Phenotype and cytokine production of *Histomonas meleagridis*-specific T cells isolated from chickens and turkeys infected with the parasite or vaccinated against histomonosis

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Contents

1. Introduction

1.1. Introduction to histomonosis

1.1.1. Transmission and pathology of histomonosis

Histomonosis (syn. histomoniasis, blackhead, infectious enterohepatitis) was first described in turkeys by Cushman (1893) and can be considered a re-emerging disease. With a worldwide occurrence it is mostly affecting chickens (*Gallus gallus*) and turkeys (*Meleagris gallopavo*) (Hess and McDougald, 2020). In addition, also other members of galliformes such as partridges and pheasants as well as ostriches are susceptible for this parasitic disease, which usually does not lead to a severe outcome in these species (Borst and Lambers, 1985; Gordo et al., 2002; Potts, 2009; Liebhart et al., 2014). In experimental settings, it was shown that birds can be infected cloacally or orally (Hu et al., 2004; Liebhart and Hess, 2009). Besides, infection can also take place indirectly by ingestion of *Heterakis gallinarum* eggs containing histomonads (Graybill and Smith, 1920). Following uptake of worm eggs infested with histomonads by the bird, parasites are released in the birds' gut after hatching of the *Heterakis* larvae (Lee, 1969; Lund and Chute, 1973).

The two primarily affected organs of host birds are the cecum and the liver. Parasites invading the cecal mucosa cause a severe tissue destruction that allows parasite infiltration into blood vessels (Tyzzer, 1934). Through the portal vein histomonads can reach the liver, which more frequently occurs in turkeys than in chickens (Tyzzer, 1920). Lesions of the ceca are usually characterized by bleedings and thickening of the mucosa as well as liquid to solid fibrinous content in the cecal lumen (Tyzzer, 1934). In the liver, lesions are round and often profound, white to yellowish spots of 2 – 10 mm in size (Tyzzer, 1920). At the final stage of the disease, parasites can be also detected in other organs including kidney, lung, spleen, brain and bursa by PCR and *in situ* hybridization (Grabensteiner et al., 2006). Macrophages incorporating histomonads that enter the blood circulation may be the reason for the broad organ distribution (Sentíes-Cué et al., 2009).

Comparing chickens and turkeys, the clinical outcome is generally different. In chickens, histomonosis leads, in the majority of cases, to an inapparent infection with the exceptions of decreased weight gain and a drop in egg production (Tyzzer, 1934; Liebhart and Hess, 2020). Lesions are often restricted to the cecum and can resolve completely in this species (Hess and McDougald, 2020). Turkeys suffer more severely with mortality rates up to 100%. Characteristic signs are listlessness, dropped wings and head, closed eyes, ruffled feathers and

sulphur-colored feces due to the liver damage (Tyzzer, 1934). Infections with other pathogens such as *E. coli* or coccidia have the potential to increase the severity of the disease (McDougald and Hu, 2001; Paudel et al., 2018).

1.1.2. Morphology of *Histomonas meleagridis*

The causative agent of histomonosis is the extracellular protozoan parasite *Histomonas (H.) meleagridis* that belongs to the order of Tritrichomonadida and the family of Dientamoebidae according to the NCBI Taxonomy database (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=135588). The unicellular parasite can vary in size ranging from 3 - 21 µm and shows a trichomonad-specific morphology including axostyle, pelta and hydrogenosomes (Schuster, 1968; Mielewczik et al., 2008). *H. meleagridis* can occur in three different forms: lumen form, tissue form and resistant form. The lumen form of the parasite can be found in the cecum and develops one or more rarely two flagella (Bishop, 1938). On the contrary, the tissue form of the parasite invades the cecal mucosa or liver and is non-flagellated with the possibility of expressing pseudopodia (Tyzzer, 1934; Schuster, 1968; Mielewczik et al., 2008). The resistant form resides in the intestinal wall, eggs and larvae of *H. gallinarum* (Tyzzer, 1934; Lee, 1969). Besides, small cyst-like stages of the parasite with a spherical shape were described for *in vitro* cultures (Munsch et al., 2009; Zaragatzki et al., 2010b; Zaragatzki et al., 2010a).

1.1.3. Limitations of therapeutic and preventive strategies

Due to food safety concerns, highly effective drugs against histomonosis have been withdrawn from the market in the 1990s (Hess et al., 2015). Among the most efficient drugs for therapeutic and preventive treatment were arsenicals, nitrofurans and nitroimidazoles (Liebhart et al., 2017). In addition to the disadvantage of potential carcinogenic residues in animal products, a negative influence on egg production and growth rates as well as decreased sensitivity of the parasite were reported for arsenical compounds (Moreng and Bryant, 1956; Abraham et al., 2014; Baynes et al., 2016). In case of an outbreak, methods of treatment are fairly limited. Under specific conditions, infected turkeys can be treated with the aminoglycoside antibiotic paromomycin sulphate, which showed variable efficacy during different outbreaks (Sulejmanovic et al., 2017). However, biosecurity and worm control remain to be one of the most important measures as intermediate hosts are the most common way of transmission (Graybill and Smith, 1920; Lund et al., 1966). Also, indirect infection of birds can occur through contaminated water or feces as survival of the parasite for several hours outside its host was reported (Lotfi et al., 2012). This underlines even more the importance of proper

cleaning and disinfection regiments. Depending on the type of housing, biosecurity has its limitations too as chickens and turkeys from free-range systems showed higher presence of *H. meleagridis*-specific antibodies in sera than birds from deep-litter systems (Grafl et al., 2011). As a result of this restriction in treatment, 13 outbreaks at eight different farms rearing commercial turkeys were reported between 2014 – 2016 in Austria. Approximately 50% of involved turkeys had to be culled highlighting the emergency of this disease (Sulejmanovic et al., 2017). Moreover, investigations of field samples indicated in general a high seroprevalence of *H. meleagridis* in chicken flocks (Grafl et al., 2011; Dolka et al., 2015; Nguyen et al., 2015).

1.1.4. Vaccination

Several attempts to achieve protection against histomonosis in chickens and turkeys have been investigated. Both, the transfer of antibodies as well as treatment with an inactivated vaccine, were not successful in protecting turkeys (Clarkson, 1963; Hess et al., 2008; Bleyen et al., 2009). Most promising for preventing histomonosis is vaccination of birds with attenuated *H. meleagridis*. First, Tyzzer (1934) discovered that an *in vitro* long-term cultivated histomonad culture lost its virulence *in vivo*. Later Hess and colleagues established xenic clonal live cultures of the parasite by micromanipulation and induced *in vitro* attenuation by up to 295 cycles of passaging (Hess et al., 2008). In the next step, monoxenic cultures were developed by exchanging the mixed fecal bacterial culture with well-defined bacterial lab strains. Studies showed that *H. meleagridis* in combination with *E. coli* DH5α gave the best results in terms of providing support for the growth of the parasite. It was suggested that the oxygen consumption by *E. coli* facilitates an anaerobic environment needed by the parasite. Comparing xenic and monoxenic *H. meleagridis* cultures with low passage numbers, clinical signs and mortality in turkeys was approximately one week delayed following infections with monoxenic-grown histomonads. It has been hypothesized that the mixed bacterial flora in the xenic culture resembles the hosts' intestinal flora better and therefore provides better growth conditions in the cecum for the parasite (Ganas et al., 2012). It was shown that the attenuated histomonad strain loses the capability to breach the cecal barrier and remains at the primary site of infection, the cecum (Liebhart et al., 2011). Vaccination with the attenuated strain prevents mortality in turkeys while in layer chickens egg drop can be reduced (Hess et al., 2008; Liebhart et al., 2013; Pham et al., 2013). No clinical signs or lesions were induced by the vaccine (Liebhart et al., 2011). Furthermore, it was shown that the parasite does not revert to virulence and is cross-protective against genetically different isolates (Sulejmanovic et al., 2013; Sulejmanovic et al., 2016).

1.1.5. Immune response towards *H. meleagridis* in the chicken and turkey

Studies on the immune response towards *H. meleagridis* are still sparse. Powell et al. (2009b) performed fundamental work on the turkeys' innate and adaptive immune response. Analysis of mRNA expression levels by qRT-PCR of a broad array of cytokines in cecal tonsils and liver was performed in infected chickens and turkeys. Additionally, changes in immune cell populations from liver and spleen of both species were investigated. Cecal tonsils and liver from infected chickens showed for most studied cytokine mRNA levels (IL-1β, IL-6, IFN-γ, IL-13, IL-10) an early increase, which was not detectable in the infected turkey. In chickens, mRNA levels of all mentioned cytokines decreased again after the initial increment with the exception of IL-13, which was up-regulated until the end of the experiment. Turkeys showed a delayed increase of cytokine mRNA levels in the cecal tonsils and liver but remained heightened similar to the chicken. Authors concluded that the early rise of innate cytokines in the cecal tonsils inhibited parasite migration to the liver and consequently limited the severity of the disease in the chicken. Apart from that, a more pronounced influx of $CD4^+$, $CD8\alpha^+$, $CD28^+$ and $CD44^+$ lymphocytes into the liver was observed for infected turkeys.

The role of type-1 and type-2 immune responses towards histomonosis was further studied by Schwarz et al. (2011). IFN-γ and IL-13 mRNA expression levels were investigated in layer chickens either mono-infected with the nematode *H. gallinarum* or co-infected with *H. meleagridis* and *H. gallinarum* at two weeks p.i. In mono-infected birds a significant increase of cecal IL-13 mRNA levels was detected while co-infection led to significant heightened levels of IFN-γ mRNA in the ceca. The authors concluded that nematode infections in birds induce type-2 immune responses similar to mammals while the protozoan co-infection with *H. meleagridis* shifted the response toward type-1 responses.

Mitra et al. (2017) investigated changes of major lymphocyte subsets (B cells, $CD4^+$ and $CD8\alpha^+$ T cells) in cecum, liver, spleen and blood comparing chickens (C) and turkeys (T) following vaccination (VC and VT, respectively), infection (IC and IT, respectively) and vaccination/infection (VIC and VIT, respectively). Infection with the virulent strain induced more pronounced alterations in the immune cell frequencies ($CD4^+$, $CD8^+$, B cells) isolated from cecum, liver, spleen and blood of both species compared to vaccination with the attenuated strain. However, changes within all major lymphocyte subsets and investigated tissues were more prominent in infected turkeys compared to infected chickens. Initial vaccination of challenged birds had a dampening effect on cell frequency changes. Noteworthy, a significant increase of all lymphocyte subsets isolated from PBMC was observed 4 days post

challenge infection (dpi) in the VIT group, which indicates a fast recall response by the vaccine in the turkey as suggested by the authors.

Another study was investigating IFN-γ and IL-13 mRNA$^+$ cells by *in situ* hybridization of cecum, liver and spleen samples. Chickens and turkeys following vaccination, infection and vaccination/infection were compared. Most strikingly, at 4 days post inoculation/challenge infection turkeys from the VT and IT groups (both inoculated at 4 weeks of life) showed an early significant decrease of IFN-γ mRNA$^+$ cecal cells compared to the VIT group (vaccinated at first day of life and challenge infected at 4 weeks of life). Three days later birds from group VT and IT responded with a strong rise of IFN-γ mRNA$^+$ cells hitting a peak at 10 dpi. A similar, although not significant, trend was seen for IFN-γ levels in the spleen. IFN-γ mRNA$^+$ cell levels in the liver were in general lower and met significance 10 dpi within the VT and IT group. On the contrary, chickens showed overall higher IFN-γ mRNA levels including control birds in cecum and spleen reaching no significance in any group. IL-13 mRNA$^+$ cell frequencies exhibited a similar trend as seen for IFN-γ in the turkey. However, IL-13 does not seem to play a key role in the immune response of the VIT group as no significant increase at the early stage post infection was detected compared to the other groups. In chickens, IL-13 mRNA levels were much lower than for IFN-γ leading to non-significance. In conclusion, it was suggested that the early rise of cecal IFN-γ mRNA$^+$ cells in the VIT group is a sign of an early activation of effector memory T cells at the local site of infection. As no such early increment was found in infected turkeys, which in contrast to the VIT group developed severe lesions leading to death, it can be hypothesized that IFN-γ plays a major role in protection against histomonosis (Kidane et al., 2018).

1.2. T-cell biology of birds

1.2.1. Avian T-cell development and major T-cell subsets

Although mammals and birds diverged phylogenetically more than 200 million years ago, basic components and functions of the immune system are well conserved. However, birds seem to have developed a less polymorphic immune system especially highlighted by the “minimal” MHC complex. Further peculiarities in birds are nucleated platelets potentially involved in phagocytosis and antigen presentation, only three classes of immunoglobulins, the absence of lymph nodes and the bursa as the primary lymphoid organ for B-cell maturation. Apart from that, avian T-cell development and antigen recognition in birds is highly similar compared to mammals (Wigley, 2017).

Based on the T-cell receptor (TCR) two major T-cell lineages can be distinguished, αβ T cells and γδ T cells, respectively (Chen et al., 1988; Sowder et al., 1988; Char et al., 1990). The TCR complex is a heterodimeric surface receptor consisting of two chains, which are connected via a disulfide bridge. All jawed vertebrates including birds are characterized by having four different TCR chains (α, γ and β, δ) (Rast et al., 1997). T-cell development in birds starts with progenitors colonizing the thymus in three consecutive waves during embryogenesis (Jotereau and Le Douarin, 1982). The T-cell lineages, γδ T cells, αVβ1 T cells and αVβ2 T cells, are sequentially generated in the thymus (Bucy et al., 1990; Char et al., 1990). All thymocytes, initially CD4/CD8 double negative, give rise to $CD4^+CD8^+$ cells. After phases of expansion and clonal selection, αβ T cells become CD4 or CD8 single positive while γδ T cells rarely express these co-receptors but have the ability to acquire CD8 expression in the periphery (Chen et al., 1989; Davidson et al., 1992; Davidson and Boyd, 1992). After exiting from the thymus, T cells migrate to the secondary lymphoid organs in the same order as they initially entered the thymus (Dunon et al., 1997).

The TCR chains are participating in antigen recognition while signal transduction is facilitated by CD3 (Clevers et al., 1988). Different to the mammalian CD3 signaling complex, avian species only have two genes, CD3ε and CD3γ/δ (Bernot and Auffray, 1991; Göbel and Fluri, 1997; Göbel and Bolliger, 2000). Previously it was suggested that the CD3γ/δ gene might be an ancestral form that is closely related to the mammalian CD3γ and CD3δ genes (Göbel and Dangy, 2000).

αβ T cells can be subdivided into $CD4^+$ T helper cells and $CD8^+$ cytotoxic cells. Both, CD4 and CD8 are essential co-receptors for the antigen recognition of the TCR. Binding of these co-receptors occurs at the extracellular domain of the MHC class I (CD8) and MHC class II (CD4) molecules. The cytoplasmatic tail of the CD4 and CD8 molecule is also associated to the protein kinase lck, which initiates a tyrosine phosphorylation cascade and assists in downstream signaling pathways. These connections are important for T-cell activation via the TCR/CD3 complex and the increase in the affinity of the TCR for MHC binding (Veillette and Ratcliffe, 1991; Murphy and Weaver, 2016). CD4 is a single chain polypeptide that is evolutionary well conserved between aves and mammals and shows high similarity in molecular structure, function and tissue distribution (Chan et al., 1988; Luhtala et al., 1993). $CD4^+$ T cells are responding to antigens processed into short fragments of at least 13 amino acids and are playing a major role in adaptive immunity by the production of various cytokines (Arstila et al., 1994; Rammensee, 1995). So far, at least seven different $CD4^+$ T-cell subtypes

based on transcription factors and cytokine production were identified in humans (Chang et al., 2010; Swain et al., 2012). Most genes of key cytokines driving T helper cell differentiation have been also discovered in the chicken but not all $CD4^+$ T helper cell subsets are elucidated yet (Kaiser et al., 2005). Th1 and Th2 $CD4^+$ T-cell subsets have been described by Degen et al. (2005) in the context of a viral and helminth infection model, respectively. Despite the presumable missing transcription factor RORγt, IL-17-producing Th17 $CD4^+$ T cells in spleen and blood could be identified by intracellular cytokine staining (ICS) (Walliser and Göbel, 2017, 2018). Earlier, a role in the immune response against *Salmonella* Enteritidis infections of chickens was reported for IL-17-expressing Th17 cells from the gut (Crhanova et al., 2011).

Besides the above-mentioned T-cell subsets, putative regulatory T cells (Tregs) were reported for the chicken (Shanmugasundaram and Selvaraj, 2011). Natural Tregs are characterized in mammals to have a $CD4^+CD25^{high}FoxP3^+$ phenotype and are capable of producing IL-10 and TGF-β leading to suppression of T-cell proliferation (Apostolou et al., 2002; Dieckmann et al., 2005). Tregs are important in protection against excessive immune responses as well as maintenance of self tolerance and mucosal tolerance (Workman et al., 2009). Although the mammalian homologue of FoxP3 has not been discovered in birds yet, a $CD4^+CD25^+$ cell subset was considered to exhibit regulatory properties, reminiscent of mammalian Tregs. Despite the existence of the transcription factor FoxP3 in various vertebrate species, it seems incomplete or entirely missing in most avian species (Denyer et al., 2016). Putative avian Tregs are mainly found in the mucosa of the intestine and lung expressing high levels of IL-10 and TGF-β mRNA (Shanmugasundaram and Selvaraj, 2011, 2012). This regulatory cell population has not been studied in avian infection models in detail yet. For Marek's disease, the phenotype of putative Tregs was described and might be involved in the suppression of anti-tumor immune responses (Gurung et al., 2017).

CD8 occurs as αβ-heterodimer or αα-homodimer, which is in coherence with the mammalian concept (Tregaskes et al., 1995). $CD8^+$ T cells are responding to antigens of shorter length (8-10 amino acids) compared to $CD4^+$ T cells and are well studied to react to various viruses, intracellular bacteria and tumor cells (Rammensee, 1995; Boon et al., 2006; Bangham, 2009; Martin and Badovinac, 2015). Within avian species, studies on the cytolytic T-cell response are scarce and mainly focused on a few viral pathogens. $CD8^{bright+}$ αβ T cells were identified as functional effector cells in infectious bronchitis virus infection of chickens (Collisson et al., 2000). For infectious bursal disease virus, an involvement of cytotoxic $CD8^+$ T cells with

increased expression levels of perforin and granzyme-A was reported in chickens (Rauf et al., 2011).

Moreover, chickens belong to the "γδ-high" T-cell species alongside with pigs, cattle, goats and sheep (Sowder et al., 1988; Mackay and Hein, 1989; McClure et al., 1989; Binns, 1994; Caro et al., 1998). The monoclonal antibody (mAb) TCR-1 can be used to identify chicken γδ T cells (Sowder et al., 1988). In chickens, γδ T cells are most abundantly found in spleen, blood as well as in the intestine and are increasing with age (Bucy et al., 1988; Arstila and Lassila, 1993). In contrast to human γδ T cells, which are mainly $CD4^-CD8^-$, in the chicken this cell subset can be subdivided according to their CD8 expression as follows: $CD8\alpha\alpha^+$, $CD8\alpha^+\beta^+$, $CD8\alpha^-\beta^-$ (Tregaskes et al., 1995). Whether antigen recognition by avian γδ T cells is not MHC restricted and a response towards non-peptide antigens can be performed, as known from mammals, remains subject of future investigations. Little is known on the functional mechanisms of γδ T cells in birds. From studies in humans and mice, it is reported that γδ T cells have a broad functional variability including cytotoxic potential against infected and tumor cells, production of cytokines such as IFN-γ, IL-17 or IL-4 in response to various pathogens as well as antigen-presenting capacities. Also, one of their key features is phagocytosis of tumor cells and regulatory roles in inflammation and tissue homeostasis (reviewed by Lawand et al., 2017).

Only a few studies have analyzed the functional role of γδ T cells in the chicken. It is known that this T-cell subset harbors cytotoxic capacities and is involved in downregulation of the immune response (Quere et al., 1990; Choi and Lillehoj, 2000). Among all γδ T-cell subsets, $CD8^+$ cells revealed the highest potential for cytotoxicity, which also differed depending on the location. Almost no cytotoxic effect was seen in γδ T cells from blood, while cells from the intestine showed a high level of cytotoxicity (Fenzl et al., 2017). In the context of infectious diseases, an increase of γδ T cells was seen in *Salmonella* Typhimurium and *Eimeria acervulina* infected chicken (Choi and Lillehoj, 2000; Pieper et al., 2008, 2011). Taken together, chicken γδ T cells might play roles in pathogen clearance by cytotoxic capacities, provide help for differentiation of $CD4^+$ T cells by production of cytokines and have immunoregulatory functions.

