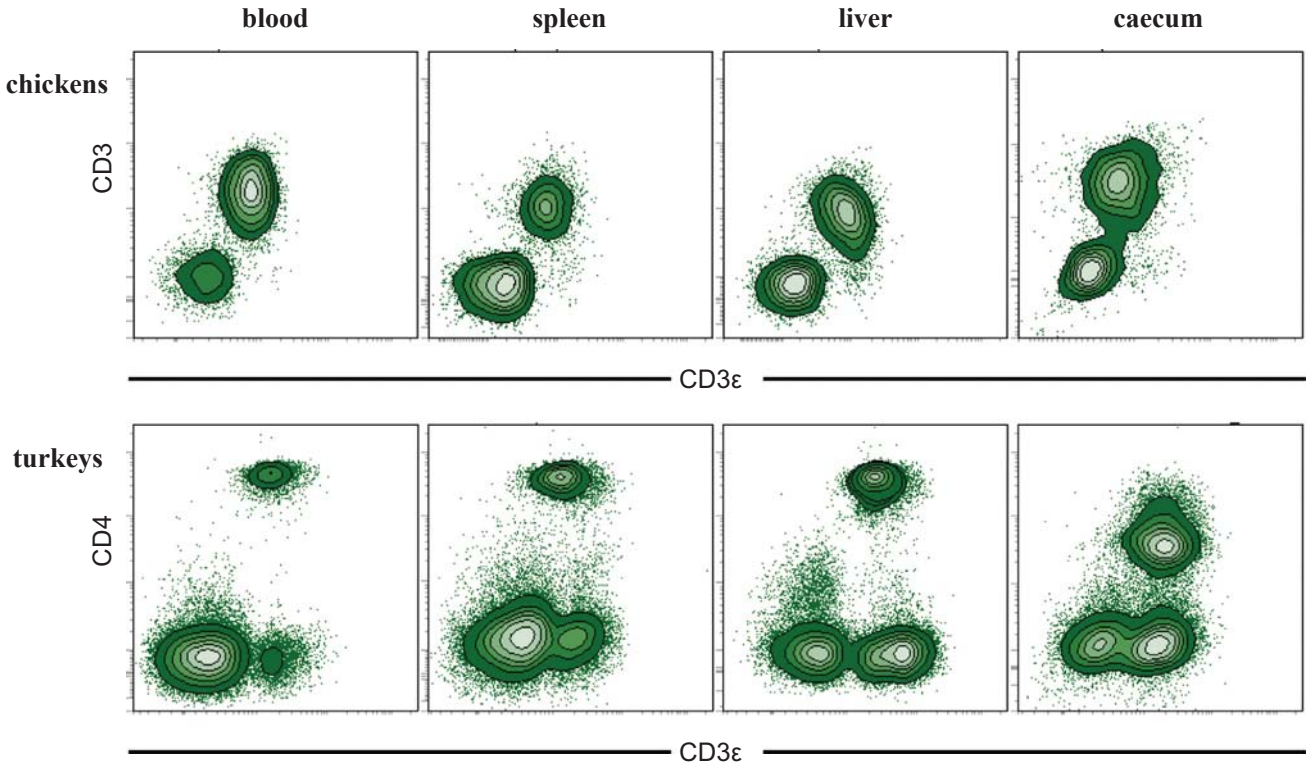

Fig. 2. Evaluation of cross-reactivity of anti-human CD3ε antibody clone CD3-12 with lymphocytes from chickens and turkeys. Lymphocytes of caecum, liver, spleen and blood of both species were isolated and stained with the anti-human CD3ε antibody clone CD3-12 (x-axes, both species) and an anti-chicken CD3 mAb (clone CT3, y-axis, top panel) or an anti-chicken CD4 mAb (clone CT4, y-axis, bottom panel). Data is representative of experiments with cells isolated from 3 different chickens or turkeys each.

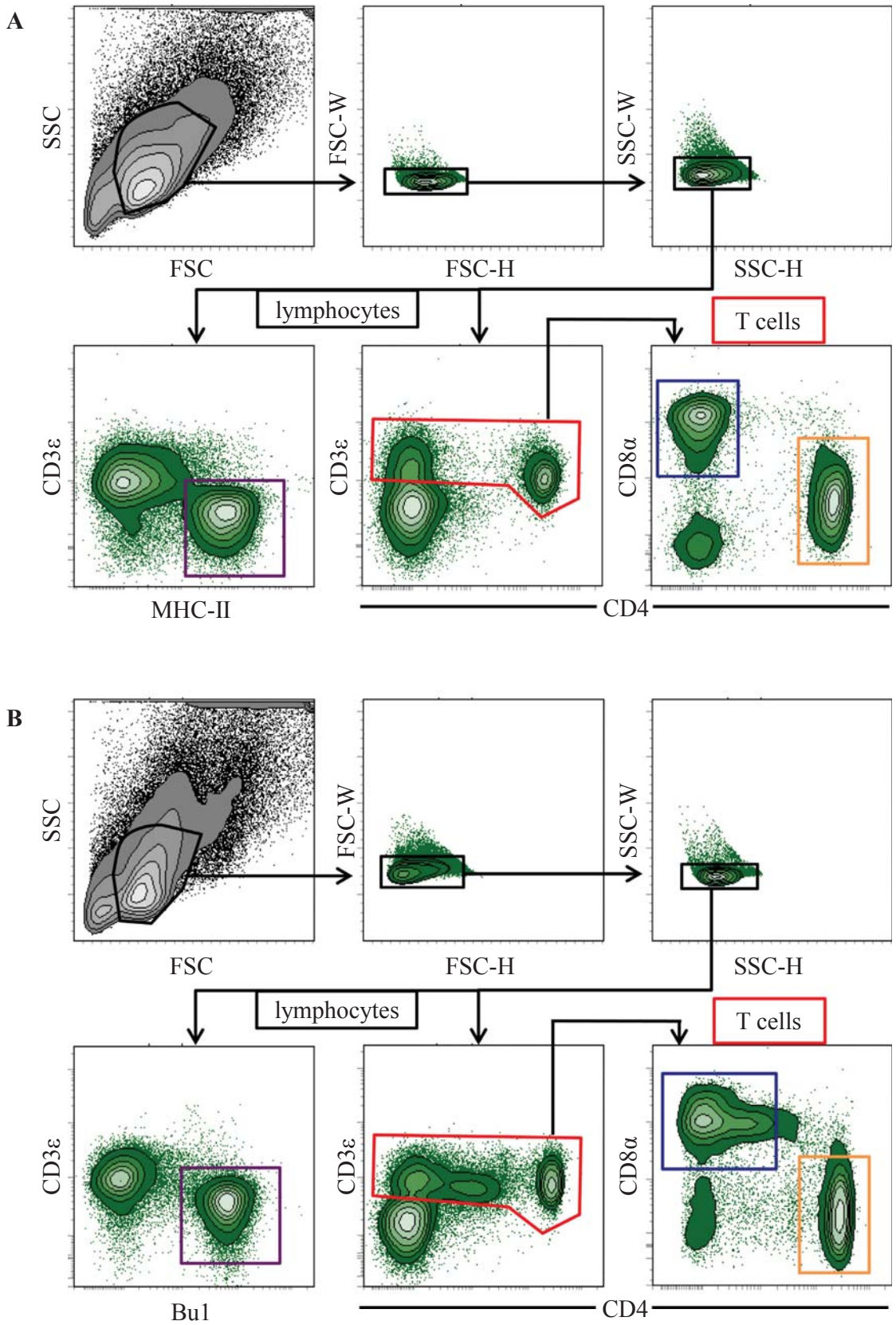

Fig. 3. Gating strategy for lymphocytes in multicolour flow cytometry analysis. Lymphocytes isolated from (A) turkeys and (B) chickens, were gated according to their light scatter properties. Potential doublet cells were excluded by consecutive FSC-H/FSC-W and SSC-H/SSC-W double discrimination gates. Single cells were analysed for CD3 expression (middle panel) and gated as CD3ε$^+$ T cells (red gate, a + b). CD3ε$^+$ T cells were further analysed for CD3ε$^+$CD4$^+$CD8α$^-$ T cells (orange gate) and CD3ε$^+$CD4$^-$CD8α$^+$ cells (blue gate). In parallel samples CD3ε$^-$MHC-II$^+$ B cells (turkey, a, purple gate) or CD3ε$^-$Bu1$^+$ B cells (chicken, b, purple gate) were identified. The gating strategy is shown as a representative example for splenocytes isolated from control birds at 10DPI and was performed accordingly for all analysed organs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly decreased number of CD8α+ T cells at 14 DPI of chickens of group IC was the only difference in chicken at the final stage of the experiment.

3.2.4. Liver

In the liver, at the early stage of infection (4 DPI), only chickens of group IC showed a significant increase of CD4+ T cells (Fig. 5). Later on at 7 DPI, elevations of all types of lymphocytes were observed in turkeys of all inoculated groups with significant changes in group IT compared to CT. Similar elevation were also visible for VIT. Infected chicken (IC) showed a robust increase of both T-cell subsets at 10 DPI. Both CD4+ and CD8α+ T cells significantly increased at 14 DPI in group VIT whereas only a higher amount of CD4+ T cells was found in the VT group. At the final sampling day the cell numbers in livers of all birds did not vary significantly from control birds.

3.2.5. Spleen

Within lymphocytes isolated from spleen (Fig. 6), most changes were observed within one week post inoculation: at 4 DPI, B cells of groups IT, VIT and IC were significantly decreased in comparison to controls, whereas T-cell subsets did not differ significantly. At 7 DPI, turkeys of group VT showed an increase of B cells as well as CD4+ T cells in all inoculated groups and CD8α+ T cells in group VT and VIT. Lymphocytes in spleen samples collected from chickens on the same day did not differ significantly but an elevation of B cells in VIC was observed at 10 DPI. On the same day in turkeys CD4+ T cells were increased as the last cellular aberrance in the time course p.i.

3.2.6. Blood

In PBMC (Fig. 7), CD4+ T and B cell populations significantly increased in groups VIT and VT together with CD8α+ T cells in