Further, 3% cells within $CD3^+$ IELs with a NKT cell-like phenotype have been described (Göbel et al., 2001). Those cells showing a $CD3^+CD8^-CD25^+$ phenotype were also identified as the main producers of IL-17A (Walliser and Göbel, 2018).

1.2.2. Potential markers for T-cell differentiation

In man and mouse, several markers have been identified for separation of T-cell differentiation stages. So far, seven stages for T cells were determined by using a combination of the following markers: CD45RO, CCR7, CD28, CD95, CD69 and CD103. Based on these markers, naïve (T_N), stem cell memory (T_{SCM}), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), terminal effector (T_{TE}) and tissue-resident memory (T_{RM}) T cells can be differentiated. Depending on the stage, an up- or down-regulation of certain markers takes place, which influences the functional capabilities of T cells. While naïve or early activated T cells show high proliferative and lymphoid homing potential, more differentiated T cells have increased effector functions and show strong peripheral homing (Mahnke et al., 2013; Mueller et al., 2013; Shin and Iwasaki, 2013).

For most of the above-mentioned differentiation markers mAbs to study their expression are not available for birds. However, a mAb against CD28 was developed by Young et al. (1994) for chickens and being cross-reactive in the turkey as well (Powell et al., 2009a). CD28 is a transmembrane glycoprotein and constitutively expressed on mature naïve T cells while getting down-regulated at late differentiation stages (Mahnke et al., 2013). Binding of CD28 to CD80 and CD86 on antigen presenting cells provides a co-stimulatory signal that is needed for T-cell activation (Jenkins et al., 1991; Greenwald et al., 2005). This functional mechanism seems to be conserved between man and aves (Koskela et al., 1998). For some infection experiments in chickens, loss of CD28 was determined as a potential phenotype for the activation of T cells. *E. coli* vaccinated chickens showed significant elevated levels of $CD8\alpha^+CD28^-$ T cells isolated from PBMC (Filho et al., 2013). Another study found increased $CD8\alpha^+CD28^-$ T-cell frequencies in PBMC from chickens infected with either IBV, Salmonella or IBDV (Beirão et al., 2012).

Another general activation marker for T cells is CD25, the α-chain of the IL-2 receptor (IL-2R). The IL-2 receptor is heterotrimeric and consists of the three protein subunits α (CD25), β (CD122) and γ (CD132). The β- and γ-chain together form a low affinity receptor complex while additional binding of the α-chain gives rise to the high-affinity IL-2R (Minami et al., 1993). IL-2 is produced by activated T cells and enhances its receptor affinity, which in turn leads to cell proliferation (Depper et al., 1984; Caruso et al., 1997). In the chicken, coherent with mammals, an up-regulation of CD25 within splenic $CD4^+$ and $CD8^+$ T cells was detected in birds upon H9N2 Avian Influenza virus infection (Teng et al., 2006b). In addition, chickens

vaccinated against *Salmonella* Enteritidis showed an increment of CD25 expression in CD8αα^{high} γδ T cells from blood (Braukmann et al., 2015).

Another promising candidate for T-cell activation is the mAb 8B1 which recognizes two distinct CD45 splice variants (Huhle et al., 2017). CD45 is expressed on all leukocytes including platelets but not erythrocytes (Paramithiotis et al., 1991; Viertlboeck and Göbel, 2007). The expression level of the 8B1 antigen was increasing on splenic and blood-derived αβ T cells as well as on splenic γδ T cells upon anti-CD3 stimulation indicating this mAb to be a marker for activation (Huhle et al., 2017).

For measuring the activation stage of CTLs, the CD107 assay turned out to be useful for human and mouse cells (Betts et al., 2003). CD107a (LAMP-1), CD107b (LAMP-2) and CD63 (LAMP-39) are lysosomal-associated membrane glycoproteins (LAMPs). The killing of infected cells is facilitated by lytic granules such as perforin and granzymes surrounded by LAMPs fusing with the plasma membrane (Peters et al., 1991). Therefore, mobilization of CD107 to the cell surface is a sign of degranulation of cytotoxic cells (Betts et al., 2003). In infectious bronchitis virus infected chickens, increased mobilization of CD107a on CTLs was identified in the respiratory tissue indicating activation of these cells (Wattrang et al., 2015).

Clearly, an expansion of immunological tools for addressing effector and memory T-cell stages in avian species is demanded.

1.2.3. Tools for phenotyping T-cell subsets comparing chickens and turkeys

Table 1 provides an overview of the most important mAbs available for studying phenotypes of different T-cell subsets and their cytokine profile in the chicken and turkey. The vast majority of the avian immunological work is done in the chicken. Hence, other bird species including turkeys are highly dependent on the development of immunological tools specific to chickens. Due to the close genetic relation of both species, some anti-chicken mAbs are cross-reactive with turkey cells. Nevertheless, the immunological toolbox in the turkey discloses tremendous gaps and represents a challenge for studying even basic T-cell subsets. Cross-reactive mAbs for the turkey are as follows: anti-human CD3ε, anti-chicken CD4, anti-chicken CD8α and anti-chicken CD28. For CD8α it has to be considered that not all anti-chicken mAbs show a full cross-reactivity in the turkey. For example, the anti-chicken CD8α mAb CT-8 seems to react only with CD8α molecules in turkeys derived from particular breeding lines (Li et al., 1999). So far, the direct identification of γδ T cells as well as CD8β^{+} cells are not possible in the turkey. In addition, αβ T cells cannot be addressed directly as the initial successful cross-

reactivity testing of clone TCR-2 was later on disproven by Meyerhoff et al. (2012). Besides detection of general T-cell markers in the turkey, staining for cytokines is even more limited in both species. Several mAbs detecting IFN-γ by ICS have been identified for the chicken. In addition, the cytokines IL-17A and IL-17F have been found to be suitable for ICS assays in the chicken (see Table 1). Whether these anti-chicken mAbs are cross-reactive in the turkey remains unknown.

1.3. Detection of cytokines by flow cytometry

1.3.1. Intracellular cytokine staining

T cells and their cytokine production profile are the key components in studies of the immune response against various pathogens including vaccine-induced responses. One of the leading-edge techniques to analyze antigen-specific T cells on a quantitative and qualitative single-cell level is the intracellular cytokine staining assay. This well-established method enables detection of cytokine production by rare antigen-specific T-cell subsets including simultaneous determination of their differentiation state (Lovelace and Maecker, 2011). In detail, incubation of T cells together with an antigen being presented by professional antigen presenting cells is performed. Thus, activated T cells start producing cytokines. To prevent cytokine secretion, Golgi export blocking substances such as Brefeldin A or Monensin are used, to restrain cytokines within the endoplasmatic reticulum. Fixation of the cell leads to cross-linking of proteins in order to preserve cell integrity during permeabilization of the cell membrane with mild detergents. This permeabilization is required to allow fluorescent antibodies to enter into the cells cytoplasm and label cytokines (Jung et al., 1993; Prussin and Metcalfe, 1995). In human and mouse immunology, ICS is a widely used method for studying T-cell immune responses. Also, for pigs ICS assays for IFN-γ, TNF-α and IL-17A are frequently used (Talker et al., 2015; Sassu et al., 2017; Schmidt et al., 2020). Due to limitations of the avian immunological toolbox, the ICS assay is not an intensively used technique in birds yet. A few studies analyzed IFN-γ-producing T cells upon polyclonal or antigen re-stimulation in the chicken (Ariaans et al., 2008; Ruiz-Hernandez et al., 2015; Andersen et al., 2017). Recently, with the development of IL-17A and IL-17F specific mAbs a protocol for IL-17-producing T cells by ICS was also established (Walliser and Göbel, 2017).

1.3.2. PrimeFlow™ RNA assay

A second method to study cytokine-producing T cells on a single-cell level is the PrimeFlow™ RNA assay, which combines RNA hybridization with flow cytometry. In case mAbs for cytokines or other molecules of interest are lacking, this method can serve as an alternative. In

principle, PrimeFlow™ RNA assays offer similar possibilities as ICS assays such as staining surface and intracellular proteins alongside detection of mRNA. A specifically designed DNA probe, the so-called target probe, consisting of oligo pairs, hybridizes to its adjacent mRNA sequence. Usually, target probes contain 20 – 40 oligo pairs. A sequence-specific hybridization of pre-amplifier, amplifier and label probe forms together with the target probe the branched DNA (bDNA), which can achieve in theory an 8000-fold signal amplification (Lai et al., 2018).

For human and mouse cells, PrimeFlow™ RNA assays are an already widely used tool with respect to various research interests. Comparing RNA and protein kinetics in one single cell, detection of microRNA or viral RNA within infected cells are just some examples for application (Bertram et al., 2019; Pekle et al., 2019; Malmhäll et al., 2020). However, for other species, it is not a widespread tool yet with only a few studies published so far. In cattle, cell-mediated immune responses by evaluating IFN-γ and IL-2 mRNA$^+$ cells following bovine viral diarrhea virus vaccination were investigated (Falkenberg et al., 2020). Another application is the validation of cross-reactive mAbs as performed for GATA-3 from canine PBMC (Früh et al., 2020).

Table 1. Available mAbs to study T-cell subsets in the chicken and turkey.

Antigen	Specificity	Clone names	Reactivity	References	Cross-reactivity	References for cross-reactivity in turkeys
Lineage markers						
CD45	all leukocytes, platelets	LT40, HIS-C7	chicken	Ratcliffe (1989), Paramithiotis et al. (1991) (LT40) Jeurissen et al. (1988) (HIS-C7)	-[1] turkey	Meyerhoff et al. (2012) (LT40) Jeurissen and Janse (1998) (HIS-C7)
CD3	T cells	CT-3	chicken	Chen et al. (1986)	- turkey	Char et al. (1990), Jeurissen and Janse (1998)
CD3ε	T cells	CD3-12	human	Jones et al. (1993)	+[2] chicken + turkey	Mitra et al. (2017)
TCRαβ/Vβ1	subset of αβ T cells	TCR-2	chicken	Lahti et al. (1991), Cihak et al. (1988)	+/- turkey	+ Char et al. (1990) - Meyerhoff et al. (2012)
TCRαβ/Vβ2	subset of αβ T cells	TCR-3	chicken	Char et al. (1990), Lahti et al. (1991)	-	Char et al. (1990)
TCRγδ	γδ T cells	TCR-1	chicken	Sowder et al. (1988)	-	Char et al. (1990)
CD4	T helper cells	CT-4, 2-35, EP96	chicken	Chan et al. (1988) (CT-4) Luhtala et al. (1993) (2-35)	+ turkey	Char et al. (1990) (CT-4) Li et al. (1999) (2-35) Meyerhoff et al. (2012) (EP96)

				Paramithiotis et al. (1991) (EP96)		
CD8α	CTL, subset of $CD4^+$ T cells, NK cells, γδ T cells	3-298, CT-8, EP72	chicken	Luhtala et al. (1995) (3-298) Chan et al. (1988) (CT-8) Tregaskes et al. (1995) (EP72)	+ turkey (3-298, CT-8) - turkey (EP72)	Li et al. (1999) (3-298) Char et al. (1990) (CT-8) Meyerhoff et al. (2012) (EP72)
CD8β	CTL	EP42	chicken	Tregaskes et al. (1995)	(+)/- turkey	- Meyerhoff et al. (2012) + (weak) Li et al. (1999)
Potential differentiation/activation markers						
CD25	activated T cells, Tregs, NK cells	AV142	chicken	Teng et al. (2006a)	n.d.[3]	
CD28	memory or naïve T cells	AV-7	chicken	Young et al. (1994)	+ turkey	Lawson et al. (2001)
CD45 splice variants	activated T cells	8B1	chicken	Huhle et al. (2017)	n.d.	
CD107a	activated CTL	5G10	chicken	Wattrang et al. (2015)	n.d.	
Cytokine analysis by ICS						

IFN-γ	Th1 cytokine	mAb80	chicken	Ariaans et al. (2008)	n.d.	
IFN-γ	Th1 cytokine	EH9	chicken	Ruiz-Hernandez et al. (2015)	n.d.	
IFN-γ	Th1 cytokine	12F7, 2B7, 7E12, 11G5, 7E3	chicken	Lagler et al. (2019)	+ turkey	Lagler et al. (2020) and own unpublished findings
IL-17A	Th17 cytokine	9F11, 10D5	chicken	Walliser and Göbel (2017)	n.d.	
IL-17F	Th17 cytokine	1E7	chicken	Walliser and Göbel (2017)	n.d.	

[1] monoclonal antibody tested negative for cross-reactivity in the chicken or turkey.

[2] monoclonal antibody tested positive for cross-reactivity in the chicken or turkey.

[3] monoclonal antibody not tested for cross-reactivity in the turkey.

2. Aims and hypotheses

As outlined above, the immune response against *H. meleagridis* in chickens and turkeys is poorly understood so far. Especially the involved antigen-specific T-cell subsets and their cytokine production profile have not been investigated at all. In this PhD project, IFN-γ and IL-13 production by *H. meleagridis*-specific T-cell subsets from spleen and liver were investigated in *in vivo* experiments with chickens and turkeys as follows: vaccination with the attenuated strain, infection with the virulent strain, vaccination and infection with both strains. The following hypotheses were addressed:

- Hypothesis 1: The T-cell immune response in chickens and turkeys against *H. meleagridis* is dominated by a type-2 immune response.

  As an extracellular parasite and according to the current immunological understanding, a type-2 polarization of T cells is hypothesized to provide protection against this disease. Analyses of the signature type-2 cytokine IL-13 in comparison to the type-1 signature cytokine IFN-γ of antigen-specific re-stimulated lymphocytes will provide information on the dominating T-cell responses in both species.

- Hypothesis 2: Turkeys suffering from severe histomonosis show an impaired stimulation of T cells.

  Turkeys infected with the virulent histomonad strain are incapable of controlling the disease, which often results in a fatal outcome. From previous studies, the pivotal role of T cells in the immune response against *H. meleagridis* is known. By addressing the cytokine-producing T-cell subsets from hypothesis 1 and comparing them between chickens and turkeys either infected or vaccinated/infected, we aim to elucidate T-cell subsets involved in protective or failing immunity.

3. Publications

- Lagler, J., Mitra, T., Schmidt, S., Pierron, A., Vatzia, E., Stadler, M., Hammer, S.E., Mair, K.H., Grafl, B., Wernsdorf, P., Rauw, F., Lambrecht, B., Liebhart, D., Gerner, W., 2019. Cytokine production and phenotype of *Histomonas meleagridis*-specific T cells in the chicken. Veterinary Research 50 (1), 1-15.

- Lagler, J., Schmidt, S., Mitra, T., Stadler, M., Wernsdorf, P., Grafl, B., Hatfaludi, T., Hess, M., Gerner, W., Liebhart, D., 2021. Comparative investigation of IFN-γ-producing T cells in chickens and turkeys following vaccination and infection with the extracellular parasite *Histomonas meleagridis*. Developmental & Comparative Immunology 116, doi: 10.1016/j.dci.2020.103949.

- Further publication not related to this thesis:

  Schmidt, S., Sassu, E.L., Vatzia, E., Pierron, A., Lagler, J., Mair, K.H., Stadler, M., Knecht, C., Spergser, J., Dolezal, M., Springer, S., Theuß, T., Fachinger, V., Ladinig, A., Saalmüller, A., Gerner W., 2021. Vaccination and infection of swine with *Salmonella* Typhimurium induces a systemic and local multifunctional $CD4^+$ T-cell response. Frontiers in Immunology 11, doi: 10.3389/fimmu.2020.603089.

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VETERINARY RESEARCH

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Cytokine production and phenotype of *Histomonas meleagridis*-specific T cells in the chicken

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Abstract

The protozoan parasite *Histomonas meleagridis* is the causative agent of the re-emerging disease histomonosis of chickens and turkeys. Due to the parasite's extracellular occurrence, a type-2 differentiation of *H. meleagridis*-specific T cells has been hypothesized. In contrast, a recent study suggested that IFN-γ mRNA$^+$ cells are involved in protection against histomonosis. However, the phenotype and cytokine production profile of *H. meleagridis*-specific T cells still awaits elucidation. In this work, clonal cultures of a virulent monoxenic strain of *H. meleagridis* were used for infecting chickens to detect IFN-γ protein and IL-13 mRNA by intracellular cytokine staining and PrimeFlow™ RNA Assays, respectively, in CD4$^+$ and CD8β$^+$ T cells. Infection was confirmed by characteristic pathological changes in the cecum corresponding with *H. meleagridis* detection by immunohistochemistry and *H. meleagridis*-specific antibodies in serum. In splenocytes stimulated either with *H. meleagridis* antigen or PMA/ionomycin, IFN-γ-producing CD4$^+$ T cells from infected chickens increased in comparison to cells from non-infected birds 2 weeks and 5 weeks post-infection. Additionally, an increase of IFN-γ-producing CD4$^-$CD8β$^-$ cells upon *H. meleagridis* antigen and PMA/ionomycin stimulation was detected. Contrariwise, frequencies of IL-13 mRNA-expressing cells were low even after PMA/ionomycin stimulation and mainly had a CD4$^-$CD8β$^-$ phenotype. No clear increase of IL-13$^+$ cells related to *H. meleagridis* infection could be found. In summary, these data suggest that *H. meleagridis* infection induces a type-1 differentiation of CD4$^+$ T cells but also of non-CD4$^+$ cells. This phenotype could include γδ T cells, which will be addressed in future studies.

Introduction

Histomonas meleagridis causes histomonosis (synonyms: enterohepatitis or blackhead disease) of chickens (*Gallus gallus*) and turkeys (*Meleagris gallopavo*) [1, 2]. The primarily affected organ is the cecum with generalized mucosal bleedings and severe fibrinous inflammation. Migration of the parasite through the portal vein leads to pathogenic changes in the liver characterized by multifocal areas of necrotic lesions. The clinical manifestation shows great variability among both species. While histomonosis in chickens usually leads to a decrease in weight gain and a drop in egg production, turkeys suffer more often with fatal outcome, as summarized previously [3]. Today, most countries impose a ban on previously applied prophylactic and therapeutic drugs due to consumer safety regulations. Because of these currently limited possibilities for medical intervention in combination with the high mortality in turkeys, histomonosis can cause severe suffering of infected birds. Hence, the disease is considered as a substantial economic threat for the poultry industry and requires the development of novel control strategies [4].

In order to protect turkeys from histomonosis, the transfer of antibodies was not effective [5]. Also,

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immunization by inactivated vaccines could not reduce mortality or lesions in turkeys [6, 7]. Instead, vaccination with a clonal in vitro attenuated *H. meleagridis* strain seems to be a promising strategy for protection of turkeys against histomonosis [7]. No reversion to virulence was observed so far for this in vitro attenuated *H. meleagridis* strain [8]. However, vaccine-induced immunity and protective traits against histomonosis are not fully understood yet. Upon *H. meleagridis* infection, a less profound influx of T cells in the liver of chickens compared to turkeys was observed [9]. A study by Mitra et al. [10] which analyzed different immune cell subsets in various organs, demonstrated distinct changes of B- and T-cell subsets after infection of chickens and turkeys, which were less pronounced in birds that had first undergone vaccination.

Due to the parasite's extracellular occurrence, a type-2 immune response is conceivable. Powell et al. [9] studied a broad set of innate pro-inflammatory and adaptive cytokines on the mRNA level in the cecal tonsil and liver. In both organs from infected chickens, IFN-γ mRNA was up-regulated during the early stage of infection. In contrast, IL-13 mRNA expression was enhanced permanently. Another study investigated the immune response after co-infection of *H. meleagridis* and *Heterakis gallinarum* in chickens [11]. Detection of cytokines in the cecum by quantitative RT-PCR showed that infection without histomonads led to a type-2 dominated response characterized by IL-13 mRNA. Contrariwise, a co-infection resulted in a shift towards a type-1 dominated response with increased cecal IFN-γ mRNA expression. Recently the immune response against *H. meleagridis* was investigated by in situ hybridization (ISH) to detect cytokine transcript containing cells. Infected compared to vaccinated and challenged turkeys showed a delayed increase of IFN-γ mRNA$^+$ cells in the cecum coinciding with severe tissue damage. Hence, an early rise in IFN-γ mRNA$^+$ cells following vaccination could be protective by a rapid activation of effector memory T cells in turkeys [12].