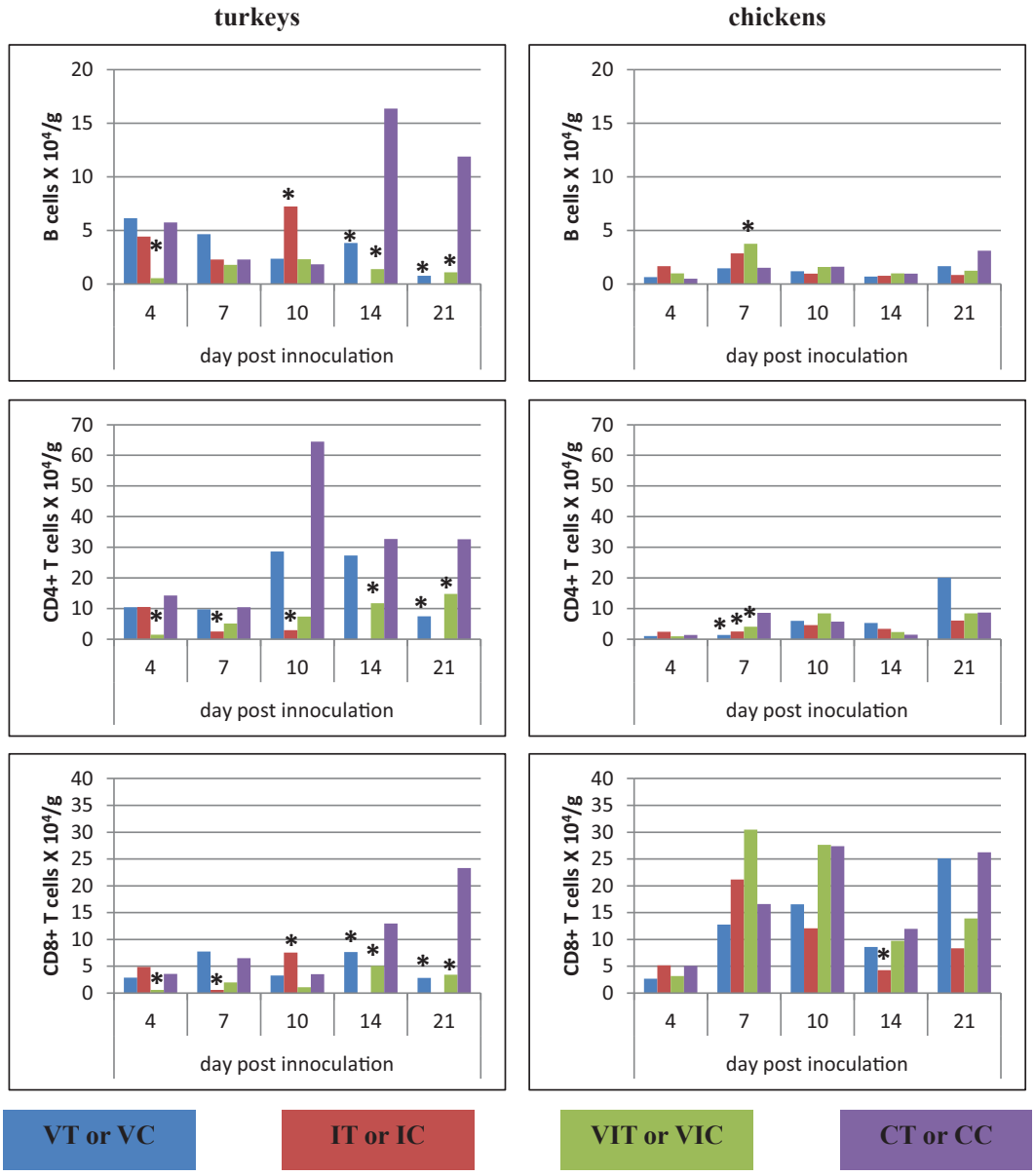

Fig. 4. Alterations of B cells, CD4+ and CD8α+ T cells following vaccination and/or infection analysed by flow cytometry in the caecum. Absolute cell numbers of B cells (CD3ε−MHC-II+, turkey; CD3ε−Bu1+, chicken), CD4+ T cells (CD3ε+CD4+CD8α−) and CD8α+ T cells (CD3ε+CD4−CD8α+) were determined in groups of vaccinated turkeys (VT), vaccinated chickens (VC), infected turkeys (IT), infected chickens (IC), vaccinated and infected turkeys (VIT), vaccinated and infected chickens (VIC), control turkeys (CT) and control chickens (CC). Data represent the mean absolute cell number of 3 birds/group. *Significant different to control group of the respective species ($P \leq 0.05$).

group VIT at 4 DPI. B cells of chickens were detected in very low numbers in the blood on 4 DPI for all groups. Three days later CD8α+ T cells were increased in group IC. At 10 DPI group IT showed a decrease of B cells as well as CD8α+ T cells which were also found in lower amounts in birds of group VIT. Birds of group IC showed an increase of all lymphocytes at 14 DPI and on the last sampling day CD8α+ T cells were decreased in group VC. The groups VT and VIT did not show changes at 14 and 21 DPI.

3.2.7. Total white blood cells of chickens

The total white blood cell analysis was performed in order to address possible changes of absolute numbers of monocytes/macrophages and heterophils. Details of the absolute cell number for heterophils and macrophages/monocytes are given in Fig. 8. In group IC monocytes/macrophages significantly increased between 4 and 14 DPI, when heterophils were found to be significantly decreased. In group VIC a reduction of heterophils in the blood at 4 DPI was observed.

4. Discussion

The rising impact of histomonosis in flocks of turkeys and chickens without preventive or therapeutic options against the disease in industrial countries increases the importance and necessity to apply new strategies to combat the disease [3]. *In vitro* attenuated *H. meleagridis* was shown to reduce lesions and clinical signs in both bird species [7,9]. However, nothing is known on the immune response following vaccination. Furthermore, no data are available on changes of absolute numbers of immune cells following infection with virulent histomonads. Especially, a comparative study

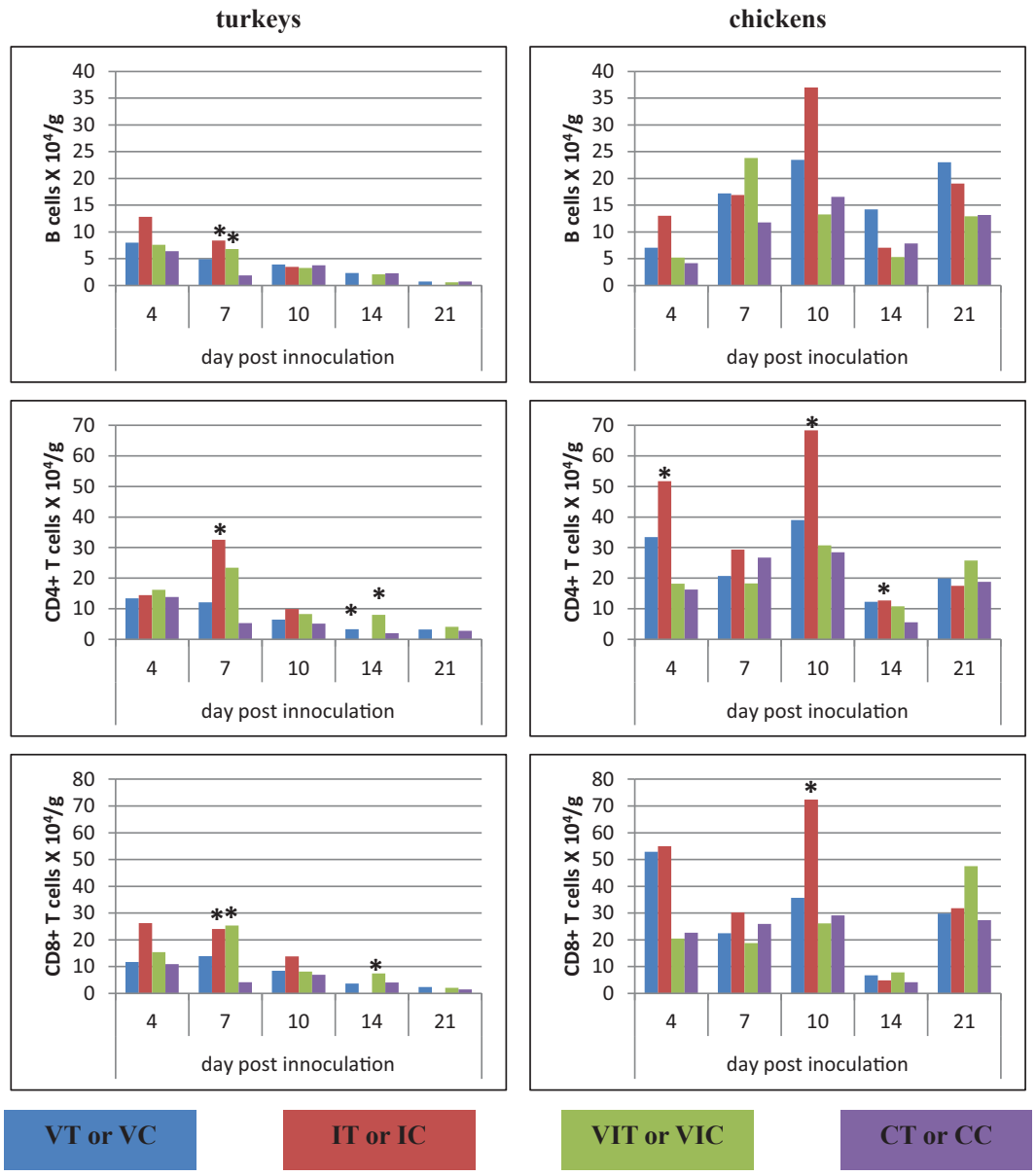

Fig. 5. Alterations of B cells, CD4+ and CD8α+ T cells following vaccination and/or infection analysed by flow cytometry in the liver. Absolute cell numbers of B cells (CD3ε−MHC-II+, turkey; CD3ε−Bu1+, chicken), CD4+ T cells (CD3ε+CD4+CD8α−) and CD8α+ T cells (CD3ε+CD4−CD8α+) were determined in different groups of turkeys and chickens at different days post inoculation. Data represent the mean absolute cell number of 3 birds/group. *Significant different to control group of the respective species ($P \leq 0.05$).

between turkeys and chickens suffering from histomonosis is of interest to elucidate cellular immune mechanisms that may contribute to the different clinical outcome of the disease in the two species.

In the present study, we focussed on major lymphocyte subsets representing cells of the adaptive immune system by investigating quantitative changes of B cells, $CD4^+$ T cells and $CD8\alpha^+$ T cells in different organs and blood of birds inoculated with attenuated and/or virulent parasites. The applied attenuated and virulent histomonads were co-cultivated with bacteria which were initially isolated from birds suffering from histomonosis and characterized as species belonging to the normal gut microbiota [19]. In the last mentioned study, it was also reported that the virulence of the same clonal strain of *H. meleagridis* cultivated with a non-pathogenic laboratory strain of *E. coli* (DH5α) is independent of the co-cultivated bacteria. Anyhow, the bacterial flora of birds from all groups consisted of normal gut bacteria for a valid comparability.

As a pre-requisite it was crucial to identify appropriate mAbs for the detection of the aforementioned lymphocyte subsets in turkeys and chickens. For chickens, the respective antibodies are commercially available but there are no specific markers for turkey cells. The cross-reactivity of anti-chicken antibodies against B cells, $CD4^+$ and $CD8\alpha^+$ of turkey was previously demonstrated [17,18,20,21], whereas anti-chicken $CD3^+$ antibody failed to react with turkey T cells. In order to identify $CD4^+$ and $CD8\alpha^+$ T cells, we tested the rat anti-human CD3-12 mAb (AbD serotec) for cross-reactivity against chicken and turkeys cells. The mAb recognizes a peptide representing an invariant cytoplasmic sequence within the CD3ε chain [22]. In several studies a broad species cross reactivity of this antibody was demonstrated in different mammals and birds like ducks and owls [22–28]. *In silico* analysis revealed a

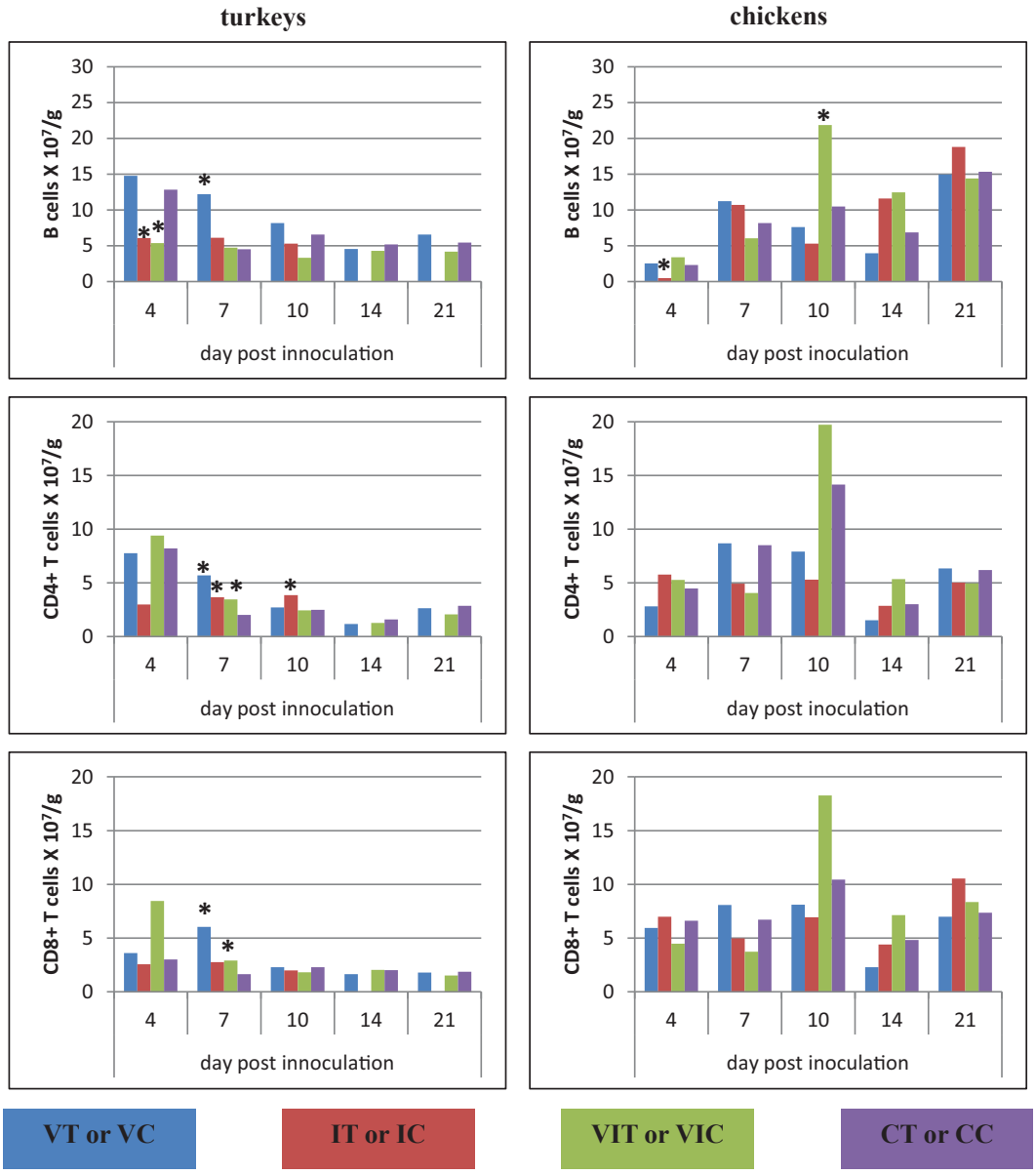

Fig. 6. Alterations of B cells, $CD4^+$ and $CD8\alpha^+$ T cells following vaccination and/or infection analysed by flow cytometry in the spleen. Absolute cell numbers of B cells ($CD3\varepsilon^-$MHC-II$^+$, turkey; $CD3\varepsilon^-$Bu1$^+$, chicken), $CD4^+$ T cells ($CD3\varepsilon^+CD4^+CD8\alpha^-$) and $CD8\alpha^+$ T cells ($CD3\varepsilon^+CD4^-CD8\alpha^+$) were determined in different groups of turkeys and chickens at different days post inoculation. Data represent the mean absolute cell number of 3 birds/group. *Significant different to control group of the respective species ($P \leq 0.05$).

difference of only a single amino acid at the N-terminal end within the entire 14 amino acid sequence recognized by the antibody. Comparative FCM investigations using the species-specific mouse anti-chicken CD3 antibody in samples of chickens verified the validity of the rat anti-human CD3ε antibody to be used as a marker for chicken T cells. Similarly, co-stainings of the anti-human CD3ε antibody with established cross-reactive CD4 and CD8α-specific antibodies in turkey lymphocytes indicated that the anti-human CD3ε antibody CD3-12 can be used for the identification of turkey T cells.

In samples from chickens and turkeys that received vaccination, vaccination and infection, or only infection divergences in the numbers of B cells, $CD3\varepsilon^{+}CD4^{+}CD8\alpha^{-}$ and $CD3\varepsilon^{+}CD4^{-}CD8\alpha^{+}$ cells were observed by FCM: in the early stage of infection (4 DPI) the main changes were observed in vaccinated and infected turkeys (group VIT) when all lymphocyte subpopulations decreased in the caecum but increased in the blood. This may indicate a rapid apoptosis of effector memory cells in the caecum which are replenished by proliferating central memory cells via the blood stream. Similar to an early time point of infection, at 4 dpi, vaccination of naive turkeys rarely caused variations which might be explained by an unresponsiveness of the adaptive immune system at this early time point post inoculation. Lymphocytes of chickens, including vaccinated and infected birds, remained largely unchanged at 4 DPI. The milder response to the challenge of vaccinated chickens compared to turkeys might be interpreted as a much lower reactivation of *Histomonas*-specific memory cells after challenge at early stage compared to turkeys.

At 7 DPI $CD4^{+}$ and $CD8\alpha^{+}$ T cells were depleted in the caecum of IT, resembling the pattern of T-cell subset changes in VIT at 4 DPI. In the liver all lymphocyte subpopulations of IT birds were increased and the same was observed for B cells and $CD8\alpha^{+}$ T cells of group VIT. This later response might be due to the delayed infiltration of the parasite into the liver compared to the caecum. In the spleen

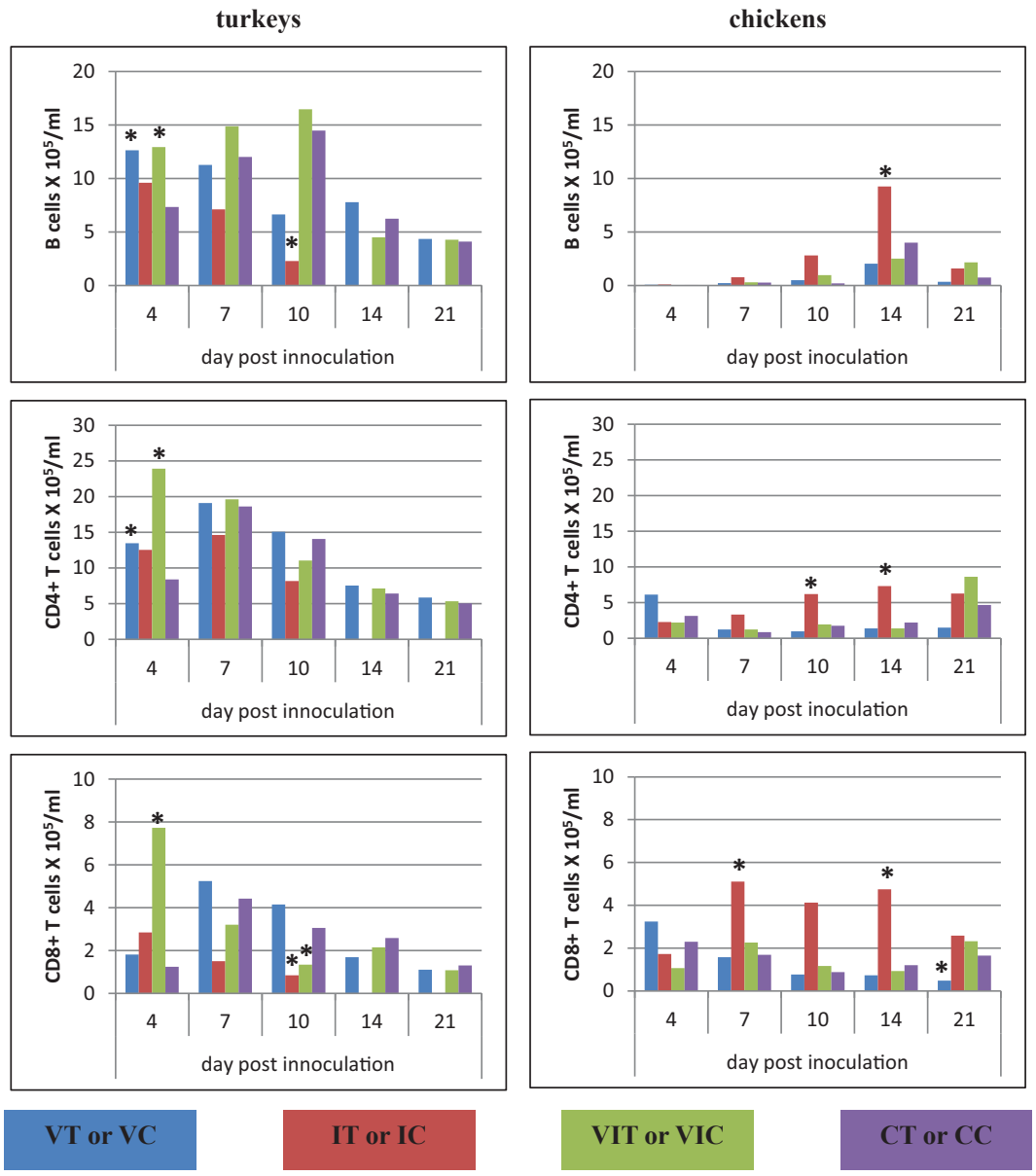

Fig. 7. Alterations of B cells, $CD4^{+}$ and $CD8\alpha^{+}$ T cells following vaccination and/or infection analysed by flow cytometry in the PBMCs. Absolute cell numbers of B cells ($CD3\varepsilon^{-}$MHC-II^{+}, turkey; $CD3\varepsilon^{-}$Bu1^{+}, chicken), $CD4^{+}$ T cells ($CD3\varepsilon^{+}CD4^{+}CD8\alpha^{-}$) and $CD8^{+}$ T cells ($CD3\varepsilon^{+}CD4^{-}CD8\alpha^{+}$) were determined in different groups of turkeys and chickens at different days post inoculation. Data represent the mean absolute cell number of 3 birds/group. *Significant different to control group of the respective species ($P \leq 0.05$).

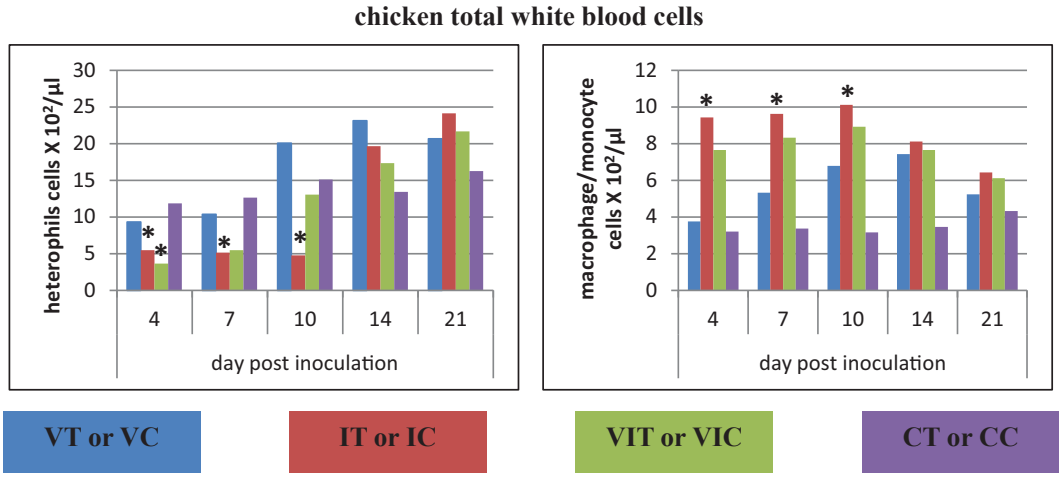

Fig. 8. Analysis of total white blood cells in chickens. Absolute numbers of heterophils and monocytes/macrophages were calculated at different days post inoculation in whole blood of vaccinated chickens (VC), infected chickens (IC), vaccinated and infected chickens (VIC) together with control chickens (CC). Data represent the mean absolute cell number of the 3 birds/group. *Significant different to control group of the respective species ($P \leq 0.05$).

only $CD4^+$ T cells increased in group IT as well as both T-cell subsets in group VIT, however, a more pronounced elevation of all cell types occurred in turkeys of group VT. This may indicate a systemic response in this group due to vaccination although this did not apply to PBMC. In chickens all cell populations were similar in number with some exceptions: most noticeable, the increase of B cells in caecum of birds in the VIC group may indicate a delayed recall response of B cells in this species compared to turkeys.