None of these studies exclusively addressed *H. meleagridis*-specific T cells by T-cell markers. Therefore, we aimed to establish assays for identifying signature Th1 and Th2 cytokines in liver and spleen of chickens infected with clonal cultures of a virulent monoxenic strain of *H. meleagridis*. IFN-γ protein$^+$ and IL-13 mRNA$^+$ CD4$^+$ and CD8β$^+$ T cells were evaluated using *H. meleagridis* antigen as well as PMA/ionomycin stimulation by intracellular cytokine staining (ICS) and PrimeFlow™ RNA Assays (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Our results indicate that *H. meleagridis* infection in chickens leads to IFN-γ but not IL-13 production in CD4$^+$ T cells as well as non-CD4$^+$ cells in liver and spleen, providing further evidence that this protozoan infection causes predominantly type-1 immune responses.

Materials and methods

Birds

Embryonated specific pathogen free (SPF) layer eggs (VALO, BioMedia, GmbH, Osterholz-Scharmbeck, Germany) were incubated and hatched at the Clinic for Poultry and Fish Medicine, University of Veterinary Medicine Vienna, Austria. After hatch, 12 chicks were placed in pens on wood shavings in rooms with filtered air under negative pressure. Feed and water were provided ad libitum.

Preparation of cultures for infection

For infection of the birds, the previously established clonal culture *H. meleagridis*/Turkey/Austria/2922-C6/04 (23 passages) obtained by micromanipulation and co-cultivated with the bacterial strain *E. coli* DH5α was used [13, 14]. For control birds, an inoculum containing the bacterial strain *E. coli* DH5α alone with a defined concentration close to the infection inoculum was prepared. For that, an average *E. coli* DH5α concentration of 1×10^8 CFU/mL (colony forming units) in three separate 72-h parasite cultures was determined. The cultivation medium contained Medium 199 with Earle's salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco™, Thermo Fisher Scientific), 15% fetal calf serum (FCS) (Gibco™, Thermo Fisher Scientific) and 0.25% sterilized rice starch (Carl Roth, Karlsruhe, Germany). Counting of viable *H. meleagridis* cells was performed using Trypan Blue and a Neubauer hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) to adjust the relevant number of the parasite for inoculation. For *E. coli* DH5α, CFUs were determined by counting *E. coli* serial dilutions on Coliform agar plates after an incubation at 37 °C for 24 h.

Infection

At an age of 28 days, the chickens were separated into two groups ($n = 6$ per group, Additional file 1) in different rooms. All chickens were subcutaneously tagged for identification (Swiftach®, Avery Dennison, Glendale, CA, USA). Prior to infection, body weight, blood samples and cloacal swabs were taken. Subsequently, one group of birds was inoculated with 6×10^5 cells of *H. meleagridis* co-cultured with 6×10^6 CFU of the bacterial strain *E. coli* DH5α in 600 µL cultivation medium. The inoculum was equally split for application via the oral and cloacal route using a syringe with crop tube or a conventional 1 mL pipette (Eppendorf AG, Hamburg, Germany), respectively. Control birds were inoculated using the same administration routes with 1×10^8 CFU of *E. coli* DH5α in 600 µL cultivation medium without

the presence of *H. meleagridis*. After inoculation, birds were kept feed and water restricted for 5 h. Three birds of each group were sacrificed 2 weeks post-infection (pi) (14 days post-infection (dpi), 15 dpi, 16 dpi) and 5 weeks pi (37 dpi, 38 dpi, 39 dpi) (Additional file 1). The infection experiment was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26 ft. of Animal Experiments Act, Tierversuchsgesetz 2012-TVG 2012 (license number 68.205/0161-WFN/3b/2017).

Clinical examination, necropsy and sampling

All birds were examined daily for clinical signs characteristic for histomonosis including depression, diarrhea and ruffled feathers. Once per week the body weight of the birds was measured and blood samples were collected, starting at the day of infection until the day of killing. Cloacal swabs were taken 3 times per week from the day of infection onwards. The cloacal swabs were subsequently transferred to 2 mL microtubes (Eppendorf AG) filled with 1.5 mL cultivation medium (composition of medium described above) for re-isolation of viable parasites and incubated at 40 °C. The re-isolations were evaluated daily using a microscope up to 5 days post sampling for the presence of *H. meleagridis*. Blood samples were stored overnight at 4 °C before centrifugation at 3300 × *g* for 12 min to obtain serum. The serum samples were stored at −20 °C before further application. For euthanasia, thiopental (medicamentum pharma GmbH, Allerheiligen im Mürztal, Austria) was applied intravenously and the birds were bled to death before necropsy and gross pathology were performed. Lesions in cecum and liver were determined according to a previously established lesion scoring (LS) system [15, 16]: LS 0 represents no lesion, whereas LS 1 to 4 indicates mild to severe pathological changes. Tissue samples of cecum and liver were preserved in formalin for detection of *H. meleagridis* using immunohistochemistry (IHC), as described below. The spleens and the remaining tissue of livers were transferred to beakers containing ice-cold phosphate buffered saline (PBS) + 2% FCS (both Gibco™, Thermo Fisher Scientific) prior to subsequent isolation of lymphocytes (see below).

Detection of *H. meleagridis* by ELISA and immunohistochemistry

For detection of antibodies against *H. meleagridis,* an indirect sandwich ELISA was performed following a previously established protocol [17]. In brief, a rabbit anti-*Histomonas* serum at a dilution of 1:10 000 was used for coating the ELISA plate. After adding a blocking buffer (Thermo Fisher Scientific) to prevent unspecific binding, the histomonad antigen was added. Subsequently, the plate was incubated with the serum samples followed by incubations with the goat anti-chicken IgG-horseradish peroxidase (Southern Biotech, Birmingham, AL, USA) and the tetramethylbenzidine substrate solution (Calbiochem, Merck KGaA, Darmstadt, Germany). After stopping the reaction with sulphuric acid the positivity of the samples was determined on a defined cut-off value set at 0.54 which is based on optical densities (OD) measured at a wavelength of 450 nm according to the before mentioned publication.

IHC for the direct detection of *H. meleagridis* in tissues was applied as described by Singh et al. [18]. Briefly, after fixation, dehydration and embedding in paraffin, cuts of 4 µm in size were prepared using a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany) and transferred to glass slides (Superfrost plus, Menzel-Gläser, Braunschweig, Germany). After dewaxing and rehydration, samples were incubated overnight at 4 °C with a purified polyclonal anti-histomonad rabbit antibody. A biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) was added after washing with PBS (Gibco™, Thermo Fisher Scientific). Following another washing step, the Vectastain ABC Kit and DAB Substrate Kit (Vector Laboratories) were used for visualizing *H. meleagridis*. The surrounding tissue was counterstained using Haematoxylin (Merck KGaA).

Preparation of *H. meleagridis* and *E. coli* antigen stocks for in vitro re-stimulation

For in vitro re-stimulation of lymphocytes, a *H. meleagridis*/*E. coli* antigen stock as well as an *E. coli* control antigen stock were prepared as follows. The *H. meleagridis* with *E. coli* antigen was generated from the same clonal culture of *H. meleagridis*/Turkey/Austria/2922-C6/04 (23 passages) as used for infection. The *E. coli* antigen was prepared from the *E. coli* inoculum for the control birds (see above for cultivation conditions). The *H. meleagridis* with *E. coli* culture and the culture with *E. coli* alone were washed and resuspended with PBS (Gibco™, Thermo Fisher Scientific) at 200 × *g* for 5 min and at 1780 × *g* for 5 min, respectively, in order to remove the cultivation medium. The concentration of the *H. meleagridis* preparation was 9.25×10^6 *H. meleagridis*/mL with 1×10^7 *E. coli* CFU/mL and the *E. coli* alone preparation was 1.3×10^9 CFU/mL. A freezing/thawing procedure at −80 °C was applied for three times. For removal of rice starch particles, a centrifugation step at 375 × *g* for 3 min was conducted. The supernatant was collected, aliquoted and frozen at −80 °C until used for stimulation.

Isolation of lymphocytes

For isolation of lymphocytes, spleen and liver initially collected in PBS + 2% FCS were transferred into petri dishes. The splenic capsule was removed and tissue teased apart by using two sterile blunt-end forceps. Liver tissue was dissected by squeezing the tissue with the end of a plunger from a 20 mL syringe. Cell suspensions from both organs were filtered through 40 µm cell strainers (BD Falcon™, BD Biosciences, San Jose, CA, USA). Spleen and liver cell suspensions were centrifuged at 350 × *g* for 10 min at room temperature and the supernatant was discarded. The cell pellet was resuspended with cold PBS + 2% FCS and layered on a double volume of Histopaque®-1077 (Sigma-Aldrich). After centrifugation at 850 × *g* for 20 min at room temperature, the interphase was collected. Following washing with PBS and centrifugation at 650 × *g* for 10 min at 4 °C, cells were resuspended in PBS and stored on ice until further processing. Cell viability and counting was performed using Trypan Blue and a Neubauer hemocytometer (Sigma-Aldrich).

Establishment of intracellular cytokine staining for chicken IFN-γ

A panel of six monoclonal antibodies (mAbs) against chicken IFN-γ were investigated for their suitability in intracellular cytokine staining. Four mouse IgG1 (2B7, 11G5, 7E3, 12F12), one mouse IgG2a (12F7) and one mouse IgG2b (12D4) mAb clone were tested. Chicken splenocytes were seeded at 5×10^5 cells per well into 96-well round-bottom microtiter plates (Greiner Bio-One, Kremsmünster, Austria) in 200 µL RPMI 1640 (PAN Biotech GmbH, Aidenbach, Germany) supplemented with stable glutamine, 10% heat inactivated FCS (Sigma-Aldrich), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (PAN Biotech GmbH). Splenocytes were cultivated overnight in a humidified incubator at 41 °C and 5% CO_2. The following day the microcultures were treated with PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) in the presence of Brefeldin A (1 µg/mL; BD GolgiPlug™, BD Biosciences) and incubated for additional 4 h. Following harvest, cells were washed twice in PBS (470 × *g* for 4 min at 4 °C; also used for all subsequent washing steps) and surface stained in 96-well plates with mAbs for CD4 (clones: 2–35, mouse IgG2b, Bio-Rad Laboratories, Hercules, CA, USA or CT-4, mouse IgG1, Southern Biotech; both FITC-conjugated) and the Fixable Viability Dye eFluor® 780 (Thermo Fisher Scientific) for 20 min at 4 °C. Afterwards, cells were washed with PBS + 2% FCS. For fixation and permeabilization, the BD Cytofix/Cytoperm (BD Biosciences) kit was applied according to the manufacturer's instructions. After this, the cells were incubated with the mAbs specific for IFN-γ mentioned above. Each antibody was tested in log2 serial dilutions, starting from 200 to 6.25 ng per sample. Following two washing steps with BD Perm/Wash™ Buffer (BD Biosciences), cells were incubated either with goat anti-mouse IgG1-RPE, IgG2a-RPE or IgG2b-RPE secondary antibodies (Southern Biotech) depending on the isotype of the IFN-γ specific mAb. Both incubation steps were performed for 30 min at 4 °C. Finally, the stained cells were washed twice, resuspended in 200 µL BD Perm/Wash™ Buffer (BD Biosciences) and transferred into 5 mL tubes for flow cytometry (FCM) analysis.

Ectopic expression of chicken IL-13 in HEK293T cells

For scrutinizing the suitability of the IL-13 PrimeFlow™ RNA Assay (Thermo Fisher Scientific) for the detection of chicken IL-13 mRNA, immortalized human epithelial 293 kidney cells (HEK293T; originally provided by K. Vanura, Medical University Vienna, Austria) were transfected with chicken IL-13 DNA inserted in a pFLAG-CMV2 expression vector (Sigma-Aldrich) by directional cloning. As control, HEK293T cells were transfected with irrelevant porcine IgE. A detailed protocol on this is given in Additional file 2.

In vitro stimulation of cells and intracellular cytokine staining

Lymphocytes from spleen and liver were cultivated under the conditions given above. For intracellular IFN-γ staining, cells were either stimulated overnight with 100 µL of *H. meleagridis* (5×10^4/mL) with *E. coli* (5.4×10^4 CFU/mL), *H. meleagridis* (5×10^3/mL) with *E. coli* (5.4×10^3 CFU/mL), *E. coli* (9.4×10^6 CFU/mL) alone, *E. coli* (9.4×10^5 CFU/mL) alone, or kept in culture medium as a negative control. An approximately 150 times higher bacterial concentration for the *E. coli* alone antigen than used for the *H. meleagridis* with *E. coli* antigen was chosen to reach similar protein levels within both preparations. Protein concentrations were determined by a Bradford Assay according to the manufacturer's instructions (Bio-Rad). The *H. meleagridis* with *E. coli* preparation had a protein concentration of 70 µg/mL. For re-stimulation, the preparation was diluted to a protein concentration of 10 µg/mL, which was equal to 5×10^4 histomonads cells/mL and 5.4×10^4 *E. coli* CFU/mL. The same calculation was performed for the *E. coli* alone preparation resulting in a concentration of 9.4×10^6 CFU/mL. As positive control, one set of wells was treated with 20 µL of PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) for 4 h. Per stimulation group, six wells with 5×10^5 cells/well were seeded. The Golgi-inhibitor Brefeldin A was added to all stimulation groups during the final 4 h of stimulation at a concentration of 1 µg/mL (BD GolgiPlug™, BD Biosciences). For the IL-13

PrimeFlow™ RNA Assay (Thermo Fisher Scientific), the same stimulation variants were prepared as described above for IFN-γ with the exception of applying two different time spans for antigen-specific re-stimulation. Cells of three birds from each inoculation group were stimulated overnight (for both infected and control group: one bird from the first necropsy 2 weeks pi and two birds from the second necropsy 5 weeks pi) whereas the cells of the remaining three birds were stimulated for 4 h (opposite distribution of birds compared to overnight stimulation). Data obtained from both time spans are grouped together in the results section, since no obvious differences related to the time of stimulation were observed.

Afterwards, cells were pooled and washed as described above. For surface staining, cells were incubated with mouse anti-chicken CD4-FITC (clone: 2–35, isotype: IgG2b, Bio-Rad Laboratories) and biotinylated CD8β (clone: EP42, isotype: IgG2a, Southern Biotech) mAbs for 20 min at 4 °C. In a second staining step, Streptavidin eFluor™ 450 (Thermo Fisher Scientific) together with Fixable Viability Dye eFluor® 780, (Thermo Fisher Scientific) was applied (20 min, 4 °C). For intracellular IFN-γ staining, the BD Cytofix/Cytoperm (BD Biosciences) kit was used for fixating and permeabilizing cells. Chicken IFN-γ-specific mAb 11G5, isotype mouse IgG1, was added and further labelled by goat anti-mouse IgG1-RPE (Southern Biotech). Washing steps were performed as described above. For IL-13 mRNA staining, the PrimeFlow™ RNA Assay kit (Thermo Fisher Scientific) was employed according to the manufacturer's instructions. The target probe specific for chicken IL-13 mRNA was designed by the company based on the sequence accession number NM_001007085 (Assay ID: VF1-4170930-PF, Thermo Fisher Scientific). An Alexa Fluor™ 647-conjugated label probe for detection of the target probe was selected. Cell surface staining as well as staining with Fixable Viability Dye eFluor® 780 (Thermo Fisher Scientific) was performed as described above for IFN-γ labelling. Thereafter, cells were fixated for 30 min at 4 °C, washed twice with permeabilization buffer and fixated for a second time for 60 min at room temperature. In between, a centrifugation step with 1000 × *g* for 4 min at 4 °C was applied. Afterwards, cells were incubated with the chicken IL-13 mRNA specific target probe for 2 h at 41 °C. Followed by two washes at room temperature and adding 100 µL PrimeFlow™ RNA Assay wash buffer (Thermo Fisher Scientific), cells were stored overnight at 4 °C in the dark. After two amplification steps for 1.5 h at 41 °C, cells were incubated with the fluorescence labelled probe for 1 h at 41 °C. After three washing steps, cells were resuspended in 200 µL PrimeFlow™ RNA Assay storage buffer (Thermo Fisher Scientific) using 5 mL tubes for subsequent FCM-analysis.

Cell analysis by flow cytometry

For measurement of IFN-γ protein stained cells and for IL-13 mRNA stained cells, a FACSAria and a FACSCanto II (both BD Biosciences) were used, respectively. Both flow cytometers were equipped with three lasers (405, 488, 633 nm). Between 2×10^5 and 6×10^5 lymphocytes (identified by light scatter properties) were acquired per sample. Flow cytometry data was acquired by FACSDiva software version 6.1.3 (BD Biosciences) and analyzed by FlowJo™ software (Version 10.5.0, Tree Star, Ashland, OR, USA).

Processing of results and statistical analysis

For a calculation of the frequency of *H. meleagridis*-specific T cells, percentages of cytokine-producing (IFN-γ or IL-13) lymphocyte subsets from *E. coli*-only stimulated samples were subtracted from percentages of *H. meleagridis*/*E. coli* co-stimulated samples. To determine significant differences in cytokine-producing cell subsets (IFN-γ or IL-13) between the two necropsies (14–16 dpi and 37–39 dpi), a Wilcoxon test was applied. Differences between stimulated and non-stimulated cytokine-producing cell subsets from the same bird were subjected to the Wilcoxon test as well. Mann–Whitney tests were applied to compare cytokine-producing cell subsets isolated from control birds with cytokine-producing cell subsets isolated from infected birds. p-values < 0.05 are indicated by * and p-values < 0.01 are indicated by **. Statistical analyses were performed by the GraphPad Prism software 7.04 (GraphPad Software Inc., San Diego, CA, USA).

Results

Establishment of test systems for the detection of IFN-γ protein by ICS and IL-13 mRNA by PrimeFlow RNA™

To expand the chicken toolbox on antibodies suitable for the detection of IFN-γ by ICS, six monoclonal antibodies, initially successfully applied in ELISA [19], were investigated. Following stimulation of splenocytes with PMA/ionomycin, four out of six monoclonal antibodies (clones: 12F7, 2B7, 11G5, 7E3) identified similar frequencies (2.39 to 2.88%) of IFN-γ-producing cells (Additional file 3, the applied gating is illustrated in Additional file 4A). Predominantly, $CD4^+$ cells produced IFN-γ. Monoclonal antibody clone 12D4 detected 0.84% IFN-γ^+ cells within live lymphocytes while clone 12F12 seemed to be not suitable for ICS since hardly any IFN-γ^+CD4^+

splenocytes were found. For all subsequent experiments clone 11G5 with a mouse IgG1 isotype was selected.

As outlined in "Materials and methods", PrimeFlow™ RNA Assays were applied in order to detect chicken IL-13 mRNA on the single cell level by flow cytometry. The test was performed according to the manufacturer's instructions but we scrutinized its specificity by the use of HEK293T cells, which were transfected for expression of chicken IL-13 (Additional files 5A and B). The gating strategy applied in these experiments is illustrated in Additional file 5A. The IL-13 specific target probe could detect IL-13 mRNA-expressing cells (33.6%), while staining without the target probe but only the label probe revealed no such population (0.02%; Additional file 5B, upper row). The labelling of HEK293T cells transfected with the same vector but containing an insert that codes for porcine IgE also led to no IL-13$^+$ cells neither in presence (0.01%) nor in absence (0%) of the target probe (Additional file 5B, lower row). Hence, both methods appeared to be reliable and were subsequently applied in a controlled infection experiment of chickens with *H. meleagridis* (see Additional file 1 for outline of the experiment).

Clinical signs, pathological score, *H. meleagridis*-specific IHC and circulating antibodies

No clinical signs were detected in any bird from the infected and control group. Pathological lesion scores (LS) of cecum and liver determined during post-mortem necropsy are summarized in Table 1. In the ceca, a maximum LS of 4 was reached for one infected bird at 2 weeks pi and overall the scores decreased for birds sacrificed 5 weeks pi to 2. Ceca collected 2 weeks pi and 5 weeks pi reached a median LS of 3 and 2, respectively. Livers of birds in the infected group were scored and the median LS was zero at both time spans, i.e. 2 weeks pi and 5 weeks pi. The non-infected control birds did not show any lesion in cecum and liver. In two out of six infected birds *H. meleagridis* could be re-isolated from cloacal swab samples (bird 11: 32 and 37 dpi; bird 12: 25, 28 and 30 dpi).