On day 10, the main cellular aberration was observed in group IT in the caecum where B cells and $CD8\alpha^+$ T cells were abundant but at the same time lower in number in the blood. This finding shows the composition of the investigated lymphocyte subsets shortly before turkeys succumb to histomonosis. Histomonosis causes severe inflammation and necrosis in caecum and liver of turkeys [2], this is confirmed in our study with highest lesion score for both the organs of infected turkeys on 10 DPI. This excessive necrosis may also result from the cytotoxic activity of $CD8\alpha^+$ T cells, acting as effector cells which was reported for the caecum of chickens following infections with the virulent protozoan parasite *Eimeria tenella* [29].

Interestingly, and in agreement with the aforementioned hypothesis infected chickens that did not contract the disease showed relatively unchanged cell numbers in the caecum. However, all lymphocytes increased (T-cell subsets significantly) in the liver. Compared to the turkey this response is again delayed and is in coherence with milder LS in the liver.

At the last two sampling days, 14 and 21 DPI, data from group IT was not available due to the fatal outcome of the infection. The remaining turkeys in group VT and VIT showed reduced numbers of all investigated lymphocytes in the caecum but increased amounts of $CD4^+$ and $CD8\alpha^+$ cells in the liver, mostly with significant relevance. These cellular changes occurred at a later time point compared to birds from the IT group. Based on this observation, it might be concluded that the lymphocyte subsets of vaccinated turkeys showed a second wave of effector activity which results in increased lesion scores and is accompanied by a rapid death of these effector cells.

In chickens the analysed immune cells in infected organs were mostly in a normal range matching with the low LS. Mainly in the blood, an increase of B cells and both T cell subpopulations in group IC was observed at 14 DPI which may indicate the ongoing recruitment of these cells due to the infection with virulent histomonads.

Analyses of white blood cells from whole blood were performed to obtain quantitative data on macrophages/monocytes and heterophils but was restricted to samples from chickens due to the lack of markers for turkey cells [16]. Within the IC group macrophages/-monocytes resulted in significant higher amounts between 4 and 10 DPI compared to the control, suggesting the activation of an innate immune response which contributes to inactivate the parasite. The heterophils were significantly lower during that time in the blood this may be due to the infiltration of these granulocytes in the local site of infection. The severe infiltration of mononuclear and polymorphonuclear cells in the infiltrated organs caecum and liver is a common histopathological finding in the course of histomonosis [30].

Overall, quantitative analyses of the present study suggest that $CD4^+$ and $CD8\alpha^+$ T cells are involved in the immune response against histomonosis which is coherent with previous results based on cytokine expression and histological assays [11]. Furthermore, it was found in the actual study that attenuated histomonads caused a much lower variation of the T-cell subsets and B cells, often equal to the control group. This reduced cellular response could also be noticed after infection of vaccinated birds with virulent histomonads. Moreover, it could be demonstrated that the fatal clinical outcome of turkeys due to histomonosis is in coherence with a more intense cellular immune response in infected organs compared to chickens.

Declarations

Ethics approval

The animal trials were discussed and approved by the institutional ethics committee and the national authority according to §26 of the Law for Animal Experiments, Tierversuchsgesetz 2012 – TVG 2012, license number: bmwf GZ 68.205/0147-II/3b/2013.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' contribution

DL, MH, AS, WG and TM conceived and designed the work. TM, DL, KFA and PW performed the animal trial and extraction of lymphocytes. TM performed data acquisition and analysis. TM, DL and

WG interpreted the data. TM, DL and WG drafted the manuscript. MH and AS revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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12. Additional works

As an additional work, RT-qPCR was performed to survey a currently established *in situ* hybridization for detecting and localizing cells positive for RNA of IFN-γ, IL-4, IL-13 in non-infected tissue samples of chickens. The manuscript has been published in Veterinary Immunology and Immunopathology.

Furthermore, in chapter 12.2. a manuscript prepared for publication has been added in which data on cytokine expressions by RT-qPCR of chickens and turkeys infected and/or vaccinated with *H. meleagridis* were generated.

12.1. *In situ* hybridization to detect and localize signature cytokines of T-helper (Th) 1 and Th2 immune responses in chicken tissues

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Short communication

In situ hybridization to detect and localize signature cytokines of T-helper (Th) 1 and Th2 immune responses in chicken tissues

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ABSTRACT

The avian immune system has been shown to possess a repertoire of cytokines directing T-helper (Th) 1 and Th2 types of immune responses similar to that in mammals. The objective of this study was to establish *in situ* hybridization (ISH) for the localization of mRNA of selected signal cytokines, chicken interferon-γ (ChIFN-γ), chicken interleukin (ChIL)-4 and ChIL-13 in fixed tissues. RNA probes were generated to hybridize to 488, 318, and 417 bp of the respective target mRNA. Probe concentrations ranging from 100 ng/ml to 400 ng/ml were shown to be suitable to label cells that expressed these cytokines. The specificity of every probe was verified using the respective sense probe. ChIFN-γ, ChIL-4 and ChIL-13 positive cells were observed in the lymphocytic infiltrations of liver and in the periarteriolar lymphatic sheaths of spleen collected from specific-pathogen-free chickens. ISH of these cytokines in a severely inflamed liver due to infiltration with the parasite *Histomonas meleagridis* revealed the expression of both ChIFN-γ and ChIL-13 mRNA in the mononuclear infiltrates. In conclusion, ChIFN-γ, ChIL-4 and ChIL-13 mRNA were efficiently localized by ISH, which supplies a valid technique to characterize immune responses in fixed tissues.

1. Introduction

The T-helper (Th) 1/Th2 paradigm, first demonstrated in murine models (Mosmann et al., 1986; Mosmann and Coffman, 1989), has been central to our understanding of adaptive immune responses as well as protective and deleterious types of immune reactions in mammals. This concept has been shown to extend to the avian immune system. Functional genes of signature cytokines hallmarking Th1- and Th2-type responses in mammals, particularly interleukin (IL)-4, IL-12, IL-13, IL-18 and interferon-γ (IFN-γ), have been identified in chicken (Avery et al., 2004; Balu and Kaiser, 2003; Degen et al., 2004; Digby and Löwenthal, 1995; Schneider et al., 2000). In addition, the existence of a Th1 like system was shown *in vitro* through IL-18-induced proliferation and IFN-γ secretion of chicken splenic $CD4^+$ T cells (Göbel et al., 2003). Another study also found Th1 and Th2 like responses *in vivo*, characterized by elevated expression of IFN-γ, IL-4 and IL-13 following infection of birds with a virus (Newcastle disease virus) and a helminth (*Ascardia galli*), representing intracellular and extracellular pathogens, respectively (Degen et al., 2005). However, the avian Th1/Th2 system has some peculiarities, for instance the absence of immunoglobulin E and functional eosinophils, which are involved in the mammalian Th2 response (Kaiser and Stäheli, 2014).

Studies that reported changes in chicken Th1/Th2 cytokine expressions following infection have mainly used quantitative reverse transcription PCR (qRT-PCR) (Kaiser and Stäheli, 2014) whereas histological assays received little attention. However, studies in mammals have benefited for long from utilizing *in situ* hybridization (ISH) to decipher associations of cytokine expression with inflammation (McNicol and Farquharson, 1997; Rutar et al., 2015). A recent study (Kuribayashi et al., 2013) showed the potentials of ISH in localizing and displaying the involvement of chicken cytokines (IL-6) in inflammatory responses using tissue sections.

Non-radioactive ISH using *in vitro* transcribed digoxigenin (DIG) labeled RNA probes is a sensitive tool to localize cells that express the mRNA of cytokines (Meltzer et al., 1998). The availability of genomic information for chicken interferon-γ (ChIFN-γ), chicken interleukin (ChIL)-4 and ChIL-13 has made establishing ISH with

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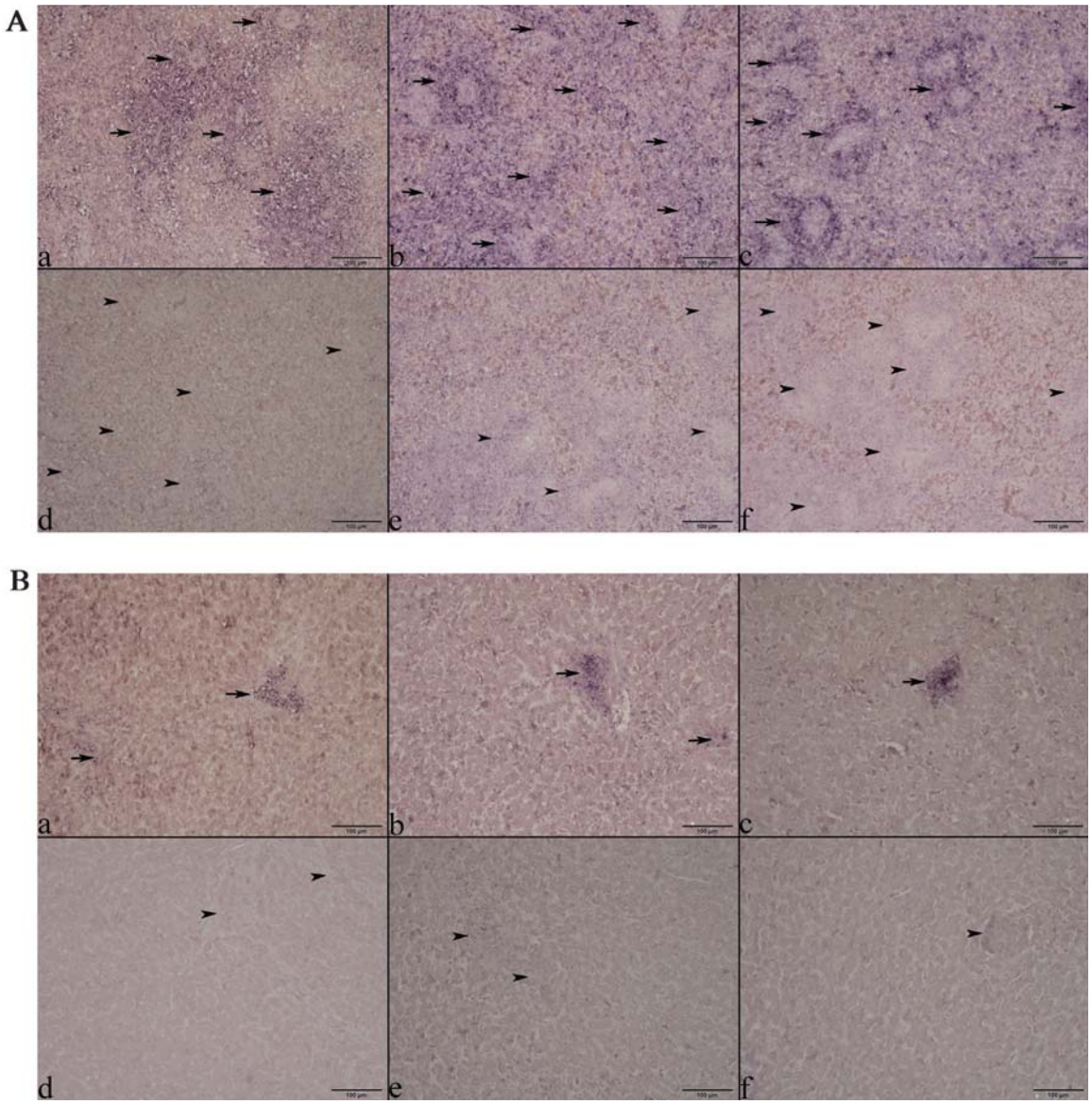

Fig. 1. ISH of ChIFN-γ (a), ChIL-4 (b) and ChIL-13 (c) mRNA in non-infected spleen (A) and liver (B) sections. Cytokine mRNA expressing cells were labeled in sections treated with antisense probes (panels a, b & c). Signals were observed in cells located mainly at the PALS of spleen and lymphoid infiltrations of liver (dark purple, shown in arrows). Sense probes used as negative control for ChIFN-γ (d), ChIL-4 (e) & ChIL-13 (f) showed no signal in same regions of the respective tissues (arrow heads). Bars = 100 μm.

such probes possible. The technique, however, depends heavily on the careful selection of probes and optimization of ISH protocols, pertinent to the target tissue and molecule. The objective of this study was therefore to test and validate DIG-labeled RNA probes and produce an optimized protocol for ISH of ChIFN-γ, ChIL-4, and ChIL-13 in formalin-fixed paraffin-embedded tissue sections in which the respective cytokine mRNA positive cells can be localized.

2. Materials and methods

2.1. Generating RNA probes for the detection of ChIFN-γ, ChIL-4 and ChIL-13

For the purpose of probe synthesis, spleen tissue was collected aseptically from a non-infected specific-pathogen-free (SPF) chicken immediately after euthanization. The tissue was stabilized with RNA*later*® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was then extracted using RNeasy® Mini kit (Qiagen). The cDNA of the respective cytokines was synthesized by conventional reverse transcription (RT)-PCR using QIAGEN OneStep RT-PCR kit and gene specific primers (Table 1). Reverse transcription PCR was performed using DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The reverse transcription was carried out at 50 °C for 30 minutes. After denaturation of the reverse transcriptase at 95 °C for 15 minutes, thermal cycling conditions were performed for 40 cycles of 95 °C for 30 seconds, gradient of 55.1 °C to 58.6 °C for 30 seconds and 72 °C for 1 minute. Final extension was allowed at 72 °C for 10 minutes. The PCR products were then separated in 1.5% Agarose gel and purified using QIAquick® Gel Extraction Kit (Qiagen). The respective cDNAs were ligated into pCR®4-TOPO® vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The sequence and orientation of the cloned products were then verified by sequencing (LGC Genomics, Augsburg, Germany).

Plasmids validated to contain the cDNA of cytokines were linearized with *Spe*I or *Not*I restriction enzymes (Thermo Scientific, Dreieich, Germany). Depending on the orientation of the inserts, T7 or T3 RNA polymerases were used to generate both sense and

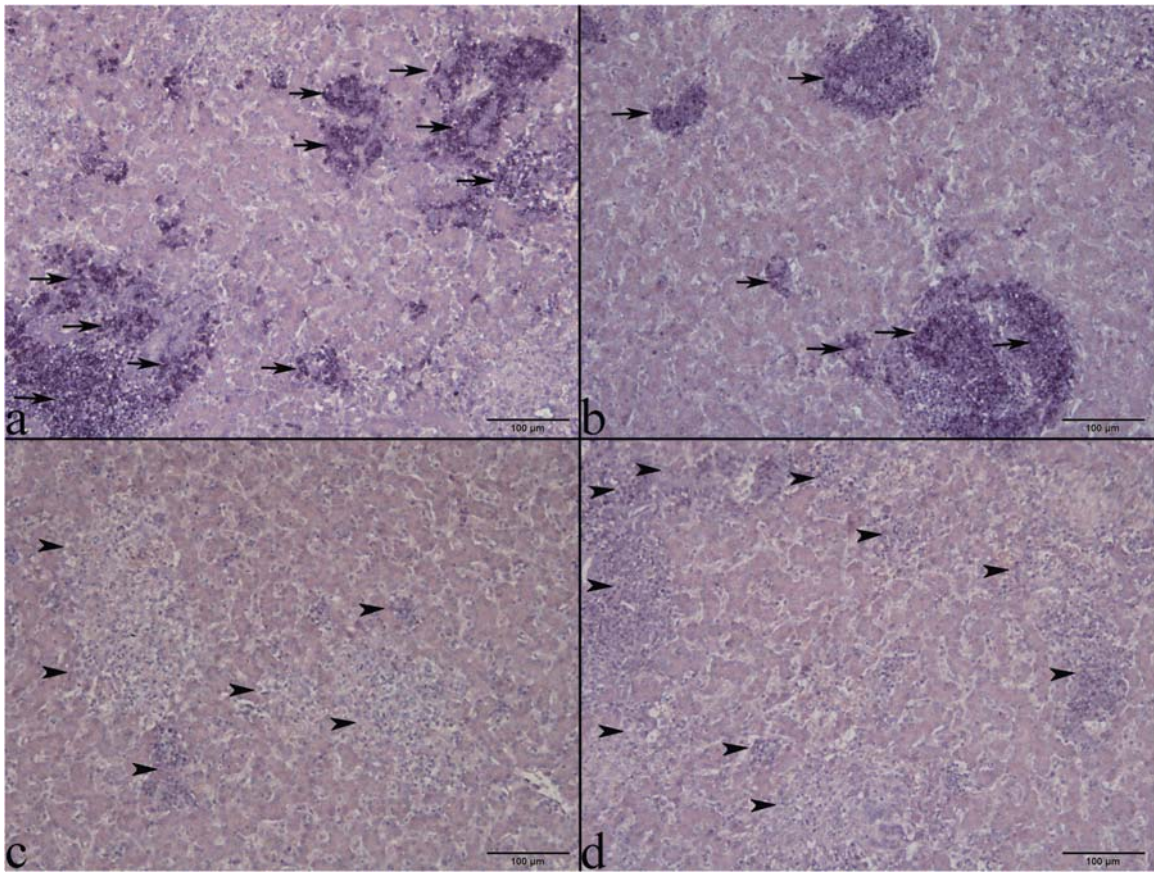

Fig. 2. ChIFN-γ (a) and ChIL-13 (b) expressing cells in *H. meleagridis* infected liver tissue. Positive cells were seen in the inflammatory infiltrates of sections treated with the antisense probe (arrows). Sense probes (c & d) showed no ISH signal in the inflammatory infiltrates (arrow heads). Bars = 100 µm.

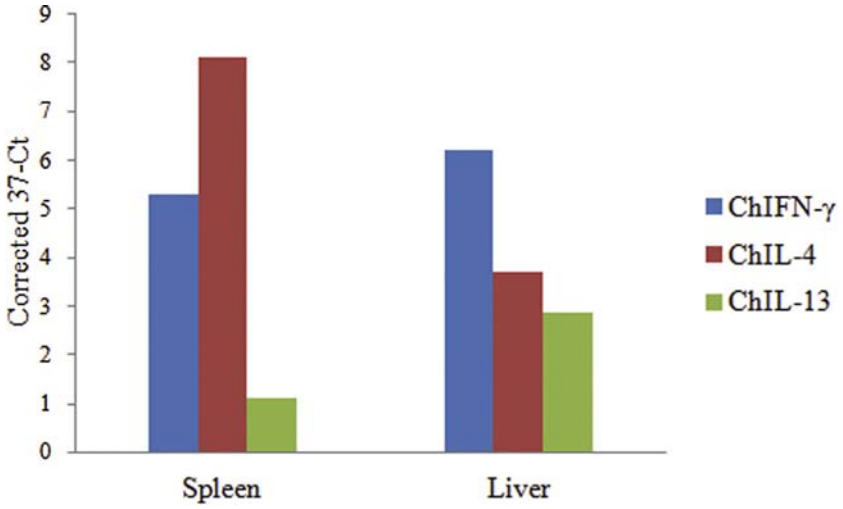

Fig. 3. Relative gene expression of ChIFN-γ, ChIL-4 and ChIL-13 mRNA determined by qRT-PCR in non-infected liver and spleen tissues. The results are expressed in 37-Ct values after normalization with 28S rRNA.

Table 1
Primers used for RT-PCR to synthesize the cDNA for RNA probe generation.

RNA Target	Primer sequence (5′-3′) [a]	Accession	Product size (bp)
ChIFN-γ	F: ACTTGCCAGACTTACAACTTGR: CAATTGCATCTCCTCTGAGAC	NM_205149	488
ChIL-4	F:GTGCTTACAGCTCTCAGTG R:TGGAAGAAGGTACGTAGGTC	NM_001007079	318
ChIL-13	F: ATGCACCGCACACTGAAG R:TCAGTTTGCAGCTGTGGC	NM_001007085	417

[a] F: forward primer; R: reverse primer.

antisense DIG-labeled RNA probes. The *in vitro* synthesis of the probes was performed using DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

The probe yield was quantified using NanoDrop spectrophotomer (Thermo Scientific) and the size of the transcribed probes was verified by gel electrophoresis. Furthermore, the efficiency of the DIG-labeling was assessed by immunoblotting dilution series of DIG-labeled RNA probes according to the manufacturer's recommended protocol given in the DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics), with some modifications. Briefly, 2 μl of serially diluted RNA solution was spotted to a positively charged nylon membrane strip and cross linked using UV. Then, the strip was incubated in a blocking solution which consisted of 50% of Buffer I (100 mM Tris-HCl pH 7.5, 150 mM NaCl), 0.3% TRITON®-X (Merck, Darmstadt, Germany) and 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in DEPC treated water, for 30 minutes. The blocking solution was then replaced by anti-DIG-AP-antibody (Roche Diagnostics) diluted 1:100 in blocking solution; the strip was allowed to incubate for 30 more minutes. Afterwards, it was rinsed two times in Buffer I diluted in equal volumes of DEPC-treated water for 15 minutes each, followed by equilibration in Buffer II (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM $MgCl_2$, final pH 9.5) for 10 minutes. For visualizing the spots, a color reaction was carried out by incubating the strip in a colorimetric substrate solution comprising of Buffer II, 0.45 mg/ml of 4-nitro blue tetrazolium chloride (NBT) and 0.175 mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics) in the dark until dark spots were observed.

2.2. *Control samples for* in situ *hybridization*

Fresh spleen and liver samples collected from five non-infected SPF chickens as well as an archived liver sample from *Histomonas meleagridis* infected chicken with liver lesions obtained from a previous infection experiment (Zahoor et al., 2011) were employed for establishing the method. Samples from non-infected SPF chicken were chosen on the basis of the respective mRNA expression confirmed by qRT-PCR in one of the non-infected birds. The tissues were fixed in 4% neutral buffered formaldehyde (SAV liquid production, Flintsbach, Germany) for 72 h at 4 °C and preceded to routine histological tissue processing and paraffin embedding under RNase-free condition. The sample from the infected bird originated from 10 days post infection, a time point when expression of ChIFN-γ, ChIL-4 and ChIL-13 mRNA by qRT-PCR in liver was observed in a previous study (Powell et al., 2009).