Detection of *H. meleagridis* by IHC in ceca and livers is also depicted in Table 1. All infected birds were found positive in the cecum except one bird which showed the highest detected cecal LS. Livers of infected birds were negative, including one bird that reached a LS of 2. The control birds were confirmed to be non-infected by IHC.

Results from the testing for circulating antibodies against *H. meleagridis* in sera from birds are shown in Table 2. All non-infected chickens were tested negative at all sampling days. Birds of the infected group killed 2 weeks pi also stayed below the threshold for positivity at every time point of sampling. In contrast, two of the remaining infected birds showed positive antibody titers already 2 weeks pi and 5 weeks pi all three infected birds showed antibody titers above the cut-off.

Table 1 Pathological changes and *H. meleagridis* detection in the cecum and liver

	Days post-infection	Animal number	Cecum		Liver	
			LS[a]	IHC[b]	LS	IHC
Control	14	1	0	−	0	−
	15	2	0	−	0	−
	16	3	0	−	0	−
	37	4	0	−	0	−
	38	5	0	−	0	−
	39	6	0	−	0	−
Infected	14	7	3	+	0	−
	15	8	4	−	0	−
	16	9	2	+	0	−
	37	10	2	+	0	−
	38	11	2	+	0	−
	39	12	2	+	2	−

[a] Lesion scoring (LS) system from 0 to 4 was applied; Cecum: 0 = no pathological changes; 1 = sporadic inflammation and/or mild thickening of the wall of one cecum; 2 = sporadic inflammation and/or mild thickening of the wall of both ceca; 3 = inflammation of both ceca and thickening of the intestinal wall with liquid fibrin or sporadic fibrinous coagula in the lumen. If only one cecum was affected, then lesion score 2 was applied; 4 = severe inflammation and necrosis in both ceca with compact fibrinous masses in the lumen of the ceca. If only one cecum was affected, then lesion score 3 was applied. Liver: 0 = no pathological changes; 1 = a few single punctiform necrosis up to 1 mm; 2 = single punctiform necrosis disseminated throughout the organ up to 1 mm or a few single punctiform necrosis more than 1 mm; 3 = single punctiform necrosis, disseminated throughout the organ more than 1 mm or some large area necrosis; 4 = confluent necrosis throughout the organ.

[b] Detection of the parasite in cecum and liver was performed by immunohistochemistry.

Table 2 *H. meleagridis*-specific antibodies in serum

	Bird no.	Week post-infection					
		0	1	2	3	4	5
Control	1	−	−	−			
	2	−	−	−			
	3	−	−	−			
	4	−	−	−	−	−	−
	5	−	−	−	−	−	−
	6	−	−	−	−	−	−
Infected	7	−	−	−			
	8	−	−	−			
	9	−	−	−			
	10	−	−	+	−	−	+
	11	−	−	−	−	−	+
	12	−	−	+	−	+	+

− Indicates O.D. values below threshold of positivity.

+ Indicates O.D. values above threshold of positivity.

T-cell response following *H. meleagridis* infection

To investigate the T-cell response following *H. meleagridis* infection, two time spans of 14–16 dpi and 37–39 dpi were initially chosen to compare an early T-cell response with a later time point, where potentially an immune memory phase may already have been reached. However, an initial analysis of the obtained frequencies of IFN-γ protein or IL-13 mRNA-producing lymphocyte subsets suggested no major differences between these two time spans. To scrutinize this, Wilcoxon tests were applied and no significant differences were found between the two time spans. Hence, in all results shown in Figures 1, 2, 3, 4, phenotypes of cytokine-producing cells from these two necropsies were grouped together and subjected to a Mann–Whitney test for significance testing. In addition, in Figures 1, 2, 3, 4 original flow cytometry data of one representative bird per group is shown in pseudocolor plots on the left while percentages of cytokine-producing cells for all birds are illustrated on the right, including a comparison of the stimulation variants between infected and control animals. Underlying values of cytokine-producing lymphocytes in these analyses are listed in Additional file 6, together with the calculated *E. coli* corrected values.

IFN-γ production in CD4/CD8β defined lymphocyte subsets in the spleens of *H. meleagridis* infected chickens

ICS analyses were performed for IFN-γ and combined with cell surface staining for CD4 and CD8β, allowing the identification of IFN-γ production in $CD4^+$, $CD8\beta^+$ and $CD4^-CD8\beta^-$ cells (gating strategy is illustrated in Additional file 4A). PMA/ionomycin stimulation led to a significant increase in IFN-γ-producing cells within total $CD4^+$ splenocytes regardless whether cells derived from control ($p < 0.05$) or *H. meleagridis* infected chickens ($p < 0.05$; Figure 1A, scatter plots on the left). A significantly elevated level of IFN-γ^+CD4^+ cells after PMA/ionomycin stimulation was found in infected birds in comparison to control birds ($p < 0.01$; Figure 1A, scatter plot on the right). *H. meleagridis*/*E. coli* stimulation in comparison to *E. coli*-only control stimulation revealed significantly higher levels ($p < 0.05$) of IFN-γ^+ cells within $CD4^+$ splenocytes in infected birds (Figure 1B, scatter plot on the left, bottom). No such difference was detectable in control birds, which showed low frequencies of IFN-γ^+ cells within the $CD4^+$ subset in *H. meleagridis*/*E. coli* stimulated cultures and *E. coli*-only stimulated control cultures (Figure 1B, scatter plot on the left, top). Percentages of *H. meleagridis*/*E. coli* stimulated IFN-γ-producing cells were significantly higher in infected than in control birds ($p < 0.01$), also after correcting the data for IFN-γ-producing splenocytes induced by *E. coli*-only stimulation ($p < 0.01$; Figure 1B, scatter plots on the right). Ten-fold lower concentrations of *H. meleagridis* antigen (5×10^3/mL) were also tested for the induction of IFN-γ-producing $CD4^+$ splenocytes. Results are shown in comparison to data obtained with 5×10^4 *H. meleagridis*/mL (Additional file 7). Although still elevated levels of IFN-γ^+CD4^+ cells in infected versus control birds were found (Additional file 7, scatter plots in right column of right panel), after correction of *E. coli* these differences did not reach significance.

For $CD8\beta^+$ splenocytes (Figure 2) in control birds, no significant rise in IFN-γ-producing cells was detected upon PMA/ionomycin stimulation in comparison to non-stimulated cells. For infected birds, a significant

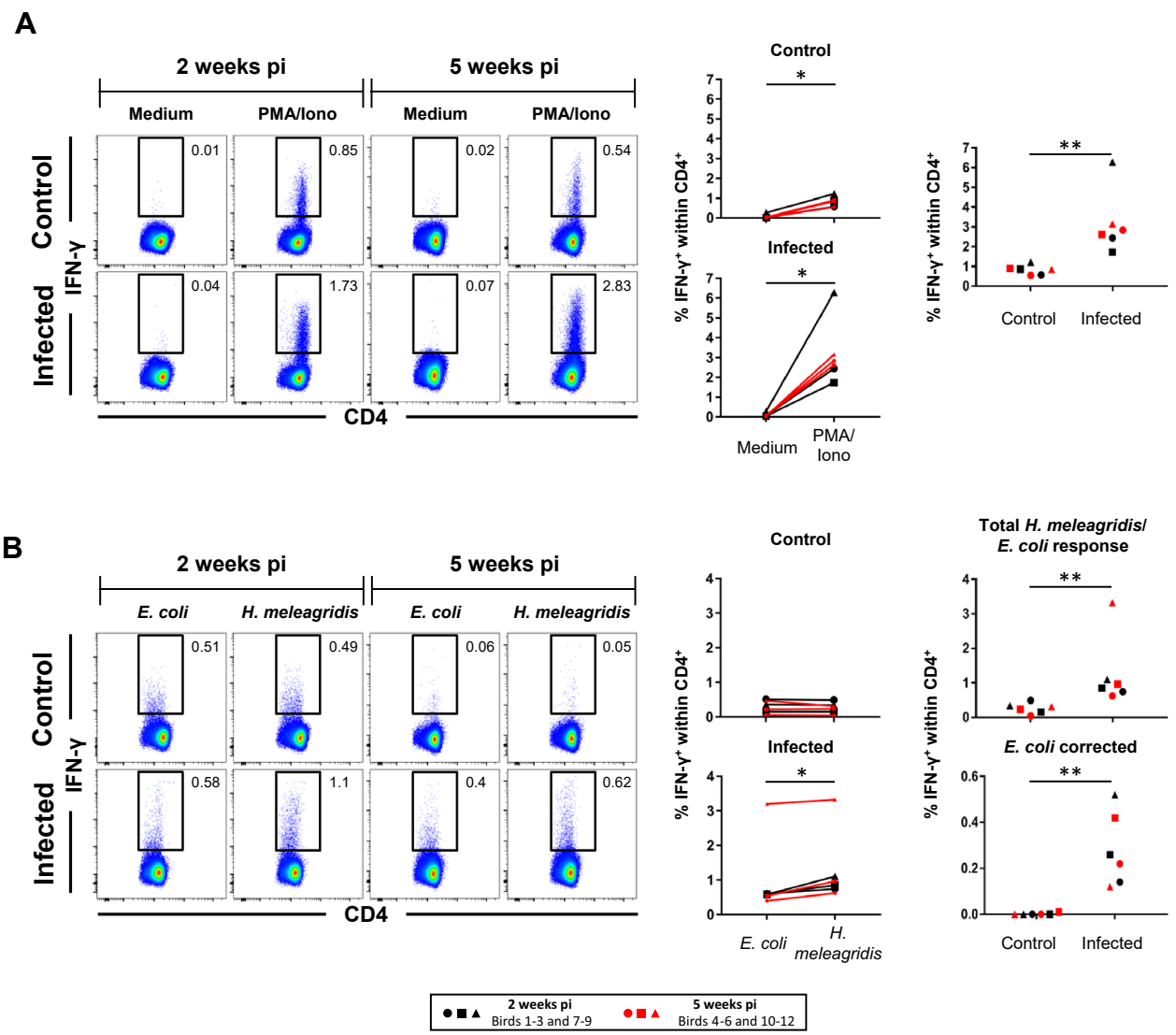

Figure 1 Frequencies of splenic IFN-γ-producing CD4$^+$ cells following stimulation with PMA/ionomycin or *H. meleagridis*. **A**, **B** Representative pseudocolor plots show IFN-γ versus CD4 expression in CD4$^+$ pre-gated (not depicted) splenocytes isolated from birds 2 weeks pi and 5 weeks pi. Approximately **A** 200 000 and **B** 150 000 CD4$^+$ cells are shown in each plot and numbers indicate frequencies of IFN-γ$^+$CD4$^+$ cells within total CD4$^+$ cells. Graphs on the right display frequencies of IFN-γ-producing CD4$^+$ cells from all birds. Each symbol represents one bird, black and red colored symbols represent birds sacrificed 2 weeks pi and 5 weeks pi, respectively. **A** Scatter plots show percent of IFN-γ$^+$ cells within the CD4$^+$ subset after PMA/ionomycin stimulation compared to medium in control and infected birds (left panel). Right panel: comparison of IFN-γ-producing CD4$^+$ cell frequencies after stimulation with PMA/ionomycin between infected and control birds. **B** Scatter plots as in **A** but after *H. meleagridis*/*E. coli* stimulation and *E. coli*-only stimulation. Right panel shows in addition percent of IFN-γ$^+$ cells after *E. coli* correction for infected and control birds. Asterisks indicate different *p*-values: *$p \leq 0.05$ and **$p \leq 0.01$.

increase for this condition was found ($p < 0.05$; Figure 2A, scatter plots on the left). However, although PMA/ionomycin-induced IFN-γ$^+$CD8β$^+$ splenocytes within total CD8β$^+$ splenocytes from the infected group were in tendency higher than those ones in the control group, these differences did not reach significance (Figure 2A, scatter plot on the right). Similarly, *H. meleagridis* re-stimulation led to no significant difference between infected and control birds for IFN-γ$^+$CD8β$^+$ splenocytes. This applied also when the data was corrected for *E. coli*-only induced IFN-γ-producing CD8β$^+$ splenocytes (Figure 2B, scatter plots on the right).

IFN-γ-producing cell frequencies within the remaining CD4$^-$CD8β$^-$ subset of splenocytes were also investigated (Figure 3). PMA/ionomycin stimulation versus medium led to a significant increase in

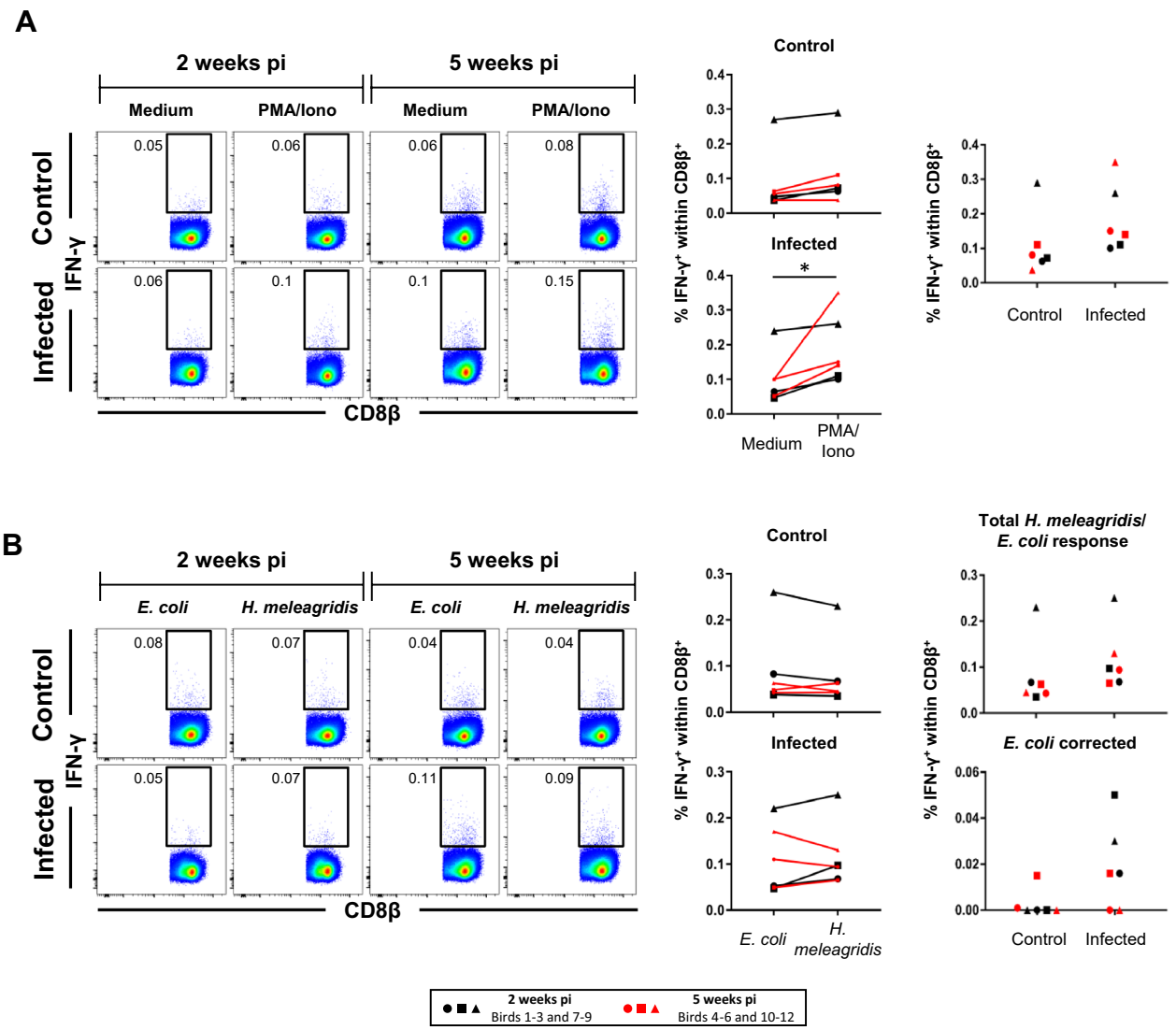

Figure 2 Frequencies of splenic IFN-γ-producing CD8β$^+$ cells following stimulation with PMA/ionomycin or *H. meleagridis*. **A**, **B** Representative pseudocolor plots show IFN-γ versus CD8β expression in CD8β$^+$ pre-gated (not depicted) splenocytes isolated from birds 2 weeks pi and 5 weeks pi. Approximately **A** 270 000 and **B** 300 000 CD8β$^+$ cells are shown in each plot and numbers indicate frequencies of IFN-γ$^+$CD8β$^+$ cells within total CD8β$^+$ cells. Graphs on the right display frequencies of IFN-γ-producing CD8β$^+$ cells from all birds. Each symbol represents one bird, black and red colored symbols represent birds sacrificed 2 weeks pi and 5 weeks pi, respectively. **A** Scatter plots show percent of IFN-γ$^+$ cells within the CD8β$^+$ subset after PMA/ionomycin stimulation compared to medium in control and infected birds (left panel). Right panel: comparison of IFN-γ-producing CD8β$^+$ cell frequencies after stimulation with PMA/ionomycin between infected and control birds. **B** Scatter plots as in **A** but after *H. meleagridis*/*E. coli* stimulation and *E. coli*-only stimulation. Right panel shows in addition percent of IFN-γ$^+$ cells after *E. coli* correction for infected and control birds. Asterisk indicates *p*-value: $*p \leq 0.05$.

IFN-γ$^+$CD4$^-$CD8β$^-$ splenocytes in control and infected birds (both $p < 0.05$; Figure 3A, scatter plots on the left). Infected compared to control birds disclosed a significantly higher level of IFN-γ$^+$CD4$^-$CD8β$^-$ cells in infected birds after PMA/ionomycin stimulation ($p < 0.01$; Figure 3A, scatter plot on the right). For most of the infected birds, higher levels of IFN-γ$^+$ cells within CD4$^-$CD8β$^-$ splenocytes were identified upon *H. meleagridis*/*E. coli* stimulation compared to *E. coli*-only stimulated controls, but this did not reach significance (Figure 3B, scatter plots on the left). However, *H. meleagridis*/*E. coli* re-stimulation induced a significant difference between control and infected birds ($p < 0.01$) but significance was lost after a correction of data for *E. coli*-only induced IFN-γ production (Figure 3B, scatter plots on the right). Overall, these results indicate the

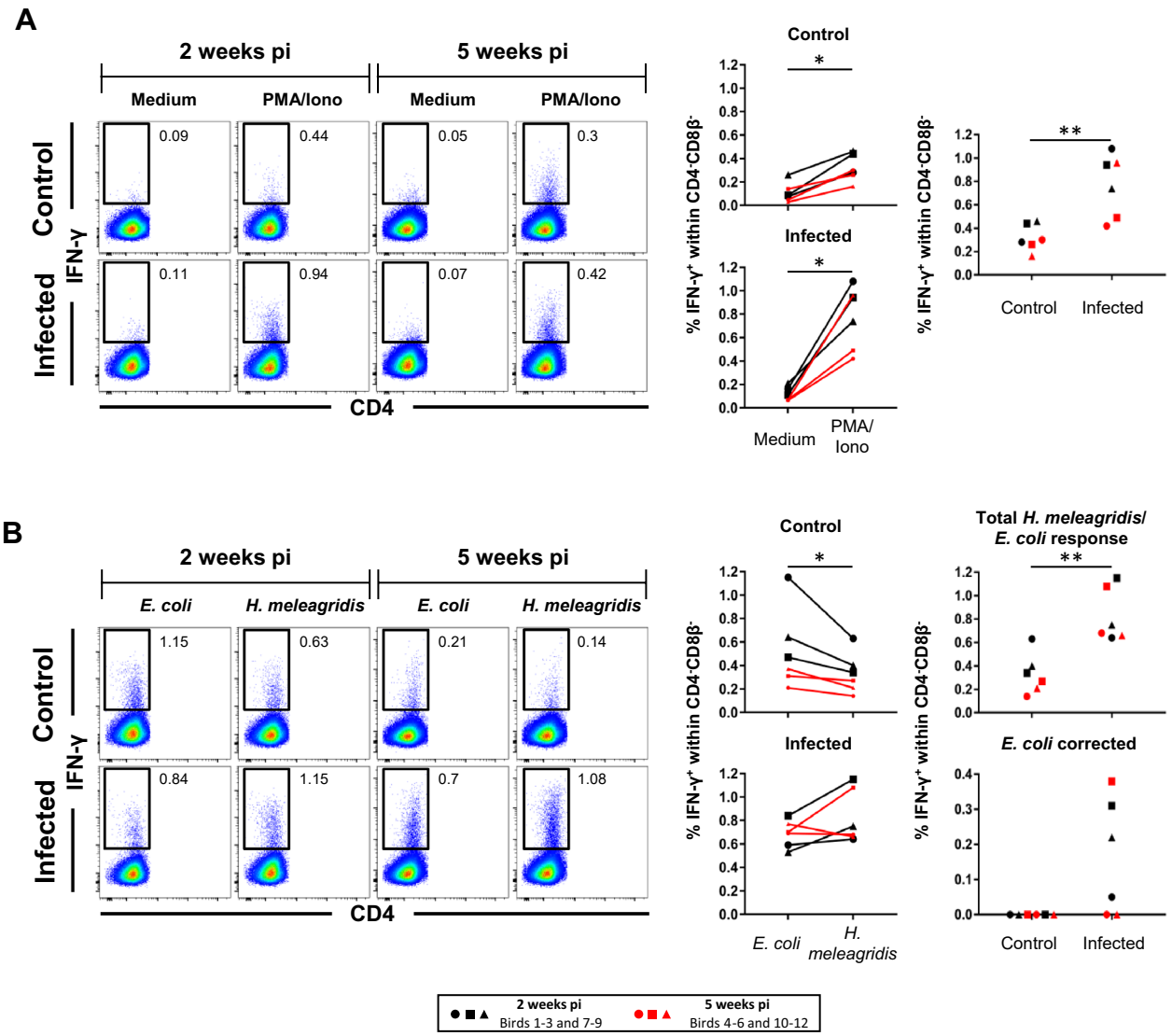

Figure 3 Frequencies of splenic IFN-γ-producing CD4$^-$CD8β$^-$ cells following stimulation with PMA/ionomycin or *H. meleagridis*. **A**, **B** Representative pseudocolor plots show IFN-γ versus CD4 expression in CD4$^-$CD8β$^-$ pre-gated (not depicted) splenocytes isolated from birds 2 weeks pi and 5 weeks pi. Approximately **A** 200 000 and **B** 180 000 CD4$^-$CD8β$^-$ cells are shown in each plot and numbers indicate frequencies of IFN-γ$^+$CD4$^-$CD8β$^-$ cells within total CD4$^-$CD8β$^-$ cells. Graphs on the right display frequencies of IFN-γ-producing CD4$^-$CD8β$^-$ cells from all birds. Each symbol represents one bird, black and red colored symbols represent birds sacrificed 2 weeks pi and 5 weeks pi, respectively. **A** Scatter plots show percent of IFN-γ$^+$ cells within the CD4$^-$CD8β$^-$ subset after PMA/ionomycin stimulation compared to medium in control and infected birds (left panel). Right panel: comparison of IFN-γ-producing CD4$^-$CD8β$^-$ cell frequencies after stimulation with PMA/ionomycin between infected and control birds. **B** Scatter plots as in **A** but after *H. meleagridis*/*E. coli* stimulation and *E. coli*-only stimulation. Right panel shows in addition percent of IFN-γ$^+$ cells after *E. coli* correction for infected and control birds. Asterisks indicate different *p*-values: *$p \leq 0.05$ and **$p \leq 0.01$.

generation of *H. meleagridis*-specific IFN-γ-producing splenocytes with a CD4$^+$ and a CD4$^-$CD8β$^-$ phenotype in *H. meleagridis*-infected chickens. Interestingly, for these two phenotypes PMA/ionomycin stimulation also caused higher frequencies of IFN-γ-producing cells in *H. meleagridis* infected chickens than in control chickens.

IFN-γ production in CD4/CD8β defined lymphocyte subsets in livers of *H. meleagridis* infected chickens

In parallel to the analyses with splenocytes, IFN-γ$^+$ cell frequencies in livers were determined for CD4$^+$, CD8β$^+$ and CD4$^-$CD8β$^-$ T-cell subsets. In contrast to data obtained with splenocytes, neither in CD4$^+$ nor in CD4$^-$CD8β$^-$ lymphocytes derived from the

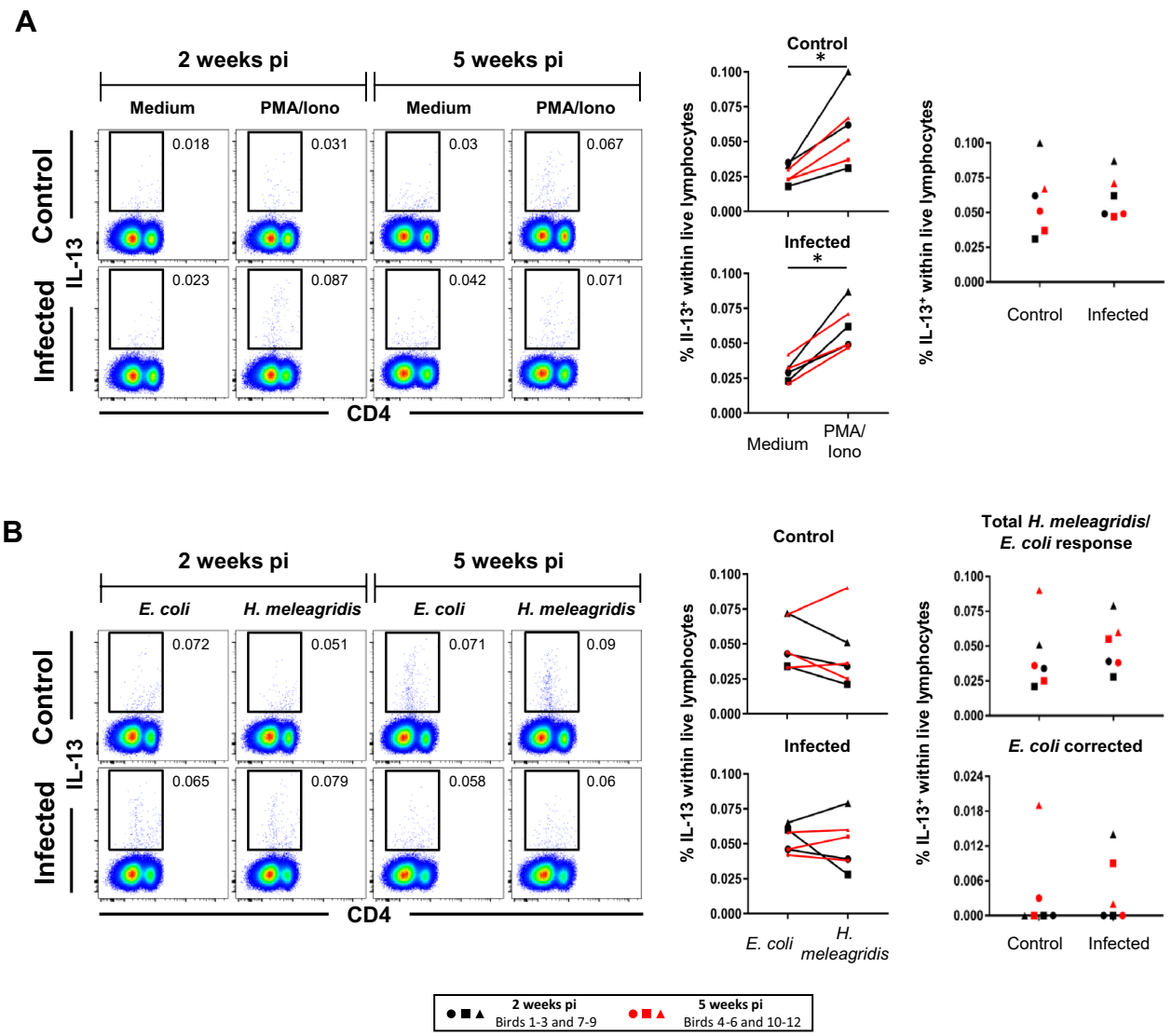

Figure 4 Frequencies of splenic IL-13 mRNA$^+$ cells following stimulation with PMA/ionomycin or *H. meleagridis*. **A**, **B** Representative pseudocolor plots show IL-13 mRNA versus CD4 expression in pre-gated (not depicted) total live splenocytes isolated from birds 2 weeks pi and 5 weeks pi. Approximately 320 000 lymphocytes are shown in each plot and numbers indicate frequencies of IL-13 mRNA$^+$ cells within total live splenocytes. Graphs on the right display frequencies of IL-13 mRNA$^+$ live lymphocytes from all birds. Each symbol represents one bird, black and red colored symbols represent birds sacrificed 2 weeks pi and 5 weeks pi, respectively. **A** Scatter plots show percent of IL-13 mRNA$^+$ cells within live lymphocytes after PMA/ionomycin stimulation compared to medium in control and infected birds (left panel). Right panel: comparison of IL-13 mRNA$^+$ lymphocyte frequencies after stimulation with PMA/ionomycin between infected and control birds. **B** Scatter plots as in **A** but after *H. meleagridis*/*E. coli* stimulation and *E. coli*-only stimulation. Right panel shows in addition percent of IL-13 mRNA$^+$ cells after *E. coli* correction for infected and control birds. Asterisks indicate *p*-value: $*p \leq 0.05$.

liver significant differences in the frequencies of IFN-γ-producing lymphocytes between *H. meleagridis* infected chickens and control chickens were found (Additional file 8, scatter plots in the lower part). This applied to both, PMA/ionomycin stimulation (Additional file 8, left columns of scatter plots) and *H. meleagridis* re-stimulation (Additional file 8, right columns of scatter plots). For CD8β$^+$ cells in the liver, hardly any IFN-γ-producing cells were identified (IFN-γ$^+$ cells ranged from 0 to 0.1%), regardless of the type of in vitro stimulation, treatment of the birds and time point of isolation post-infection (data not shown).

IL-13 mRNA production of lymphocytes in the spleen of *H. meleagridis* infected chickens

Frequencies of IL-13 mRNA-producing lymphocytes were investigated by PrimeFlow™ RNA Assay (Thermo Fisher Scientific) in combination with cell surface staining for CD4 and CD8β. A gating strategy as depicted in Additional file 4B was applied for identifying IL-13 mRNA$^+$ cell frequencies within live splenocytes. No further sub-gating for CD4 or CD8β expression was applied since IL-13 mRNA$^+$ cells appeared to have a CD4$^{dim/-}$CD8β$^-$ phenotype (Additional file 4B and Figures 4A and B, pseudocolor plots on the left).

Stimulation with PMA/ionomycin compared to medium significantly increased the frequency of IL-13 mRNA$^+$ splenocytes in infected ($p < 0.05$) and control birds ($p < 0.05$; Figure 4A, scatter plots on the left). Comparing values of PMA/ionomycin stimulated IL-13 mRNA$^+$ cells revealed no obvious difference between infected and control birds (Figure 4A, scatter plot on the right). *E. coli*-only stimulated control cultures induced slightly higher levels of IL-13 mRNA$^+$ cells than *H. meleagridis*/*E. coli* stimulated cultures in control birds, but this did not reach significance (Figure 4B, scatter plot on the left, top). Also in infected birds, no major difference in IL-13 mRNA$^+$ cells was detected between *H. meleagridis*/*E. coli* and *E. coli*-only stimulation (Figure 4B, scatter plot on the left, bottom). Frequencies of IL-13 mRNA$^+$ splenocytes in infected birds did not significantly differ from frequencies in control animals upon *H. meleagridis*/*E. coli* stimulation and after correcting data from *E. coli* background (Figure 4B, scatter plots on the right). Overall, frequencies of IL-13 mRNA-producing cells detected by flow cytometry were very low and showed no clear phenotype for CD4 or CD8β expression.

Discussion

In avian species, the immunological toolbox for studying cytokine production of different T-cell subsets, especially antigen-specific T cells, is still limited. Flow cytometry-based intracellular cytokine staining is a powerful tool for investigating the frequency and phenotype of cytokine-producing immune cells [20]. The detection of IFN-γ by ICS following in vitro stimulation has only been applied in a limited number of studies using chicken IFN-γ specific mAb clones mAb80 [21], EH9 [22] or the coating and detection antibody of the IFN-γ CytoSet™ ELISA (Thermo Fisher Scientific) [23]. In the present study, six IFN-γ monoclonal antibodies were screened for their capability of detecting chicken IFN-γ by ICS following PMA/ionomycin stimulation (Additional file 3). Four of them detected similarly high frequencies of IFN-γ-producing splenocytes and one of them has a mouse IgG2a phenotype, making it an attractive candidate in multicolor staining panels where isotype-specific secondary antibodies are in use. Hence, the aforementioned studies and our data indicate that there is a panel of IFN-γ-specific antibodies, which are all suitable for ICS in chicken lymphocytes.

In our study, detection of IFN-γ by ICS was tested alongside to detection of IL-13 mRNA by PrimeFlow™ RNA Assay (Thermo Fisher Scientific). Both assays were applied to investigate cytokine production in spleen and liver derived lymphocytes from *H. meleagridis* infected chickens. *H. meleagridis*-specific antibodies, which were investigated in parallel to the T-cell response, showed some degree of variability among infected chickens (Table 2). However, this variability is in accordance with previously published data from Windisch and Hess [15], where a very similar infection model as in our study was applied. Rather, this variability might be seen as an indication that analyses on the T-cell response will contribute to a better understanding of the immune response in chickens against *H. meleagridis*. Indeed, against the hypothesis of a dominating type-2 immune response following an extracellular parasitic infection, which might also support antibody production, our data suggest a type-1 response mainly driven by IFN-γ-producing CD4$^+$ T cells (see also summary of significantly increased cytokine-producing lymphocytes subsets in Additional file 9). Significant rises of putative *H. meleagridis*-specific T cells in the spleen with an IFN-γ$^+$CD4$^+$ phenotype could be found upon antigen re-stimulation in infected compared to non-infected control birds. This did not apply to CD8β$^+$ splenocytes, in which after both, PMA/ionomycin and *H. meleagridis* stimulation, only low frequencies of IFN-γ-producing cells were found and frequencies in infected chickens were only slightly above control chickens, not reaching significance. This suggests that at least for chickens, conventional CD8 T cells are not involved in the sytemic immune response against *H. meleagridis*, which might be explained by the extracellular occurrence of the parasite.

Next to CD4$^+$ cells, significant enhancements of IFN-γ-producing splenocytes within the CD4$^-$CD8β$^-$ subset upon both stimulation approaches in infected birds could be identified. This finding suggests an involvement of putative γδ T cells or NK cells. Chicken γδ T cells are highly abundant in blood as well as in lymphatic organs and can be divided according to their CD8 expression into CD8$^-$, CD8α^{hi}β$^+$ and CD8αα^{hi+} subpopulations [24, 25]. Splenic CD8αα$^+$ γδ T cells seem to be the most potent IFN-γ producers among all γδ T-cell subsets [26]. Published data on the transcription levels of IFN-γ in *Salmonella* Typhimurium infected chickens identified γδ T cells as a possible source of IFN-γ as well [27]. In the last mentioned study, significant increases of IFN-γ mRNA in

γδ T cells with CD8αα$^+$ and CD8α$^+$β$^+$ phenotypes from blood and spleen were observed. Besides γδ T cells, NK cells are known to be major IFN-γ producers in various mammalian species [28, 29]. However, in chickens, NK cells seem to account only for a minor lymphocyte subpopulation in blood and spleen with frequencies below 3%; albeit some of them have the capacity for IFN-γ production [30, 31]. Clearly, a more precise phenotyping of the identified *H. meleagridis*-specific IFN-γ-producing CD4$^-$CD8β$^-$ lymphocytes is something that we will aim to address in future studies.

On the contrary, frequencies of IL-13-producing lymphocytes derived from spleen and liver were extremely low and did not rise following stimulation with *H. meleagridis* antigen. So far, only studies on the gene expression of various cytokines from *H. meleagridis* infected chickens and turkeys were published. Powell et al. [9] found overall higher levels of IL-13 than IFN-γ mRNA in the liver of chickens while our studies did not show a significant increase in frequencies of IFN-γ or IL-13-producing liver cells from infected birds. In another study, which analyzed cytokine mRNA expression by ISH, spleens and livers of infected chickens showed no distinct elevated levels of IFN-γ or IL-13 mRNA in comparison to controls [12]. On the contrary, we found significant differences in IFN-γ-producing CD4$^+$ and CD4$^-$CD8β$^-$ splenocytes comparing infected and control chickens. A possible explanation for this discrepancy might be that ISH analyses identify actively IFN-γ mRNA expressing cells, whereas the in vitro re-stimulation assay applied in this study re-activates putative memory and effector T cells, as indicated by the extremely low frequencies of IFN-γ-producing cells found in medium cultures.

The liver is besides the cecum one of the main affected organs in the course of a *H. meleagridis* infection [32]. It was suggested that in chickens, an up-regulation of pro-inflammatory cytokines in the cecal tonsils limits the migration of parasites from the cecum towards the liver and explains the lower prevalence of liver lesions compared to turkeys [9]. In our analyses on IFN-γ-producing lymphocytes isolated from this organ only low frequencies of *H. meleagridis*-specific cells were found and differences between infected and control birds did not reach significance. This finding coincides with the fact that only one of the chickens in the infected group showed lesions in this organ and all birds were negative by IHC. Although it can be assumed that the negative findings by IHC were a result of the investigation of a non-*H. meleagridis* infested part of the organ, for the analysis of cytokine production lymphocytes of the entire liver were isolated (only a small portion of approximately $0.5 \times 0.5 \times 0.5$ cm was devoted to IHC slide preparation). Hence, it is conceivable that local liver-resident *H. meleagridis*-specific T cells were barely induced, whereas the spleen harbors re-circulating effector and memory T cells, which might contribute also to immune responses in the gut.

In summary, our study is the first that indicates that *H. meleagridis* infection in chickens induces a systemic T-cell related immune response against *H. meleagridis* that is dominated by IFN-γ-producing CD4$^+$ and CD4$^-$CD8β$^-$ splenocytes, whereas no hints for an IL-13 mediated type-2 immune response were found. With the established functional assays, we aim to address in future studies a comparison of the cytokine production in chickens infected with virulent *H. meleagridis* cultures and chickens vaccinated with attenuated *H. meleagridis* cultures as well as vaccinated and challenged birds. This will include a more detailed T-cell phenotyping, addressing specifically CD4$^+$, CD8$^+$ and γδ T cells.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-019-0726-z.

Additional file 1. Design of the animal infection experiment. Twelve 28-day-old chickens were equally distributed to an infected and a control group at the day of infection. The birds were infected via the oral and cloacal route with an equally split inoculum of 6×10^5 virulent *H. meleagridis* cells (23 passages) in combination with 6×10^6 CFU *E. coli*, strain DH5α (infected group, $n = 6$). Birds of the control group ($n = 6$) were sham-infected with the *E. coli* strain DH5α (1×10^8 CFU) only. For organ collection, three birds from each group were sacrificed on 3 consecutive days 2 weeks pi (X symbol) and 5 weeks pi, respectively.

Additional file 2. Transfection of HEK293T cells with chicken IL-13 for scrutiny of the IL-13 mRNA PrimeFlow RNA™ Assay. It contains a detailed methodological description on the transfection of HEK293T cells for the ectopic expression of chicken IL-13.

Additional file 3. Suitability of chicken IFN-γ-specific monoclonal antibodies specific for intracellular cytokine staining. A panel of six mAbs with either mouse IgG1 isotype (2B7, 11G5, 7E3, 12F12), or mouse IgG2a isotype (12F7) and mouse IgG2b isotype (12D4) was tested on PMA/ionomycin stimulated splenocytes. For each antibody, results are shown for the optimal quantity (clone 12F7: 150 ng, 2B7: 50 ng, 11G5: 12.5 ng, 7E3: 100 ng, 12D4: 250 ng, 12F12: 100 ng), initially identified in experiments with serial dilutions. Goat-anti-mouse isotype specific RPE-conjugated antibodies were applied afterwards for fluorescence labelling. Cells were pre-gated as described in Additional file 4A. Results are representative of four experiments with splenocytes from three different chickens.

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Additional file 6. Frequencies of cytokine-producing lymphocyte subsets for all investigated organs and stimulation variants. Frequencies of cytokine-producing lymphocyte subsets for all investigated organs and stimulation variants are given in this table. In addition, all calculated *E. coli* corrected values for control and infected birds are listed.