2.3. In situ *hybridization*

Paraffin sections of 4 μm thickness were prepared using Microme HM360 rotary microtome (Microme, Walldorf, Germany), mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and allowed to adhere overnight at 37 °C. For ISH, the sections were first deparaffinized in Neo-Clear® (Merck), and rehydrated in a series of graded alcohols (100%, 96% and 70%), for 5 minutes in each solvent. They were then pre-treated with 2.8 μg/ml of proteinase K (Invitrogen, Carlsbad, CA, USA) in 0.05 M Tris-HCl (pH 7.5), at 40 °C for 30 minutes. Following this, rinsing and dehydration was performed in DEPC-treated water followed by 95% and 100% ethanol. After a brief period of air drying, the sections were covered with a hybridization mix composed of 50% formamid, 4x standard saline citrate buffer (SSC), Herring's Sperm DNA (500 μg/ml), 1x Denhardt's solution, 10% dextran sulfate, and the digoxigenin (DIG)-labeled RNA probe diluted in DEPC treated water. For obtaining an optimal staining of cytokine mRNA expressing cells, a varying concentration of RNA probes ranging from 5–1000 ng per milliliter of the hybridization mix were used. Finally, the slides were heated at 95 °C, for six minutes, followed by a quick cooling on ice. Hybridization was allowed to continue overnight at 40 °C, in a humidified chamber.

On the next day, post-hybridization stringency washes were performed in four steps: first by incubating the slides in 2x SSC at room temperature for 30 minutes, followed by RNase A (20 μg/ml) (Roche Diagnostics) treatment in a solution mix of 0.5 M NaCl, 5 mM Tris-HCl (pH 7.5) and 1 mM EDTA, at 40 °C for 30 minutes. Further washing steps for two times in 1x and 0.1x SSC at room temperature for 10 minutes in each.

For immunological detection of the hybridized probes, the sections were first incubated in a blocking solution of 50% of Buffer I, 0.3% TRITON®-X and 5% normal goat serum in distilled water, for 30 minutes in a humidified chamber. The blocking solution was then replaced by anti-DIG-AP-antibody diluted 1:100 in the blocking solution mix, incubation was allowed for 1 hour at room temperature. Afterwards, post-immunological-hybridization wash and equilibration was performed in three steps. First unbound antibody was removed by washing the slides in 1 to 1 dilution of Buffer I in distilled water, two times, 15 minutes each and lastly equilibration in Buffer II for 10 minutes.

Chromogenic detection of the hybrid-immune-complexes was conducted by incubating the sections with colorimetric substrate solution composed of Buffer II, 0.45 mg/ml NBT, 0.175 mg/ml BCIP and Levamisol (240 μg/ml) (Sigma, Deisenhofen, Germany). Reaction was allowed in a humidified chamber under dark conditions. Coloring was monitored every 30 minutes until 2 hrs and 4 hrs, 6 hrs, 8 hrs and 16 hrs afterwards. After overnight incubation, the reaction was stopped in Tris-EDTA (pH 8.0) for at least 10 minutes. The slides were then rinsed in distilled water and briefly counterstained with Haematoxyline (Merck). They were mounted under cover slips (Menzel-Gläser) with Aquatex® aqueous mounting medium (Merck). The sections were inspected under Olympus BX53 microscope and documented with an Olympus DP72 camera (Olympus Corporation, Tokyo, Japan).

2.4. *Quantitative reverse transcription polymerase chain reaction*

To examine the expression of ChIFN-γ, ChIL-4, and ChIL-13 in non-infected tissue samples, a pilot qRT-PCR analysis of the cytokines was performed. Spleen and liver samples were stabilized, and total RNA was extracted as described in the probe generation work flow. Amplification and detection of specific products was performed using one step qRT-PCR with TaqMan chemistry. The reaction was performed on Agilent Mx3000 P qPCR system (Agilent Technologies, Santa Clara, CA, USA) using Brilliant III Ultra-Fast QRT-PCR Master Mix kit (Agilent Technologies). Previously published primers and probes were employed for amplification and quantification of ChIFN-γ (Rothwell et al., 2004), ChIL-4, ChIL-13 and 28S rRNA (Avery et al., 2004). The thermal profile was one cycle at 50 °C for 10 min and 95 °C for 3 min followed by 37 cycles at 95 °C for 5 sec and 60 °C for 20 sec. Fluorescence was detected and reported at each cycle during the 60 °C step. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold (ΔR_n). The Ct values of the cytokines were normalized for every sample with the Ct value of the reference gene 28S rRNA by using the following formula: $Ct + ((N't - C't) \times S/S')$ where $N't$ is the mean Ct for 28S rRNA among all samples, $C't$ is the mean Ct for 28S rRNA in the sample, S and S' are the slopes of the regressions of the standard plots for the cytokine mRNA and the 28S RNA, respectively (Chrzastek et al., 2014; Powell et al., 2009).

3. Results and Discussion

Digoxigenin labeled RNA probes designed to hybridize to ChIFN-γ, ChIL-4 and ChIL-13 mRNA were efficient to localize cells expressing the cytokines in spleen and liver. Cells expressing ChIFN-γ mRNA were stained at a probe concentration of 400 ng/ml whereas ChIL-4 and ChIL-13 mRNA expressing cells could be labeled at a probe concentration of 100 ng/ml. As illustrated in Figs. 1 and2, all the probes showed distinctive and specific staining results, however, an overnight incubation of the color substrate was necessary to find clearly identifiable positively labeled cells. DIG-labeled sense RNA probes showed no signal. In addition, same tissue sections incubated only with DEPC–treated water in place of the probes were used as an additional negative control and showed no signal (data not shown).

In spleen sections (Fig. 1A), ChIFN-γ, ChIL-4 and ChIL-13 mRNA positive cells were mainly distributed in the periarteriolar lymphoid sheaths (PALS). Chicken spleen in general harbors various T-cell subtypes and the PALS in particular is enriched with $CD3^+$ lymphocytes that mostly express CD4 (Oláh et al., 2014), one of the cellular sources of Th1/Th2 cytokines in naive organisms (Mosmann and Coffman, 1989). In liver sections (Fig. 1B), ChIFN-γ, ChIL-4 and ChIL-13 mRNA positive cells were present exclusively among the mononuclear cells surrounding the portal areas. These leukocytes in the liver which have the ability to express IFN-γ, IL-4 and IL-13 mRNA presumably represent similar lymphocyte populations as found in the spleen. Nonetheless, various cell types have been shown to be the physiological sources of these cytokines in the mammalian immune system. Double staining approaches using cell surface markers will be a prospective approach to identify the cell subsets which express the mRNA of these cytokines in the avian tissue.

The expression of these cytokines in leukocytes of non-infected birds is somewhat similar to previous observations. Spontaneous secretion of ChIFN-γ protein was observed in unstimulated splenocyte cultures from some SPF birds (Prowse and Pallister, 1989; Weiler and von Bülow, 1987). Moreover, another study showed comparable levels and simultaneous expressions of ChIFN-γ, ChIL-4 and ChIL-13 mRNA in spleen tissues collected from non-infected birds by semi-quantitative RT-PCR (Degen et al., 2005). Karr et al. (1995) also observed *in vitro* murine Th0 cells that co-express Th1 (IFN-γ) and Th2 (IL-4) mRNA simultaneously, by ISH in cellular preparations.

In this study, the qRT-PCR assay substantiated the ISH staining by confirming the detection of all the target cytokines' mRNA in the non-infected spleen and liver tissues (Fig. 3). Nevertheless, the results obtained by qRT-PCR and ISH should not be quantitatively compared in a direct way due to the intrinsic difference in the detection system of both techniques. There is a likelihood of variation in the expression assays which can be either due to fewer presence of the target cell population in a structurally heterogeneous tissue sample (De et al., 2010; Rosa et al., 2013) or low expression of the mRNA in individual cells.

Furthermore, ISH was performed in *H. meleagridis* infected liver tissue sections. As shown in Fig. 2, ChIFN-γ and ChIL-13 mRNA positive cells were observed in inflammatory infiltrates comprising areas in the liver sections with lesions. Cells expressing ChIL-4 mRNA could not be detected (data not shown). In chickens, *H. meleagridis* can cause severe inflammation of the liver and the caecum following extracellular infiltration of the tissue. In this study, a marked cellular infiltration of both ChIFN-γ and ChIL-13 mRNA expressing cells were noticed. In an affected liver, increased levels of cytokine mRNA expression could reflect the elevated number of immune cells (Powell et al., 2009). In addition to T-lymphocytes, macrophages and heterophils are also known to infiltrate into the liver after infection with the protozoa (McDougald, 2008). In a relevant but not directly related study, investigating delayed type hypersensitivity, eosinophils have also been shown to modulate inflammatory responses in chicken (Abdul-Careem et al., 2008). As mentioned earlier, immunohistochemical and special stain approaches can be a potential area for further investigation to gain an insight on the cellular sources of the cytokines as well as cells infiltrating lesions.

The absence of ChIL-4 ISH signal in the infected liver sections could be due to the low expression level of the cytokine in this infection model. Using qRT-PCR, it was previously shown that IL-13 mRNA was the main Th2 marker cytokine expressed during *H. meleagridis* infection, not IL-4 which usually predominates a Th2 response in mammals (Powell et al., 2009). Infection experiments with *Ascardia galli* or *Heterakis gallinarum* also revealed IL-13 as the dominantly expressed cytokine in chickens compared to IL-4 by semiquantitative RT-PCR and qRT-PCR (Degen et al., 2005; Schwarz et al., 2011a; Schwarz et al., 2011b).

Chromogenic ISH as a technique to study cytokine responses provides a unique approach by localizing cells expressing the cytokine genes. Through the application of counterstaining, lesions and cellular relationships associated with cytokine mRNA expressing cells and the general alteration of tissues in response to an infectious agent can be correlated. The technique also allows quantification of positively labeled cells and is generally applicable to computer-based imaging analyses such as TissueFAXS (TissueGnosticsGmbH, Vienna, Austria), by which leukocytes can be enumerated on the basis of labeling (Paudel et al., 2015). By using appropriate reagents and standardized protocols, multiple label co-analysis can be employed to identify the phenotype of the cytokine mRNA expressing cells. Apart from spleen and liver sections reported in this study, the technique may be adapted to other organs.

In conclusion, ISH for the detection of ChIFN-γ, ChIL-4 and ChIL-13 mRNA was employed and will be useful to show the role of these cytokines in the organ/tissue response to homeostatic or different infectious stimuli and their relation to physiological or pathological changes in tissue sections.

Conflict of interest

None

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12.2. Expression patterns of IFN-γ and IL-13 differ with the virulence of *Histomonas meleagridis* in turkeys and chickens

Introduction

The mammalian immune system can be divided into the type 1 and type 2 pathways (Mosmann et al., 1986), depending on the causative pathogen. Later on, mammalian immune system was additionally divided into Th17, Tfh, Treg (Dong, 2010). The separation into a Th1 or Th2 pathway in birds was previously shown by Degen et al., (2005) who demonstrated the respective polarization initiated by intracellular (viral) and extracellular (helminth) infection in chickens. Powel *et al* indicate that infection with *H. meleagridis* triggered Th2 pathways (Powell et al., 2009). In this study we compared the effect of virulent and attenuated histomonads at different time points in caecum, liver and spleen samples of infected turkeys as well as chickens for the Th1 and Th2 key cytokines.

Materials and methods

Experimental setup

The birds were housed separately in four different rooms with feeding bowl and water supply. Water and feed (un-medicated turkey and chicken starter feed) were provided; except for a period of 5 hrs feed restriction directly after inoculation. Four different groups of chickens and turkeys were i) infected with virulent histomonads ii) challenged following vaccination, iii) vaccinated with attenuated histomonads and iv) kept as negative control. Each group consisted of 15 birds per species. Birds of group ii were vaccinated on day 1 of life and challenged after 28 days of life birds along with non-vaccinted birds of the infected group (group i). On the same day (28^{th} day of life) birds from the only vaccinated group (group iii) were inoculated with the attenuated strain and control birds were sham infected with culture medium. Three birds from each group were sacrificed in ascending order of the tags at 4, 7, 10, 14, 21 day post inoculation

(DPI). The expression levels of IFN-γ and IL-13 mRNA at 10 and 21 DPI was measured using RT-qPCR. These time points were chosen due to the fact that histomonosis was seen reaching its peak of severity both clinically and pathologically at 10 DPI. Additionally, IFN-γ and IL-13 are regarded as adaptive response-signature cytokines, hence observing their mRNA level at 10 and 21 DPI was convincing.