Additional file 7. Influence of different *H. meleagridis* concentrations on the frequency of IFN-γ-producing CD4$^+$ splenocytes. Intracellular cytokine staining for IFN-γ was performed following 18 h antigen specific re-stimulation either with *H. meleagridis* at 5×10^4/mL and *E. coli* (9.4×10^6 CFU/mL) or a 10-fold lower concentration of *H. meleagridis* (5×10^3/mL) and *E. coli* (9.4×10^5 CFU/mL). Plots on the left of each stimulation variant compare frequencies of IFN-γ-producing CD4$^+$ cells after combined *H. meleagridis/E. coli* stimulation or stimulation only with *E. coli* in infected and control chickens. Plots on the right compare frequencies of IFN-γ-producing CD4$^+$ cells between infected and control chickens after stimulation with *H. meleagridis/E. coli* antigen with or without correction for the response against *E. coli* alone. Each symbol represents one bird, black and red colored symbols show birds sacrificed 2 weeks pi and 5 weeks pi, respectively, as percent of total CD4$^+$ splenocytes. Asterisks indicate different *p*-values: $*p \leq 0.05$, and $**p \leq 0.01$.

Additional file 8. Frequencies of IFN-γ-producing CD4$^+$ and CD4$^-$CD8β$^-$ cells in the liver. The upper panel shows frequencies of IFN-γ-producing CD4$^+$ and CD4$^-$CD8β$^-$ cells after PMA/ionomycin or *H. meleagridis/E. coli* stimulation compared to medium or *E. coli*-only stimulation in control and infected birds. The lower panel compares frequencies of IFN-γ-producing CD4$^+$ and CD4$^-$CD8β$^-$ cells after stimulation with PMA/ionomycin, *H. meleagridis/E. coli* or after correction for *E. coli* between infected and control birds. Each symbol represents one bird, black and red colored symbols show birds sacrificed 2 weeks pi and 5 weeks pi, respectively, as percent of total CD4$^+$ or CD4$^-$CD8β$^-$ intrahepatic lymphocytes. Asterisks indicate *p*-value: $*p \leq 0.05$.

Additional file 9. Summary of significant differences between cytokine-producing lymphocyte subsets isolated from control and *H. meleagridis* infected chickens. In case of *H. meleagridis/E. coli* stimulated results only significance of *E. coli* corrected values are displayed. Asterisks indicate *p*-value levels ($*p \leq 0.05$, and $**p \leq 0.01$).

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Authors' contributions
Conceived the study and designed the experiments: DL, WG. Performed necropsy including sample collection: JL, TM, DL. Performed in vitro stimulation and flow cytometry experiments: JL, SS, AP, EV, MS. Performed ELISA experiments: BG. Performed IHC experiments: PW. Performed IL-13 transfection experiments: JL, SEH, KHM. Analyzed and interpreted the data: JL, WG, DL, FR, BL. Wrote the manuscript: JL, DL, WG. All authors read and approved the final manuscript.

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

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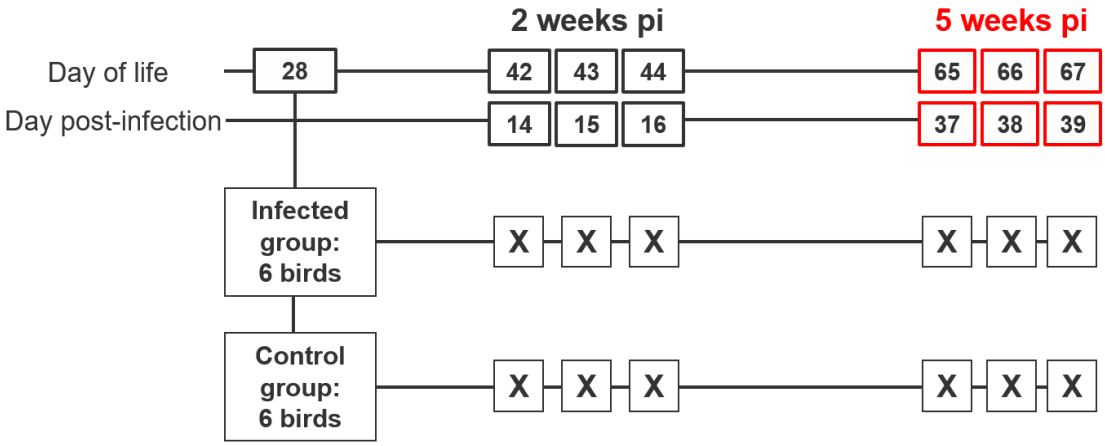


Additional file 1. Design of the animal infection experiment. Twelve 28-day-old chickens were equally distributed to an infected and a control group at the day of infection. The birds were infected via the oral and cloacal route with an equally split inoculum of 6×10^5 virulent *H. meleagridis* cells (23 passages) in combination with 6×10^6 CFU *E. coli*, strain DH5α (infected group, $n = 6$). Birds of the control group ($n = 6$) were sham-infected with the *E. coli* strain DH5α (1×10^8 CFU) only. For organ collection, three birds from each group were sacrificed on 3 consecutive days 2 weeks pi (X symbol) and 5 weeks pi, respectively.

Additional file 2: Transfection of HEK293T cells with chicken IL-13 for scrutiny of the IL-13 mRNA PrimeFlow RNA™ Assay.

For extraction of chicken IL-13 mRNA, PMA (50 ng/mL) and ionomycin (500 ng/mL; both Sigma-Aldrich) stimulated (2 h at 41 °C in 5% CO_2) splenocytes isolated from a 72-week-old chicken were used. Lymphocyte isolation and stimulation was performed as in Materials and Methods. After harvest of stimulated cells, total RNA was extracted by using the Direct-zol RNA MiniPrep Kit (Zymo Research Corp., CA, USA) according to the manufacturer's instructions, followed by RNA concentration measurement on the NanoDrop 2000c (Thermo Fisher Scientific). Subsequently, 500 ng total RNA were subjected to oligo-dT primed cDNA synthesis using the SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. For subcloning IL-13 into the pFLAG-CMV2 vector, appropriate restriction sites had to be introduced by proof-reading RT-PCR reactions using a EcoRI-tagged forward primer together with a XbaI-tagged reverse primer. Therefore, RT-PCR reactions were set-up in a total volume of 50 µL by combining 2 µL cDNA, 10 pM of each primer (Eurofins Genomics GmbH, Ebersberg, Germany), 1.25 U KAPAHiFi™ Polymerase (Thermo Fisher Scientific), 10 µL 5x PCR buffer (KAPAHiFi™ High Fidelity Buffer containing $MgCl_2$ at a 1x concentration of 2.0 mM; Thermo Fisher Scientific), 1.5 µL dNTP mix (10 mM each dNTP; Thermo Fisher Scientific) and 5 µL 10x CoralLoad (Qiagen GmbH, Hilden, Germany). PCR cycling conditions were 2 min at 95 °C, followed by 20 s at 94 °C, 15 s at 60 °C, 30 s at 72 °C, for 35 cycles, followed by a final extension step for 3 min at 72 °C, using a T-gradient thermal cycler (Biometra, Göttingen, Germany). Obtained IL-13 PCR products were run on 1.5% standard agarose gels and further gel purified using the QIAquick Gel Cleanup Kit (Qiagen) according to the manufacturer's instructions. Note: DNA concentrations of consecutive samples were measured on the NanoDrop 2000c (Thermo Fisher Scientific). Purified PCR products were ligated into the pJET1.2/blunt cloning vector (GeneJET™ PCR cloning kit; Thermo Fisher Scientific) by combining in a total volume of 20 µL, 14.4 ng of purified PCR product (= vector to insert molar ratio of 1:3), 10 µL 2x Rapid Ligation buffer, 50 ng pJET1.2/blunt cloning vector and 5 U T4 DNA Ligase. Next, 4 µL ligated IL-13 products were transformed into 50 µL competent *E. coli* cells (JM109; Promega, Madison, WI, USA) and incubated on ice for 20 min. Thereafter, samples were incubated for 1 min at 42 °C and put back on ice for ≥2 min. After adding 500 µL SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose; Sigma-Aldrich) they were incubated for 60 min at 37 °C with shaking at 700 rpm. Finally, 150 µL each were plated onto two LB/Amp^{100} plates (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 15 g/L bacterial agar) being supplemented with Ampicillin (100 µg/mL) and incubated overnight at 37 °C.

Successful ligation and transformation of the tagged IL-13 inserts was confirmed by gene-specific PCR reactions on randomly selected *E. coli* colonies. Therefore, 6.25 µL 2x TopTaq® HotStart DNA Polymerase Master Mix (Qiagen), 2.5 pM each of the tagged IL-13 primer and 1.25 µL 10x CoralLoad were added to 4.5 µL of *E. coli* colonies being resuspended in 100 µL PCR-grade water. Cycling conditions were 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, followed by a final extension step of 5 min at 72 °C using a T-Gradient thermal cycler. Colony PCR reactions were screened on 1.5% standard agarose gels and positively identified cell suspensions were inoculated into 3 mL LB/Amp^{100} medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 100 µg/mL Ampicillin) and incubated overnight at 37 °C with shaking at 250 rpm. Following overnight incubation, pJET1.2-IL-13 plasmid DNA was extracted according to the protocol of the Plasmid Mini-Prep classic kit (Zymo Research Corp.). Purified plasmids were analyzed for correct insert size by BglII digest by combining

200 ng plasmid DNA, 1 µL 10x BglII buffer (Thermo Fisher Scientific), 1 µL 10x CoralLoad (Qiagen) and 10 U of BglII (Thermo Fisher Scientific) in a total volume of 10 µL. After 1 hour incubation at 37 °C, samples were run on 1.5% standard agarose gels and positive clones were sent for bidirectional sequencing (Eurofins Genomics) for further confirmation.

For generating IL-13 expression constructs, the IL-13 insert was subcloned via EcoRI/ XbaI restrictions sites into the pFLAG-CMV2 vector. In separate reactions, the IL-13 insert was retrieved from positive pJET1.2-IL-13 plasmids and compatible pFLAG-CMV2 vector overhangs were generated by restriction digest with EcoRI and XbaI. Therefore, in a total volume of 20 µL, 500 ng plasmid DNA, 6 µL 10x Tango buffer (Thermo Fisher Scientific), 2 µL 10x CoralLoad, 10 U of EcoRI and 20 U of XbaI (both Thermo Fisher Scientific) were combined and incubated overnight at 37 °C. On the next day, samples were run on 1.5% standard agarose gels to further gel purify the EcoRI/ XbaI tagged IL-13 insert and the pFLAG-CMV2 vector by using the QIAquick Gel Cleanup Kit according to the manufacturer's instructions. Next, IL-13 inserts were ligated into the pFLAG-CMV2 vector at a vector to insert molar ratio of 1:3. Therefore, in a total volume of 20 µL, 2 µL 10x T4 DNA Ligase buffer (Thermo Fisher Scientific), 50 ng pFLAG-CMV2 vector, 19 ng of purified IL-13 insert and 10 U T4 DNA Ligase (Thermo Fisher Scientific) were combined and incubated overnight at 4 °C. Transformation of ligated pFLAG-CMV2-IL-13 expression constructs, subsequent screening and sequence confirmation of positive clones were performed as described above. To obtain sufficient DNA of the IL-13-expression constructs for the subsequent transfection experiments, 200 mL of transformed *E. coli* cell culture were grown overnight (16-21 h until OD_{600} = 2-4) and subjected to Plasmid Midiprep (PureYield™ Plasmid Midiprep System; Promega) following the manufacturer's instructions. Finally, correct orientation and intact reading-frame of pFLAG-IL-13-CMV expression constructs were performed by bidirectional sequencing being followed by generating bacterial glycerol stocks of the plasmids for long-term storage.

Prior to transfection, HEK293T cells were cultivated in T75 cell culture flasks with DMEM supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 0.1 mg/mL streptomycin (all PAN-Biotech) and 10% heat-inactivated FCS (Sigma-Aldrich). 2.8×10^6 cells were seeded and incubated at 37 °C in 5% CO_2 for 48 hours. At a confluence of 80% cells were transfected with pFLAG-CMV2-IL-13 or as control with an irrelevant porcine IgE insert in a pFLAG-CMV2 expression vector using the PolyFect® transfection reagent (Qiagen) according to the manufacturer's instructions. In brief, 15 µg of DNA (pFLAG-CMV2-IL-13 or pFLAG-IgE) were incubated with 130 µL PolyFect® reagent for 10 min at room temperature. Subsequently, 280 µL of the DNA complex mix was added to the cells and incubated for 24 h at 37 °C with 5% CO_2. After adding 5 mL pre-warmed trypsin (PAN-Biotech) for 10 min at 37 °C with 5% CO_2, cells were washed and subjected to intracellular IL-13 mRNA staining using the PrimeFlow™ RNA assay according to manufacturer's instructions (Thermo Fisher Scientific). Briefly, cells were stained with Fixable Viability Dye eFluor® 506 (Thermo Fisher Scientific) for 20 min at 4 °C. After incubations for fixation and permeabilization the chicken IL-13 target probe was added to the cells for two hours at 41 °C. Two amplification steps were followed by an incubation with the fluorescence labelled probe. Cells were subsequently analyzed by flow cytometry.

Primers used in this study

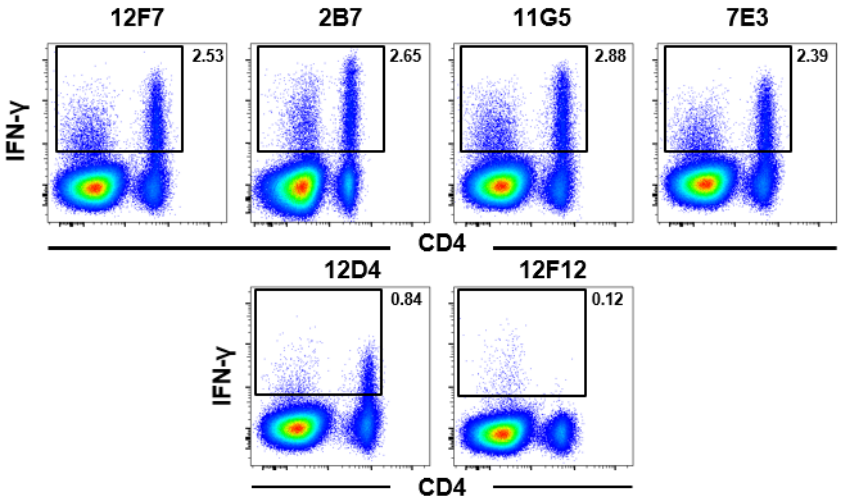
Primer name	Primer sequence (5'→ 3')	Primer position (5'→ 3')	Accession Number or web resource
EcoRI-IL-13	*GAA TTC* G**AT G**CA CCG CAC ACT GAA GGC	nt 1 to 20 (forward primer)	NM_001007085
IL-13-XbaI	*TCT AGA* **TCA** GTT TGC AGC TGT GGC CGA	nt 397 to 417 (reverse primer)	NM_001007085
pJET1.2-fw	CGA CTC ACT ATA GGG AGA GCG GC	nt 310 to 332 (forward primer)	CloneJET™ PCR Cloning Kit Manual[1]
pJET1.2-rev	AAG AAC ATC GAT TTT CCA TGG CAG	nt 428 to 405 (reverse primer)	CloneJET™ PCR Cloning Kit Manual[1]
N-CMV-30	AAT GTC GTA ATA ACC CCG CCC CGT TGA CGC		nt 825 to 854 (forward primer)E7398 (Adgene Vector Database)[2]
C-CMV-24	TAT TAG GAC AAG GCT GGT GGG CAC	nt 1080 to 1103 (reverse primer)	E7398 (Adgene Vector Database) [2]

EcoRI and XbaI restriction sites are in italics.

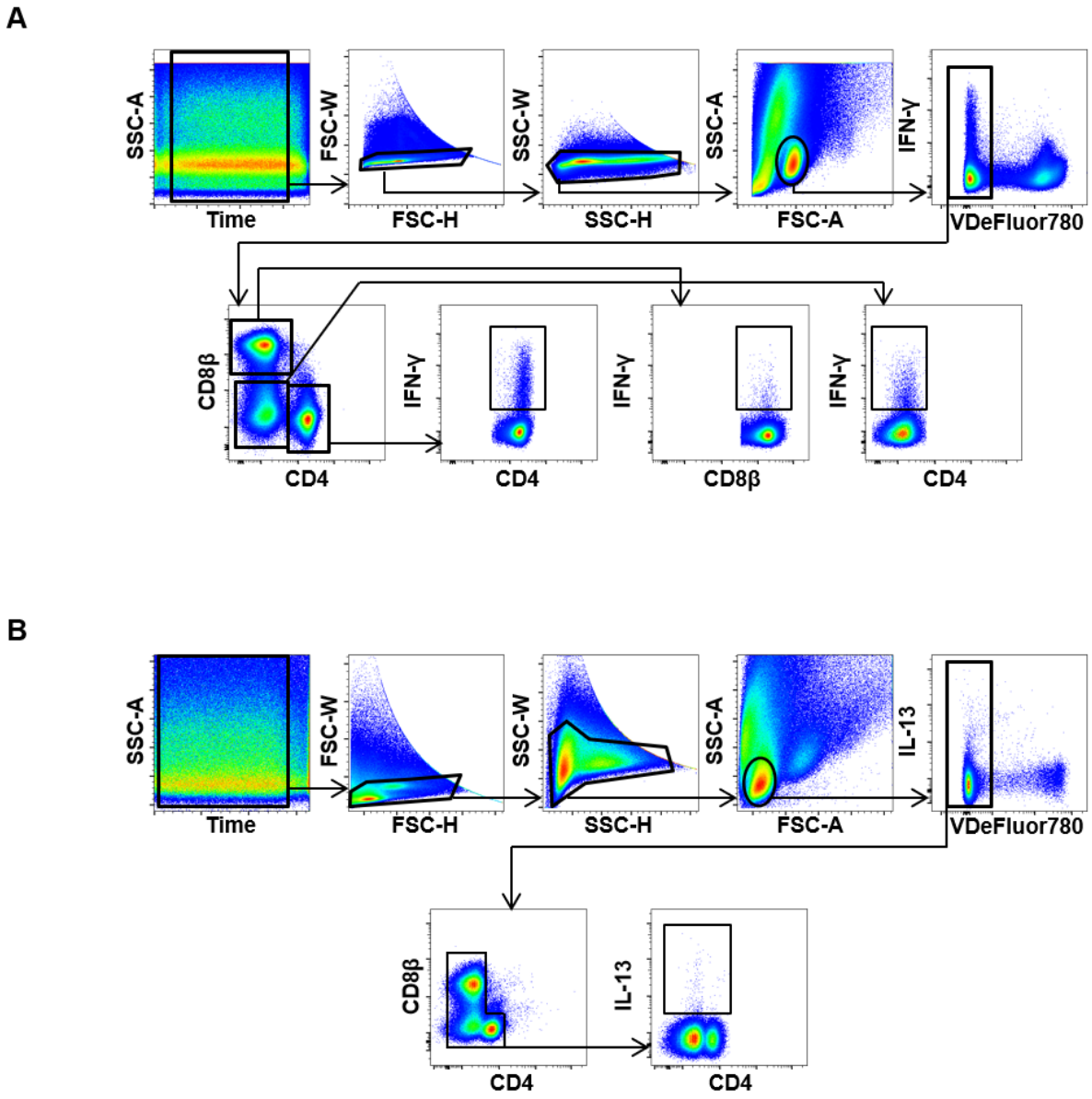
ATG start and TGA stop codons are indicated by bold letters.

[1] https://www.thermofisher.com/

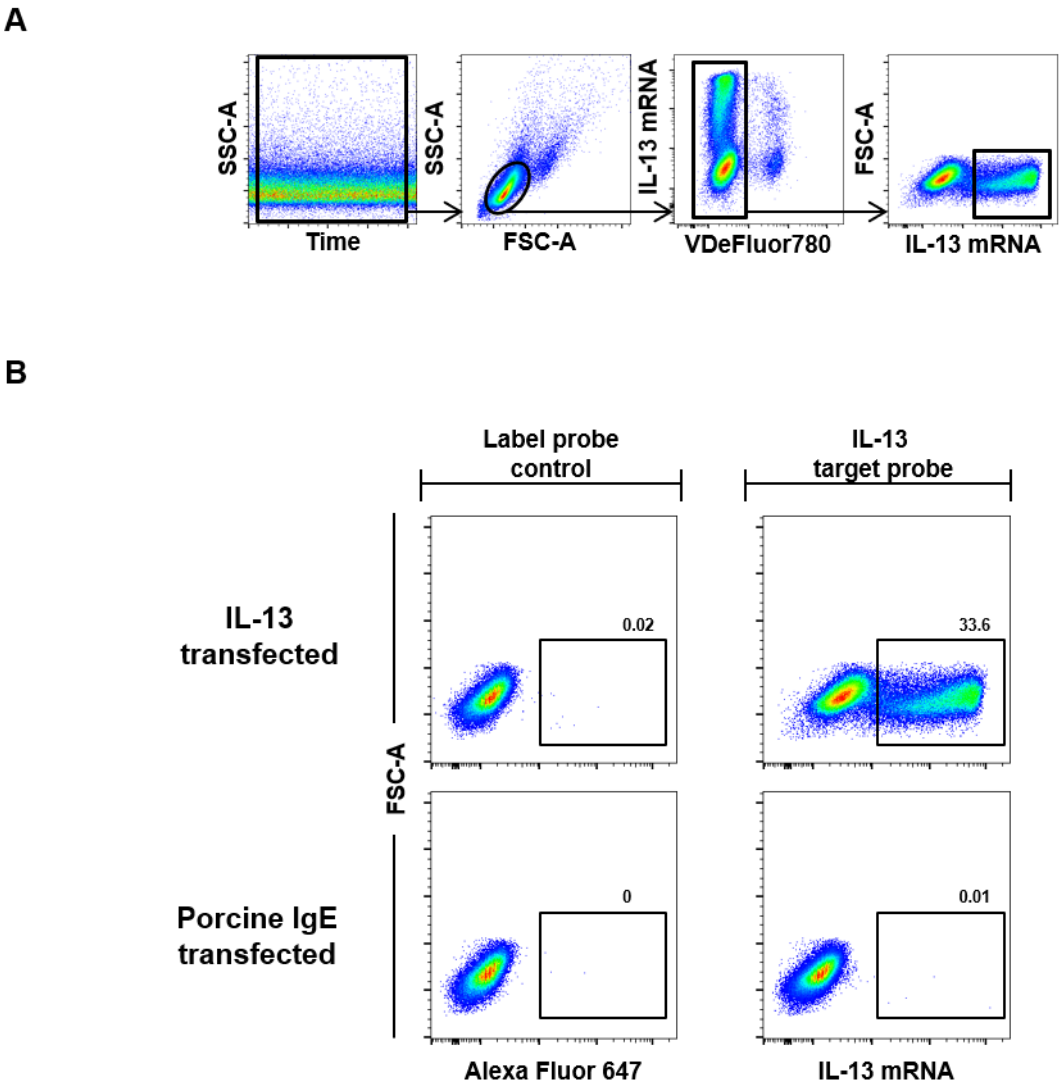
[2] https://www.addgene.org/vector-database/2770/



Additional file 3. Suitability of chicken IFN-γ-specific monoclonal antibodies specific for intracellular cytokine staining. A panel of six mAbs with either mouse IgG1 isotype (2B7, 11G5, 7E3, 12F12), or mouse IgG2a isotype (12F7) and mouse IgG2b isotype (12D4) was tested on PMA/ionomycin stimulated splenocytes. For each antibody, results are shown for the optimal quantity (clone 12F7: 150 ng, 2B7: 50 ng, 11G5: 12.5 ng, 7E3: 100 ng, 12D4: 250 ng, 12F12: 100 ng), initially identified in experiments with serial dilutions. Goat-anti-mouse isotype specific RPE-conjugated antibodies were applied afterwards for fluorescence labelling. Cells were pre-gated as described in Additional file 4A. Results are representative of four experiments with splenocytes from three different chickens.



Additional file 4. Gating strategy for lymphocytes from spleen and liver in multicolor flow cytometry. For lymphocytes subjected to intracellular IFN-γ staining (A) and PrimeFlow™ RNA Assay (Thermo Fisher Scientific) staining for IL-13 mRNA (B) a time gate as well as FSC-H/FSC-W and SSC-H/SSC-W doublet discrimination gates were applied consecutively. Lymphocytes were then selected within a FSC-A/SSC-A plot followed by a dead cell exclusion gate using the Fixable Viability Dye eFluor® 780. (A) Frequencies of IFN-γ$^+$ cells within CD4$^+$, CD8β$^+$ and CD4$^-$CD8β$^-$ subgates were determined. (B) Percentages of IL-13 mRNA$^+$ cells were determined within total live lymphocytes after excluding cells stained with putative dye aggregates in the CD4/CD8β plot. The gating strategy is shown for splenocytes from representative experiments and was applied for both organs from all birds.



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Additional file 6. Frequencies of cytokine-producing lymphocyte subsets for all investigated organs and stimulation variants.

Spleen

% of **IFN-γ$^+$** cells within total live **CD4$^+$** subset

		Animal Nr.	Medium	PMA/ Ionomycin	*H. meleagridis*	*E. coli*	*E. coli* corrected	*H. meleagridis* 10^{-1} fold lower concentration	*E. coli* 10^{-1} fold lower concentration	*E. coli* corrected 10^{-1} fold lower concentration
First necropsy week	Control	1	0,021	0,560	0,490	0,510	0,000	0,110	0,360	0,000
		2	0,011	0,850	0,150	0,150	0,000	0,059	0,110	0,000
		3	0,260	1,220	0,340	0,360	0,000	0,220	0,400	0,000
	Infected	7	0,061	2,440	0,740	0,600	0,140	0,310	0,320	0,000
		8	0,036	1,730	0,850	0,590	0,260	0,210	0,280	0,000
		9	0,270	6,280	1,100	0,580	0,520	1,100	0,550	0,550
Second necropsy week	Control	4	0,016	0,540	0,045	0,058	0,000	0,019	0,032	0,000
		5	0,034	0,890	0,230	0,220	0,010	0,075	0,120	0,000
		6	0,008	0,850	0,310	0,470	0,000	0,300	0,240	0,060
	Infected	10	0,067	2,830	0,620	0,400	0,220	0,340	0,260	0,080
		11	0,061	2,610	0,960	0,540	0,420	0,530	0,260	0,270
		12	0,038	3,150	3,320	3,200	0,120	2,950	2,140	0,810

Liver

% of **IFN-γ$^+$** cells within total live **CD4$^+$** subset

		Animal Nr.	Medium	PMA/ Ionomycin	*H. meleagridis*	*E. coli*	*E. coli* corrected
First necropsy week	Control	1	0,043	0,100	0,029	0,024	0,005
		2	0,012	0,019	0,006	0,010	0,000
		3	0,130	0,200	0,095	0,170	0,000
	Infected	7	0,082	0,380	0,065	0,033	0,032
		8	0,039	0,160	0,030	0,013	0,017
		9	0,270	2,950	0,460	0,140	0,320
Second necropsy week	Control	4	0,160	0,220	0,067	0,027	0,040
		5	0,120	0,190	0,120	0,039	0,081
		6	0,011	0,033	0,027	0,025	0,002
	Infected	10	0,080	0,300	0,057	0,031	0,026
		11	0,200	0,120	0,099	0,072	0,027
		12	0,066	1,030	0,090	0,031	0,059

Spleen

% of **IL-13 mRNA$^+$** cells within total live lymphocytes

		Animal Nr.	Medium	PMA/ Ionomycin	*H. meleagridis*	*E. coli*	*E. coli* corrected
First necropsy week	Control	1	0,035	0,062	0,034	0,043	0,000
		2	0,018	0,031	0,021	0,034	0,000
		3	0,033	0,100	0,051	0,072	0,000
	Infected	7	0,029	0,049	0,039	0,046	0,000
		8	0,023	0,062	0,028	0,060	0,000
		9	0,032	0,087	0,079	0,065	0,014
Second necropsy week	Control	4	0,023	0,051	0,036	0,033	0,003
		5	0,023	0,037	0,025	0,044	0,000
		6	0,030	0,067	0,090	0,071	0,019
	Infected	10	0,032	0,049	0,038	0,042	0,000
		11	0,021	0,047	0,055	0,046	0,009
		12	0,042	0,071	0,060	0,058	0,002

Spleen

% of **IFN-γ$^+$** cells within total live **CD8β$^+$** subset

		Animal Nr.	Medium	PMA/ Ionomycin	*H. meleagridis*	*E. coli*	*E. coli* corrected
First necropsy week	Control	1	0,047	0,063	0,067	0,083	0,000
		2	0,037	0,072	0,035	0,038	0,000
		3	0,270	0,290	0,230	0,260	0,000
	Infected	7	0,064	0,100	0,068	0,052	0,016
		8	0,046	0,110	0,097	0,047	0,050
		9	0,240	0,260	0,250	0,220	0,030
Second necropsy week	Control	4	0,055	0,081	0,043	0,042	0,001
		5	0,063	0,110	0,063	0,048	0,015
		6	0,037	0,037	0,045	0,063	0,000
	Infected	10	0,100	0,150	0,094	0,110	0,000
		11	0,051	0,140	0,065	0,049	0,016
		12	0,100	0,350	0,130	0,170	0,000

Spleen

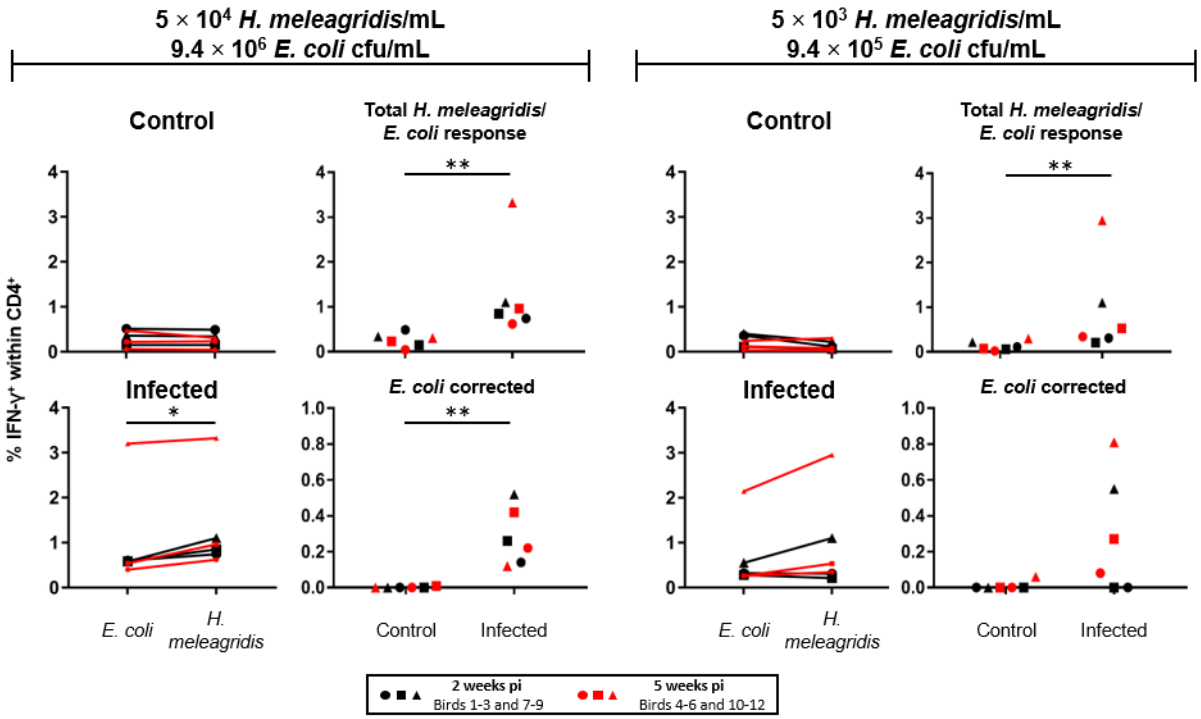
% of **IFN-γ$^+$** cells within total live **CD4$^+$CD8β$^-$** subset

		Animal Nr.	Medium	PMA/ Ionomycin	*H. meleagridis*	*E. coli*	*E. coli* corrected
First necropsy week	Control	1	0,076	0,280	0,630	1,150	0,000
		2	0,086	0,440	0,340	0,470	0,000
		3	0,260	0,460	0,400	0,640	0,000
	Infected	7	0,160	1,080	0,640	0,590	0,050
		8	0,110	0,940	1,150	0,840	0,310
		9	0,210	0,740	0,750	0,530	0,220
Second necropsy week	Control	4	0,047	0,300	0,140	0,210	0,000
		5	0,140	0,260	0,270	0,310	0,000
		6	0,029	0,160	0,210	0,370	0,000
	Infected	10	0,065	0,420	0,680	0,690	0,000
		11	0,069	0,490	1,080	0,700	0,380
		12	0,068	0,960	0,660	0,770	0,000

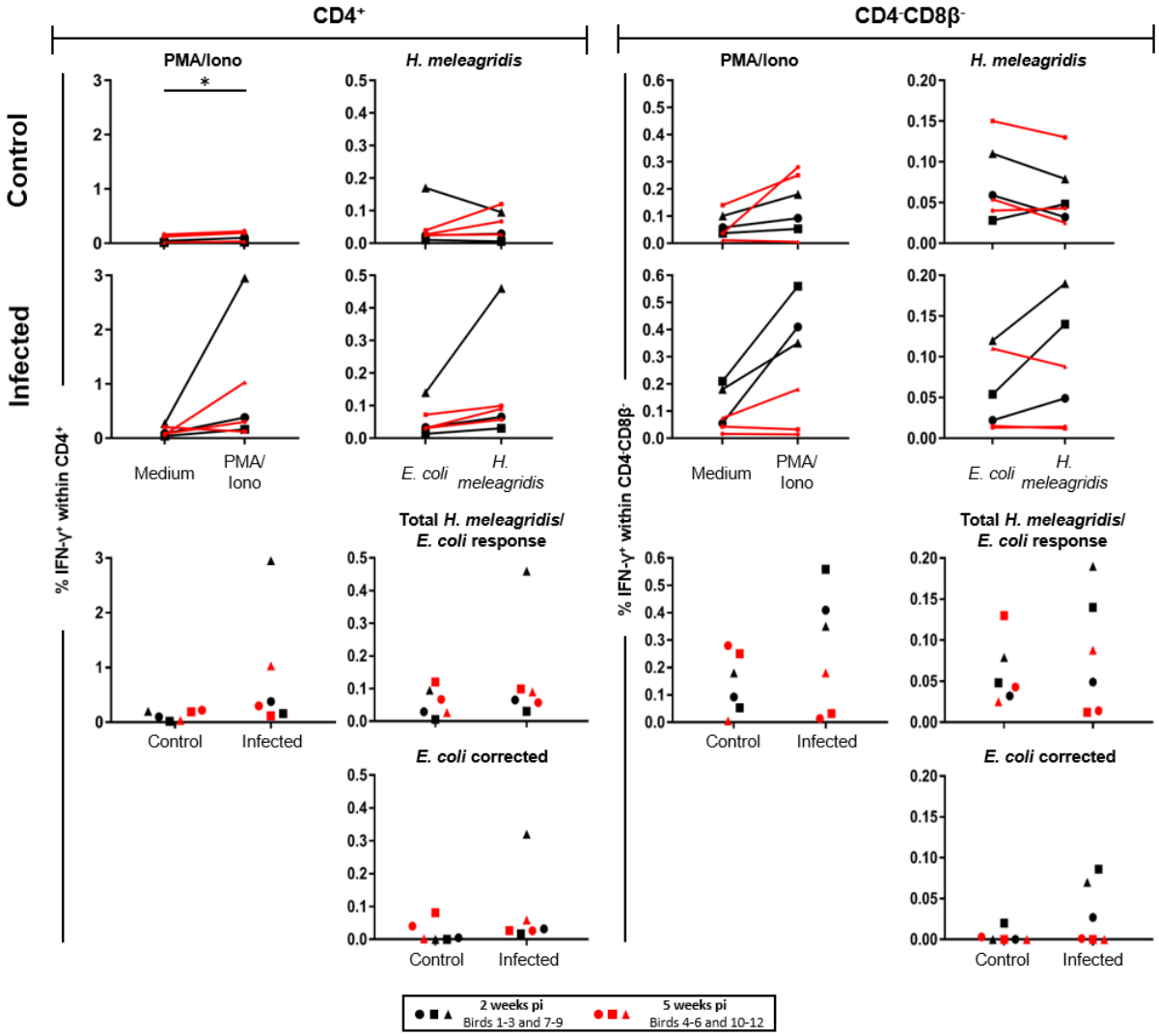
Liver

% of **IFN-γ$^+$** cells within total live **CD4$^+$CD8β$^-$** subset

		Animal Nr.	Medium	PMA/ Ionomycin	*H. meleagridis*	*E. coli*	*E. coli* corrected
First necropsy week	Control	1	0,058	0,092	0,032	0,059	0,000
		2	0,037	0,053	0,048	0,028	0,020
		3	0,100	0,180	0,079	0,110	0,000
	Infected	7	0,055	0,410	0,049	0,022	0,027
		8	0,210	0,560	0,140	0,054	0,086
		9	0,180	0,350	0,190	0,120	0,070
Second necropsy week	Control	4	0,036	0,280	0,043	0,040	0,003
		5	0,140	0,250	0,130	0,150	0,000
		6	0,011	0,005	0,025	0,054	0,000
	Infected	10	0,016	0,014	0,014	0,013	0,001
		11	0,043	0,032	0,012	0,015	0,000
		12	0,074	0,180	0,088	0,110	0,000



Additional file 7. Influence of different *H. meleagridis* concentrations on the frequency of IFN-γ-producing $CD4^+$ splenocytes. Intracellular cytokine staining for IFN-γ was performed following 18 h antigen specific re-stimulation either with *H. meleagridis* at 5×10^4/mL and *E. coli* (9.4×10^6 CFU/mL) or a 10-fold lower concentration of *H. meleagridis* (5×10^3/mL) and *E. coli* (9.4×10^5 CFU/mL). Plots on the left of each stimulation variant compare frequencies of IFN-γ-producing $CD4^+$ cells after combined *H. meleagridis*/*E. coli* stimulation or stimulation only with *E. coli* in infected and control chickens. Plots on the right compare frequencies of IFN-γ-producing $CD4^+$ cells between infected and control chickens after stimulation with *H. meleagridis*/*E. coli* antigen with or without correction for the response against *E. coli* alone. Each symbol represents one bird, black and red colored symbols show birds sacrificed 2 weeks pi and 5 weeks pi, respectively, as percent of total $CD4^+$ splenocytes. Asterisks indicate different *p*-values: $*p \leq 0.05$, and $**p \leq 0.01$.



Additional file 8. Frequencies of IFN-γ-producing $CD4^+$ and $CD4^-CD8\beta^-$ cells in the liver. The upper panel shows frequencies of IFN-γ-producing $CD4^+$ and $CD4^-CD8\beta^-$ cells after PMA/ionomycin or *H. meleagridis*/*E. coli* stimulation compared to medium or *E. coli*-only stimulation in control and infected birds. The lower panel compares frequencies of IFN-γ-producing $CD4^+$ and $CD4^-CD8\beta^-$ cells after stimulation with PMA/ionomycin, *H. meleagridis*/*E. coli* or after correction for *E. coli* between infected and control birds. Each symbol represents one bird, black and red colored symbols show birds sacrificed 2 weeks pi and 5 weeks pi, respectively, as percent of total $CD4^+$ or $CD4^-CD8\beta^-$ intrahepatic lymphocytes. Asterisks indicate *p*-value: $*p \leq 0.05$.

Additional file 9. Summary of significant differences between cytokine-producing lymphocyte subsets isolated from *H. meleagridis* infected birds compared to control birds (corrected values for *H. meleagridis* / *E. coli;* Asterisks indicate *p*-value levels (*$p \leq 0.05$, and **$p \leq 0.01$)).