Total RNA extraction and analysis for purity and integrity

Total RNA was extracted from RNA*later*® (Qiagen, Hilden, Germany) stabilized liver, spleen, caecum and caecal tonsil tissues. Each tissue sample was at first homogenised using QIAshredders (Qiagen, Hilden, Germany) and then RNA was extracted by RNeasy® mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Every sample was assessed for their purity by using NanoDrop 2000 (ThermoFisher scientific, Vienna, Austria) to ascertain that A260/280 and additionally A260/230 ratio. Furthermore, every sample was surveyed by chip-based capillary electrophoresis machine Tapstation 4200 (Agilent technologies, Waldbronn, Germany).

RT-qPCR

The cytokines' mRNA expression levels were quantified using TaqMan one step real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Brilliant III Ultra-Fast QRT-PCR master mix kit (Agilent technologies, Waldbronn, Germany) was used for one step RT-qPCR. Amplification and quantification of mRNA were performed using AriaMx real-time PCR system (Agilent Technologies, Waldbronn,Germany) together with the Agilent AriaMx1.0 software (Agilent Technologies, Waldbronn, Germany). Thermal cycle profile for RT-qPCR was as follows: 1 cycle of reverse transcription at 50°C for 10min followed by 95°C for 3min to hot

start, 40 cycles of amplification at 95°C for 5 sec and 60°C for 10sec. Previously published sequences of primers and probes for reference genes and cytokines were used (Table 1) (Mitra et al., 2016; Powell et al., 2009). Every sample was analysed in replicate. With every plate different types of controls such as NRT (non reverse transcriptase) and NTC (non template control) were additionally examined to exclude variations due to genomic DNA contamination or reagent contamination.

The cytokines average CT values for every organ was normalized using geometric mean CT of reference genes to exclude technical variations which can be introduced during sampling and RT-qPCR. The normalized mean CT value for cytokines from each group was used to measure fold change of cytokines $2^{(-\Delta\Delta CT)}$ (Livak and Schmittgen, 2001).

Table 1: RT-qPCR primers and probes

gene symbol	primer and probe sequences (F-forward primer; R-reverse primer; P-probe)	efficiency (%)	reference paper
chicken-IL-13	F: CACCCAGGGCATCCAGAA R: TCCGATCCTTGAAAGCCACTT P: CY5-CATTGCAAGGGACCTGCACTCCTCTG- BHQ1	98.7 (chicken)	Powell et al., 2009
chicken-IFN-γ	F: GTGAAGAAGGTGAAAGATATCATGGA R: GCTTTGCGCTGGATTCTCA P: HEX-TGGCCAAGCTCCCGATGAACGA-BHQ1	100.7 (chicken)	
turkey-IFN-γ	F: AACCTTCCTGATGGCGTGAA R: CTTGCGCTGGATTCTCAAGTC P: HEX-AAAGATATCATGGACCTGGCCAAGCTTCA-BHQ1	99.2 (turkey)	
turkey-IL-13	F: CCTGCACGGCCAGATGA R: GGCAAGAAGTTCCGCAGGTA P: CY5-TGCCAGCTGAGCACCGACAACG-BHQ1	96.9 (turkey)	
RPL13	F: GGAGGAGAAGAACTTCAAGGC R: CCAAAGAGACGAGCGTTTG P: HEX-CTTTGCCAGCCTGCGCATG-BHQ1	95.1 (chicken) 96 (turkey)	Mitra et al., 2016
TBP	F: CTGGGATAGTGCCACAGCTA R: GCACGAAGTGCAATGGTTT P: ROX-TGCAACCAAGATTCACCGTGGA-BHQ2	100.4 (chicken) 99.2 (turkey)	
TFRC	F: AGCTGTGGGTGCTACTGAA R: GGCAGAAATCTTGACATGG P: ROX-CTCTGCCATGCTGCATGCCA-BHQ2	97.3 (chicken) 96.8 (turkey)	

Results and discussion

All RNA samples included in the present work were within the range of 1.5 and 2.3 ratio of 260/280 value and secondary measures of nucleic acid purity with 260/230 value was equal or above 2 by NanoDrop 2000 (ThermoFisher Scientific). The integrity of each RNA sample considered for RT-qPCR analysis was ensured by reaching a RIN value of 6.5 to 10 (Table 2), measured by TapStation 4200 (Agilent Technologies)

At 10 dpi turkeys of the infected group showed an increased expression of IFN-γ in every organ and an up-regulated of IL-13 except from liver (Figure 1.a). Infected turkeys exhibit a much higher IL-13 expression (>100 fold) in the caecum whereas IFN-γ increased moderately (>10 fold). These findings are consistent with previous results reporting the development of a Th2 response against histomonosis (Powell et al., 2009). However, no turkey from the mentioned group survived until the second sampling day (21 dpi) due to histomonosis. Turkeys that were vaccinated before the challenge exhibit an up-regulation for both cytokines only in the caecum. Only vaccinated turkeys responded with a down regulation of the cytokines in all organs, except from a low up-regulation of IFN-γ in caecum. The results indicate that turkeys vaccinated with attenuated histomonads caused a much less up-regulation of cytokines compared to the turkeys that received virulent histomonads.

Infected chickens showed an up-regulation of cytokines in every organ compared to birds of the control group, except from a down regulation of IL-13 in caecum. Only vaccinated chickens and birds that were vaccinated and challenged showed a similar expression profile as turkeys with the exception of an up-regulation of IL-13 in the liver in both the groups (Figure 1.a).

At 21 dpi, vaccinated or vaccinated and challenged turkeys exhibited a low up-regulation in the expression of both cytokines in caecum and liver (Figure 1.b). In the spleen, IL-13 was found to be up-regulated in the same groups whereas IFN-γ was down-regulated. Chickens displayed an up-regulation of the cytokines in the caecum in birds of every group that received attenuated or virulent histomonads. In contrast, IL-13 and IFN-γ were found to be lower expressed in the liver of chickens of the same groups, except from a very mild up-regulation of IL-13. In the spleen, only infected birds showed up-regulated IL-13 and IFN-γ expression. Overall, the results are consistent with the occurrence of inflammation and the expression profiles were found to be related to the virulence of the parasite (Hess et al., 2013).

Table 2: RNA integrity number measured with TapStation 4200. The RIN values of all the samples used in the experiment are given.

		10DPI				21DPI			
species	**organ**	**infected**	**vaccinated and infected**	**vaccinated**	**control**	**infected**	**vaccinated and infected**	**vaccinated**	**control**
chickens	spleen 1	9.9	6.5	8.7	9.7	8.4	6.7	7.2	6.7
	liver 1	8.9	8.5	10	8.9	10	7.5	8.1	9.9
	caecum 1	6.6	7.3	7.3	9.1	8.8	9	6.7	9.7
	caecal tonsil 1	10	7.9	7.6	10	10	8.4	6.5	9.7
	spleen 2	9.5	9.7	8.1	8.6	8	8.2	6.8	8.4
	liver 2	6.8	6.8	9.6	7.4	9.1	7.2	7.6	7.1
	caecum 2	7.8	8.6	6.5	8.2	9	8.7	6.7	9.1
	caecal tonsil 2	9.8	6.7	7.1	10	9.4	10	6.5	10
	spleen 3	9.9	7.5	6.7	7.1	9.7	7.3	7.1	7.7
	liver 3	8	9	7.3	7.6	7.8	7.6	6.7	7.8
	caecum 3	7.5	6.6	6.6	9.4	7.7	8.1	7.5	9.1
	caecal tonsil 3	9.5	6.7	6.7	9.8	9.7	9.6	6.8	10
turkeys	spleen 1	7.6	10	10	6.8	X[n.a]	8.3	10	7.5
	liver 1	6.5	7.2	10	7.3	X	6.5	10	8.5
	caecum 1	7	7.1	10	8.8	X	10	8.5	6.6
	caecal tonsil 1	8.1	8.2	8.3	9.2	X	10	9.7	10
	spleen 2	6.5	9.3	6.9	9.7	X	6.5	10	7.2
	liver 2	6.7	10	10	10	X	7.4	10	6.9
	caecum 2	7.3	7.4	10	10	X	6.7	7.4	6.7
	caecal tonsil 2	8.7	9.8	6.5	8.3	X	7.3	7.6	8.6
	spleen 3	8.6	7.7	9.3	10	X	10	7.2	8.8
	liver 3	6.6	8	9.8	9.4	X	7.2	6.7	9.4
	caecum 3	6.9	7.5	8	9.3	X	7.8	6.6	10
	caecal tonsil 3	7.1	10	6.9	10	X	7.3	10	8.7

n.a: not applicable

Figure 1.a

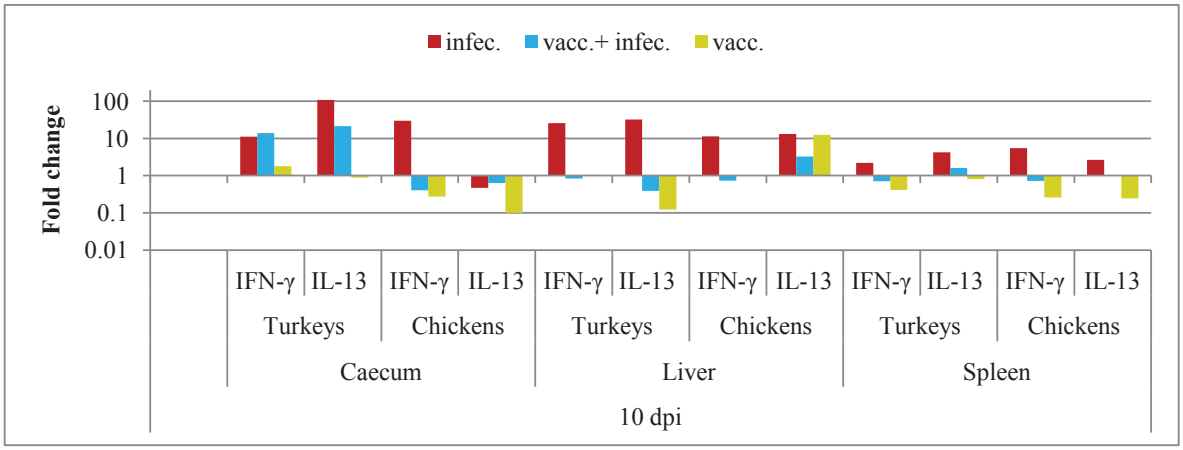

Figure 1.b

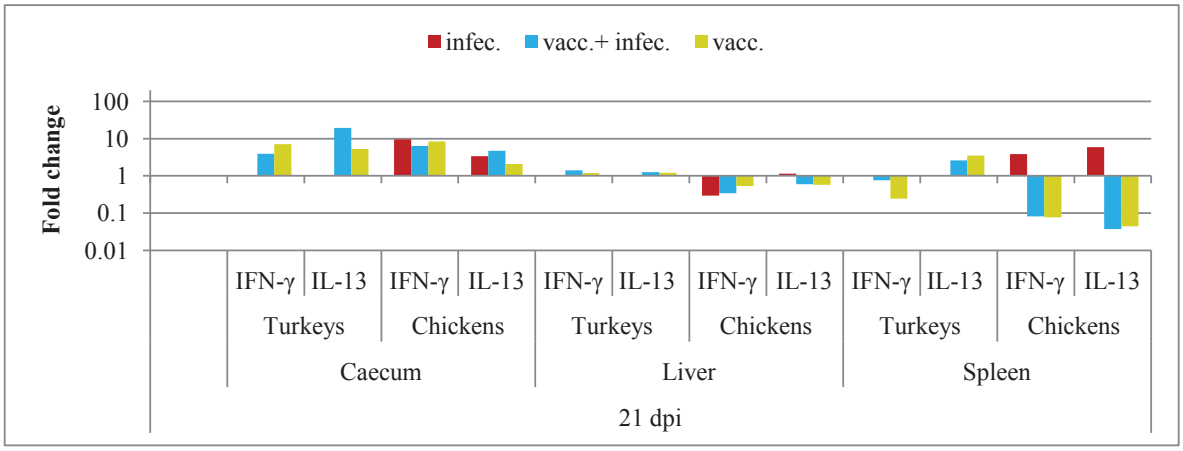

Figure 1: Cytokine mRNA expression levels in the caecum, livers and spleens of chickens and turkeys following inoculation with attenuated and/or virulent *H. meleagridis*, shown as fold change in mRNA expression levels relative to those in age-matched controls. **(a)** Cytokine mRNA expression levels at 10 days post inoculation; **(b)** Cytokine mRNA expression levels at 21 days post inoculation.

13. Discussion

The outcome of histomonosis depends on the species of galliform bird which is infected with the causative agent, *H. meleagridis*. Chickens mostly recover to become healthy carriers whereas high mortality rates are seen in the turkey (McDougald, 2005). However, the application of attenuated histomonads does not cause clinical signs in both species (Liebhart et al., 2011). Host depended differences in the immunological reaction against infection and vaccination with *H. meleagridis* of the two different galliform birds are so far not elucidated. Therefore, the immune responses of both, the chicken and the turkey to experimental infection with virulent and attenuated *H. meleagridis* were investigated in the present work. For that, the expression of different key cytokines involved in the adaptive immune response together with major leukocyte populations were quantified at the sites of parasite infiltration, in the spleen as immune organ and in the blood.

The most obvious differences between the two species were the severity of lesion during infection with virulent histomonads in the caecum and liver, which was considerably milder in organs of chickens compared with organs of turkeys, consistent to previous studies (Hess et al., 2006). The severe inflammation in the liver of infected turkeys (lesion score average of 3.5) compared to a lower affection of infected chicken livers (lesion score average of 2) can be interpreted as a varying colonization of the parasite in the liver depending on the host species. This suggests that the chicken is able to control the infection by limiting the parasite's progression to the liver more effectively than the turkey.

In order to investigate host-related differences in the adaptive immune response, the expression of key cytokines of Th1/Th2 have been analysed by RT-qPCR in turkeys and chicken following vaccination and/or infection at different time points post inoculation. For that, it was necessary to normalize the expression of genes by reference genes to obtain

accurate results. The lack of validated reference genes for gene expression studies in bird species necessitates a comprehensive evaluation of possible candidate genes. According to MIQE guidelines the normalization procedures require more than one appropriate reference genes (Bustin et al., 2009). Historically, the ribosomal RNA (rRNA) gene, especially the 28S rRNA, was frequently used as reference gene to normalize RT-qPCR results (Avery et al., 2004; Huggett et al., 2005). However, the 28S rRNA gene was considered to be suboptimal as a normalization reference in gene expression studies because their expression is regulated by the RNA polymerase I enzyme, whereas the synthesis of mRNAs to be measured is processed by the RNA polymerase II (Kozera and Rapacz, 2013; Suzuki et al., 2000). Furthermore, the 28S rRNA is lacking of any introns which implicates the amplification of genomic DNA, if not removed properly. In order to obtain a valid result by using the 28s rRNA gene as reference, it is necessary to reduce the number a cycles for calculations as the amplification of genomic DNA was observed to begin from the 38th cycle, even after DNase I treatment (Kidane et al., 2016). This raised the question to identify more suitable reference genes to measure gene expression during parasitic infection in different organs of turkeys and chickens. Hence, the aim of the second study was to evaluate reference genes to be used in spleen, liver, caecum and caecal tonsils of chickens and turkeys infected with *H. meleagridis* (Mitra et al., 2016). The results of the study determined the genes RPL13 and TBP in organs of chickens and RPL13 and TFRC in organs of turkeys as most suitable for normalization. RPL13 and TBP were further investigated in tissues of non-infected and infected broiler chickens with fowl avian adenovirus. These additional investigations confirmed the stable expression of both genes in chicken spleen, liver, caecum and caecal tonsil samples. The results demonstrated that neither the genetic background of chickens nor the pathogen used for infection caused remarkable variations of RPL13 and TBP.

The established reference genes were then used to investigate T helper (Th1/Th2) key cytokines IL-13 and IFN-γ expression in caecum, liver, spleen in turkeys and chickens infected with attenuated and/or virulent *H. meleagridis*. A much lower expression of cytokines for chickens compared to turkeys were observed, which correlated with the severity of inflammation specified by lesion scores. Furthermore, a balanced Th1/Th2 response was observed in both the species. Although, higher up regulation of IL-13 compared to IFN-γ mRNA expression levels in the infected turkey at 10 DPI may gave an indication towards Th2 type response, nevertheless a clear manifestation of one of the pathways was not observed.

Another focus of the PhD thesis was to investigate major lymphocyte subsets representing cells of the adaptive immune system by investigating quantitative changes of B cells, $CD4^+$ T cells and $CD8\alpha^+$ T cells in different organs and blood of birds inoculated with attenuated and/or virulent parasites. As a pre-requisite it was necessary to investigate cross reactive mAbs for turkeys due to the lack of such markers for immune cells in this species. The cross-reactivity of anti-chicken antibodies against B cells, $CD4^+$ and $CD8\alpha^+$ of turkey was previously demonstrated (Chan et al., 1988; Luhtala et al., 1997; Meyerhoff et al., 2012; Windau et al., 2013). However, the respective marker to detect total T cells in turkeys was not available and the anti-chicken $CD3^+$ antibody failed to react with turkey T cells. Therefore, the rat anti-human CD3 monoclonal antibody (mAb) raised against the peptide representing an invariant cytoplasmic sequence within CD3ε chain was surveyed for its cross reactivity with T cells of chickens and turkeys. *In silico* analysis and FCM analysis confirmed that the antibody targets specific mononuclear cells of different organs and blood in both species.

Afterwards, selected cross-reactive mAbs were used for a detailed investigation of immune cells during vaccination and infection. Most noticeable, in the early stage of infection (4 DPI)

the main changes were observed in vaccinated and challenged turkeys when all lymphocyte subpopulations decreased in the caecum but increased in the blood. This may indicate a rapid apoptosis of effector memory cells in the caecum which are replenished by proliferating central memory cells and dispersed via the blood stream. Lymphocytes of chickens, including vaccinated and challenged birds, remained largely unchanged at that time point. This milder response to the challenge of vaccinated chickens compared to turkeys, which might be interpreted as a much lower re-activation of *Histomonas*-specific memory cells after the challenge at the early stage compared to turkeys. By 10 DPI in infected turkey caeca were severely affected with highest lesion score and $CD8\alpha^+$ T cells were abundant. This shows that excessive necrosis may also result from the cytotoxic activity of $CD8\alpha^+$ T cells, acting as effector cells which was reported for the caeca of chickens following infections with the virulent protozoan parasite *Eimeria tenella* (Vervelde et al., 1996). $CD8\alpha^+$ lymphocytes have both cytotoxic and suppressor effects (Sprent et al., 1986). Therefore, a predominance of $CD8\alpha^+$ T cells could cause local suppression of other immune responses, including the inhibition of $CD4^+$ T cells proliferation (Vukmanovic-Stejic et al., 2001). This could explain the significant decrease of $CD4^+$ T cells in the caecum as well as lack of parasite elimination and the fatal outcome of the infection in turkeys after 10 DPI. The phenomena of $CD8^+$ T cells suppressing $CD4^+$ T cells was also reported for *Entamoeba histolytica*, a closely related parasite that causes amoebiasis in humans characterized by hepatic granulomas (Ventura-Juárez et al., 2003). In chickens the analysed immune cells in infected organs were mostly in a normal range matching with the low LS. At the last two sampling days, 14 and 21 DPI, the remaining turkeys in group VT and VIT showed reduced numbers of all investigated lymphocytes in the caecum but increased amounts of $CD4^+$ and $CD8\alpha^+$ cells in the liver. As these cellular changes occurred at a later time point compared to birds from the IT group, it might be concluded that the lymphocyte subsets of vaccinated turkeys showed a second wave

of effector activity which results in increased lesion scores and is accompanied by a rapid death of these effector cells. In blood of chicken, an increase of B cells and both T-cell subpopulations in the infected group was observed at 14 DPI which may indicate the ongoing recruitment of these cells due to the infection with virulent histomonads. Moreover, the increased lymphocytes population in the blood stream supports the view that histomonads spreads via hematogenous route (Clarkson, 1962; Farmer et al., 1951; McGuire and Morehouse, 1958). Analyses of chicken white blood cells from whole blood revealed a significant higher amount of macrophages/monocytes between 4 and 10 DPI compared to control birds, suggesting the activation of an innate immune response which contributes to inactivate the parasite. The heterophils were significantly lower during that time in blood this may be due to the infiltration of these granulocytes in the local site of infection. The severe infiltration of mononuclear and polymorphonuclear cells in the infiltrated organs caecum and liver is a common histopathological finding in the course of histomonosis (Hess et al., 2013). Comprehensively, compared to the turkey this response is again delayed and is in coherence with milder LS.

Overall, quantitative analyses of the cytokines together with leukocytes suggest that T cell and B cell immune reactions in the caecum with higher induction of the Th2 cytokine IL-13 dominate the immunopathogenesis in turkeys in contrast to the chickens. Thus, the pattern and time needed for disease progression and the severity of the disease resulted in varying profiles of cytokines and lymphocytes at different time points in turkeys and chickens. Furthermore, it was found that attenuated histomonads caused a much lower variation of the T-cell subsets, B cells and both the cytokines, often equal to the control group. This reduced cellular response could also be noticed after infection of vaccinated birds with virulent histomonads. Hence, it was demonstrated that the extensive changes of lymphocyte subpopulations can be limited by vaccination with attenuated *H. meleagridis*.

14. Conclusion

The present PhD thesis demonstrated, for the first time, the divergence of adaptive and cellular immune responses of *Meleagris gallopavo* and *Gallus gallus* species against virulent and attenuated *Histomonas meleagridis* over a certain time course on local and systemic level. In conclusion, the extensive changes of cytokines and lymphocyte subpopulations contribute to severe inflammation during histomonosis of turkeys compared to chickens and can be reduced by vaccination with attenuated *H. meleagridis*.

15. Prospective studies

Results obtained within this thesis provide new information on the role of adaptive cytokines, B cells and $CD4^+$ and $CD8\alpha^+$ T cells, in shaping immune parameters in turkeys and chickens in coincidence with the virulence of *H. meleagridis*. As a lack of specific and sensitive mAbs for turkeys and chickens is an obstacle to study in-depth immune response of avian species. Generation of selected cross reactive monoclonal antibodies for chickens and turkeys to study cytokine, TLRs and different immune cells would prove to be a powerful tool in advancing basic knowledge of avian immune function and immune responses to infection.

Additionally, a natural and logical direction of future studies would be to investigate the role of innate, regulatory cytokines and Toll-Like Receptors (TLRs), in modulation of immune function after vaccination or infection. Functional studies on the cells responsible for the secretion of innate, adaptive, regulatory cytokines from those cells would also be mandatory to understand the mechanisms of the immune response against *H. meleagridis*.

Furthermore, analysing the paradigm of memory cells and activated T cells following vaccination and challenge at different time points in turkeys and chickens would give an indication on the time graph for vaccine to be immunologically responsive.

16. References

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