		Spleen			Liver		
		$CD4^+$	$CD8\beta^+$	$CD4^-$ $CD8\beta^-$	$CD4^+$	$CD8\beta^+$	$CD4^-$ $CD8\beta^-$
IFN-γ	PMA/Iono	**	-	**	-	-	-
	H. meleagridis / *E. coli*	**	-	-	-	-	-
		Total lymphocytes					
IL-13	PMA/Iono		-				
	H. meleagridis / *E. coli*		-				

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Full Length Article

Comparative investigation of IFN-γ-producing T cells in chickens and turkeys following vaccination and infection with the extracellular parasite *Histomonas meleagridis*

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ABSTRACT

The re-emerging disease histomonosis is caused by the protozoan parasite *Histomonas meleagridis* that affects chickens and turkeys. Previously, protection by vaccination with *in vitro* attenuated *H. meleagridis* has been demonstrated and an involvement of T cells, potentially by IFN-γ production, was hypothesized. However, comparative studies between chickens and turkeys on *H. meleagridis*-specific T cells were not conducted yet. This work investigated IFN-γ production within $CD4^+$, $CD8\alpha^+$ and $TCR\gamma\delta^+$ (chicken) or $CD3\varepsilon^+CD4^-CD8\alpha^-$ (turkey) T cells of spleen and liver from vaccinated and/or infected birds using clonal cultures of a monoxenic *H. meleagridis* strain. In infected chickens, re-stimulated splenocytes showed a significant increase of IFN-γ^+CD4^+ T cells. Contrariwise, significant increments of IFN-γ-producing cells within all major T-cell subsets of the spleen and liver were found for vaccinated/infected turkeys. This indicates that the vaccine in turkeys causes more intense systemic immune responses whereas in chickens protection might be mainly driven by local immunity.

1. Introduction

The parasitic disease histomonosis (syn. histomoniasis, blackhead, infectious enterohepatitis) is caused by the extracellular protozoan pathogen *Histomonas meleagridis* (Tyzzer, 1920). Gallinaceous birds such as chickens (*Gallus gallus*) and turkeys (*Meleagris gallopavo*) are mostly affected (Hess and McDougald, 2020). As a result of banning highly effective therapeutics, histomonosis can be considered as a re-emerging disease which leads to an increase of outbreaks worldwide (Hess et al., 2015; Clark and Kimminau, 2017; Liebhart et al., 2017). Flagellated histomonads can be found in the cecal lumen and non-flagellated within tissues, mainly the cecal mucosa and liver. In addition, cyst-like stages were described to be more resistant (Tyzzer, 1920; Munsch et al., 2009; Zaragatzki et al., 2010).

Comparing chickens and turkeys histomonosis can cause an eminently different clinical manifestation. Chickens usually display an inapparent progression showing no or only mild clinical signs (Tyzzer, 1934). However, it is of economic concern in layers since the infection with the parasite leads to a reduced weight gain and a drop in egg production (Liebhart and Hess, 2020). Pathological lesions are predominantly restricted to the ceca but may also progress to severe mucosal destruction including thickening and ulceration. An important feature in chickens is the often noticed colibacillosis (Paudel et al., 2018). In turkeys, an infection with *H. meleagridis* is much more severe and often fatal with mortality rates up to 100%. Infected turkeys can exhibit distinct clinical signs including listlessness, ruffled feathers, dropped wings and sulphur-colored diarrhea (Tyzzer, 1934). This is caused by histomonads invading the cecal mucosa with later migration via the portal vein to the liver causing round, often profound, lesions (Tyzzer, 1920).

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To prevent histomonosis new control strategies are currently under investigation considering limitations of new drug developments (Regmi et al., 2016). Previous studies on inactivated vaccines or passive immunization by transferring antibodies did not show a protective effect in turkeys (Clarkson, 1963; Hess et al., 2008; Bleyen et al., 2009). However, it could be demonstrated that vaccination with attenuated histomonads prevents mortality in turkeys and reduced a drop in egg production of layer chickens following challenge (Hess et al., 2008; Liebhart et al., 2011; Pham et al., 2013). In addition, applying an *in vitro* attenuated clonal culture of the parasite, neither a negative effect on the birds' performance nor a reversion of histomonads to virulence was found (Liebhart et al., 2010; Sulejmanovic et al., 2013).

Comparative studies on the cellular immune response following a *H. meleagridis* infection in chickens and turkeys are sparse and mainly focused on gene expression analyses of cytokines and *in situ* hybridization to detect cytokine transcripts. In infected chickens, Powell et al. (2009) demonstrated an early rise of pro-inflammatory cytokines including IFN-γ mRNA in the cecal tonsils and liver, which decreased at later stages of infection. In the same study, increasing IL-13 mRNA levels were also shown in both organs and remained elevated until the end of the experiment. The authors suggested that this rapid innate immune response limits the severity of the disease in chicken. A study on IFN-γ mRNA$^+$ and IL-13 mRNA$^+$ cell detection by *in situ* hybridization revealed only minor changes in infected, vaccinated and vaccinated/infected chickens (Kidane et al., 2018). On the contrary, infected turkeys failed to mount an early innate immune response but showed cytokine mRNA to increase at later time points, which might explain the spread to the liver and a more severe progression compared to chickens (Powell et al., 2009). In addition, a comparison of cecal IFN-γ mRNA$^+$ cells in chickens and turkeys suggested that a rise of such cells in vaccinated/infected turkeys correlates with protection (Kidane et al., 2018).

Recently, investigations on the cytokine production of T cells after *in vitro* re-stimulation revealed significantly elevated levels of *H. meleagridis* antigen-specific IFN-γ-producing CD4$^+$ and non-CD4$^+$ splenocytes in infected chickens (Lagler et al., 2019). However, the T-cell response in vaccinated and vaccinated/infected chickens was not addressed. Also, the T-cell immune response as such in turkeys, following *H. meleagridis* vaccination and/or infection, remains largely unexplored. Hence, in the actual study we report the establishment of an intracellular cytokine staining assay for IFN-γ in turkeys. This tool was applied in a vaccination as well as a challenge infection experiment using clonal cultures of a monoxenic *H. meleagridis* strain. Frequencies of *H. meleagridis* re-stimulated IFN-γ-producing CD4$^+$, CD8α$^+$ and non-CD4$^+$/CD8α$^+$ (in chickens γδ T cells) T cells from spleen and liver of both species were evaluated. Our results confirmed that *H. meleagridis* leads to a significant IFN-γ production in CD4$^+$ T cells isolated from spleens of infected chickens (Lagler et al., 2019). Most important, it could be shown that in vaccinated/infected turkeys IFN-γ-producing cells were induced in CD4$^+$, CD8α$^+$ and non-CD4$^+$CD8α$^+$ T-cell subsets from spleen and liver, suggesting a role of those lymphocytes for protection.

2. Materials and methods

2.1. Birds

For chickens, incubation and hatching of embryonated specific pathogen free (SPF) layer eggs (VALO, BioMedia, GmbH, Osterholz-

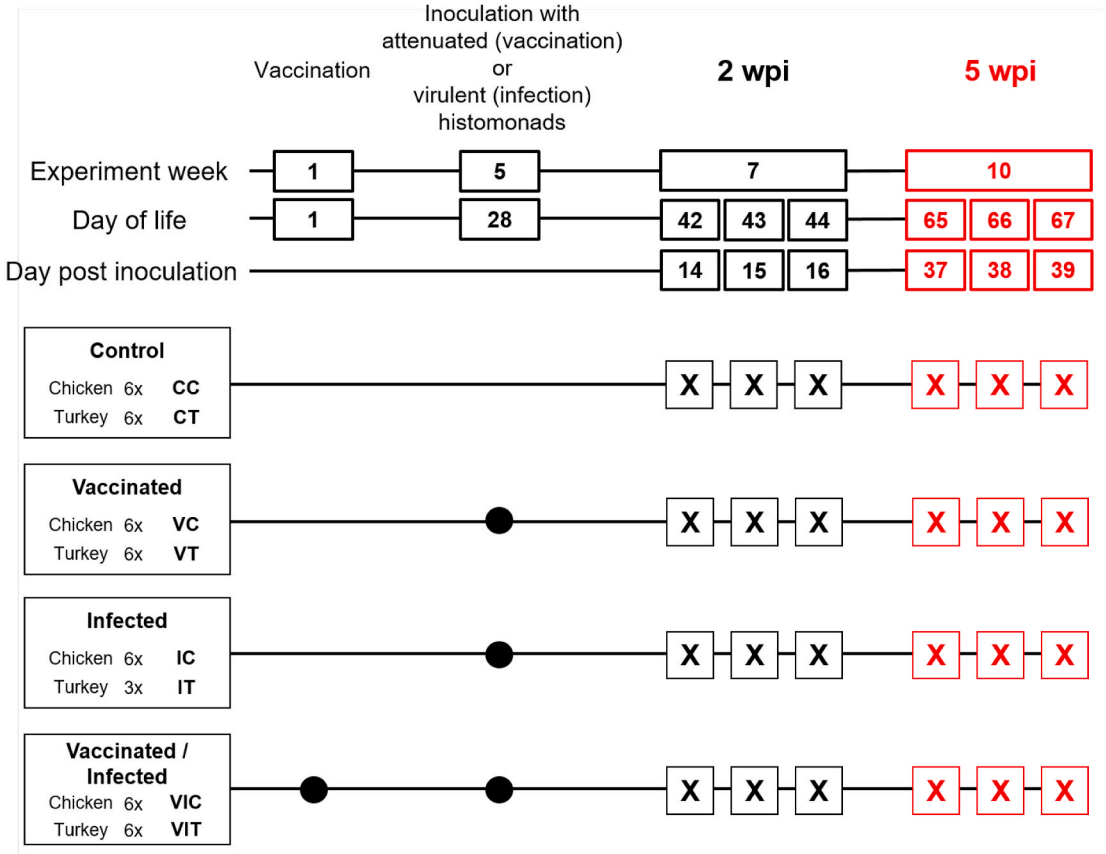

Fig. 1. Design of the animal trial. Six birds ($n = 6$) of the control groups (chicken: CC, turkey: CT), vaccinated groups (chicken: VC, turkey: VT), infected chicken group (IC), vaccinated/infected groups (chicken: VIC, turkey: VIT) and three infected turkeys ($n = 3$; IT) were kept in separate rooms from their first day of life. Black dot symbolizes day of inoculation with *H. meleagridis* of the respective groups. Three birds from each group were euthanized (X symbol) for organ collection on three consecutive days two wpi and five wpi, respectively.

Scharmbeck, Germany) was carried out at the Clinic for Poultry and Fish Medicine, University of Veterinary Medicine Vienna, Austria. After hatch, 24 chicks were divided into four groups of six birds each (Fig. 1). For turkeys, 1-day-old commercial Hybrid Converter poults (Hendrix Genetics, Boxmeer, The Netherlands) were separated into four groups of three (infected group) or six (control, vaccinated, vaccinated/infected group) birds each. All pens were supplied with wood shavings and filtered air under negative pressure. Feed and water were provided *ad libitum*. The trial was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26 ft of Animal Experiments Act, Tierversuchsgesetz 2012 -TVG 2012 (license number 68.205/0161-WF/V/3b/2017).

2.2. Preparation of cultures for inoculation

The clonal culture *H. meleagridis*/Turkey/Austria/2922-C6/04 was used for infection (maximum 33 passages) or vaccination (minimum 301 passages). The histomonads were co-cultivated with the bacterial strain *E. coli* DH5α as supplement for propagation of the parasite (Ganas et al., 2012). All preparations were cultivated in Medium 199 with Earle's salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco™, Thermo Fisher Scientific, Walham, MA, USA), 15% fetal calf serum (FCS) (Gibco™, Thermo Fisher Scientific) and 0.25% sterilized rice starch (Carl Roth, Karlsruhe, Germany). Viable *H. meleagridis* cells were stained and counted using trypan blue and a Neubauer hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) to calculate the required cell numbers for preparation of the infection and vaccination inoculum. Colony-forming units (CFUs) of *E. coli* DH5α from serial dilutions on Coliform agar plates after an incubation at 37 °C for 24 h were counted for preparation of the *E. coli* DH5α control inoculum.

2.3. Inoculation

On the first day of life chickens ($n = 6$ per group) and turkeys ($n = 6$ for all groups except $n = 3$ for the infected group) were separated into eight groups and placed in different rooms (Fig. 1). A subcutaneous tag was applied to every bird for identification (Swiftach®, Avery Dennison, Glendale, CA, USA). Subsequently, chickens and turkeys from the vaccinated/infected group (VIC and VIT, respectively) were vaccinated with 6×10^5 cells of attenuated *H. meleagridis* (chicken: passage 303, turkey: passage 302) co-cultured with 1×10^7 CFU (chicken) or 1.2×10^8 CFU (turkey) of *E. coli* DH5α. Control animals were inoculated with an *E. coli* DH5α only preparation containing a bacterial concentration close to the infection and vaccination inoculum. Therefore, animals from the control group were inoculated with 6.2×10^6 CFU (chickens) or 7.2×10^6 CFU (turkeys) of *E. coli* DH5α (CC and CT, respectively). Four weeks later all birds from the vaccinated/infected and only infected groups (IC and IT) were infected with 6×10^5 cells of virulent *H. meleagridis* (chicken: passage 33, turkey: passage 28) co-cultured with 2.4×10^7 CFU (chickens) or 9.9×10^7 CFU (turkeys) of *E. coli* DH5α. Birds from the vaccinated groups (VC and VT) were vaccinated with 6×10^5 cells of attenuated *H. meleagridis* (chicken: passage 311, turkey: passage 301) co-cultured with 2.2×10^6 CFU (chickens) or 1.2×10^8 CFU (turkeys) of *E. coli* DH5α. Control chickens were inoculated with 6.5×10^6 CFU and control turkeys with 1×10^7 CFU of *E. coli* DH5α. All inocula were administered in a total volume of 600 μL cultivation medium (composition of medium see above in section 2.2.) per bird, split in equal amounts of 300 μL for oral and cloacal application using a syringe with crop tube or a conventional 1 mL pipette (Eppendorf AG, Hamburg, Germany), respectively. Following inoculation, the birds were kept feed restricted for 4 h. Three birds of each group were sacrificed two weeks post inoculation (wpi) (14 days post inoculation (dpi), 15 dpi, 16 dpi) and five wpi (35 dpi, 36 dpi, 37 dpi) (Fig. 1).

2.4. Clinical examination, necropsy and sampling

Birds from all groups were examined daily for clinical signs. From birds of the vaccinated/infected and control group, blood and cloacal samples were collected once and three times per week, respectively, during the first four weeks of life. From four weeks of life onwards, all birds were sampled for blood and cloacal swabs in the mentioned setup. To investigate the parasite shedding, cloacal swabs were transferred to 2 mL microtubes (Eppendorf AG) containing 1.5 mL cultivation medium (composition of medium described above in section 2.2.). After two days of incubation at 40 °C, the cultures were screened for viable *H. meleagridis* cells under light microscopy. To obtain serum, blood samples were kept overnight at 4 °C followed by centrifugation at $3300 \times g$ for 12 min and stored until further processing at −20 °C. Euthanasia was performed by intravenously administered thiopental (medicamentum pharma GmbH, Allerheiligen im Mürztal, Austria) and subsequent bleeding to death. During the ensuing necropsy, the lesion scores (LS) of cecum and liver were determined. For this, an already established scoring system of pathological changes in cecum and liver was applied (Windisch and Hess, 2010; Zahoor et al., 2011): LS 0 represents no lesion whereas LS 1 to 4 indicates mild to severe pathological changes. For detecting *H. meleagridis* by immunohistochemistry (IHC) (see section 2.5.), tissue samples from cecum and liver were preserved in formalin. For isolation of mononuclear cells (see section 2.7.), spleens and livers were placed in ice-cold phosphate buffered saline (PBS) + 2% FCS (both Gibco™, Thermo Fisher Scientific) filled beakers.

2.5. Detection of H. meleagridis by ELISA and immunohistochemistry

H. meleagridis-specific antibodies in sera were measured by an indirect sandwich ELISA following a protocol previously established by Windisch and Hess (2009). Briefly, ELISA plates were coated with a 1:10 000 diluted rabbit anti-*Histomonas* serum. To avoid unspecific binding a blocking buffer (Thermo Fisher Scientific) followed by the histomonas antigen was added. Subsequently, chicken and turkey sera were pipetted into the plates. Final incubation steps included goat anti-chicken IgG-horseradish peroxidase (Southern Biotech, Birmingham, AL, USA) and tetramethylbenzidine substrate solution (Calbiochem, Merck KGaA, Darmstadt, Germany) as well as sulphuric acid for stopping the reaction. On the basis of optical densities (OD) measured at a wavelength of 450 nm, OD-values above 0.54 (chicken) and 0.36 (turkey) were considered as positive (+).

Direct detection of *H. meleagridis* in ceca and livers was performed by IHC (Singh et al., 2008). In brief, tissue samples were fixed in formalin, dehydrated and embedded in paraffin. Using a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany), 3 μm sized pieces were cut. After transferring to glass slides (Superfrost plus, Menzel-Gläser, Braunschweig, Germany), samples were dewaxed and rehydrated. An overnight incubation step with a purified polyclonal anti-histomonad rabbit antibody at 4 °C was applied. Following a washing step with PBS (Gibco™, Thermo Fisher Scientific), a biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) was added. To visualize the parasite, the Vectastain ABC Kit and DAB Substrate Kit (Vector Laboratories) were applied and Haematoxylin (Merck KGaA) was used to counterstain the surrounding tissue.

2.6. Preparation of H. meleagridis and E. coli antigen stocks for in vitro re-stimulation

Three antigen preparations were used for *in vitro* re-stimulation of lymphocytes: virulent *H. meleagridis* with *E. coli* (chicken: passage 33, turkey: passage 28), attenuated *H. meleagridis* with *E. coli* (chicken: passage 303, turkey: passage 302) as wells as *E. coli*-only. All antigen stocks were generated from the corresponding inoculation batches (see section 2.2. above used for cultivation) according to a protocol established by Lagler et al. (2019). In brief, cultures used for inoculation were

centrifuged at 200×*g* for 5 min (*H. meleagridis* with *E. coli*) or at 1780×*g* for 5 min (*E. coli*-only) and washed with PBS (Gibco™, Thermo Fisher Scientific). *H. meleagridis* cell concentrations and *E. coli* CFU were determined. For re-stimulation of chicken lymphocytes, the concentration of the virulent *H. meleagridis* was 4.3×10^6/mL with 3.4×10^7 *E. coli* CFU/mL, the attenuated *H. meleagridis* was 7.1×10^6/mL with 5.7×10^6 *E. coli* CFU/mL and the *E. coli*-only preparation was 1.9×10^9 *E. coli* CFU/mL. For re-stimulation of turkey lymphocytes, stock concentrations were 2.3×10^6 *H. meleagridis*/mL with 2.7×10^7 *E. coli* CFU/mL for virulent *H. meleagridis,* 6.6×10^6 *H. meleagridis*/mL with 1×10^7 *E. coli* CFU/mL for attenuated *H. meleagridis* and 1×10^8 *E. coli* CFU/mL for the *E. coli*-only preparation. Subsequently, all antigen stocks were subjected to three freezing/thawing cycles at −80 °C. Afterwards a centrifugation step at 375×*g* for 3 min was applied to remove the remaining rice starch. The collected supernatant was frozen at −80 °C until further use in stimulation assays.

2.7. Cell isolation

Mononuclear cells from spleen and liver were isolated in petri dishes filled with ice-cold PBS +2% FCS (both Gibco™, Thermo Fisher Scientific). Splenocytes were obtained by removing the splenic capsule and tearing the tissue apart with two sterile blunt-end forceps. For isolation of mononuclear cells from livers, the tissue was dissected using the end of a 20 mL syringe plunger. Cell suspensions obtained from minced tissues were filtered through 40 μm cell strainers (BD Falcon™, BD Biosciences, San Jose, CA, USA) and centrifuged at 350×*g* for 10 min at room temperature. Following resuspension of the cell pellets in cold PBS +2% FCS, cell suspensions were layered on Histopaque®-1077 (Sigma-Aldrich). Interphases were collected after centrifugation at 850×*g* for 20 min at room temperature. After two washing steps (650×*g*, 10 min, 4 °C) and resuspension in PBS + 2% FCS, mononuclear cell suspensions were stored on ice until subsequent *in vitro* cultivation. Counting of viable cells was performed on a Cellometer® X2 fluorescent viability cell counter using the Cellometer® ViaStain™ AOPI staining solution and Cellometer® cell counting chambers (Nexcelom Bioscience LLC).

2.8. Identification of an anti-chicken IFN-γ mAb for cross-reactivity with turkey splenocytes

An alignment of the amino acid sequence of IFN-γ from chicken and turkey was performed before scrutinizing the suitability of mouse anti-chicken IFN-γ monoclonal antibodies (mAbs) for cross-reactivity to turkey IFN-γ. IFN-γ protein sequences of chicken (accession number: NP_990480) and turkey (accession number: XP_003202096) were analyzed using the BioEdit sequence alignment editor software (version 7.2.5. available at https://bioedit.software.informer.com/7.2/).

Cross-reactivity of several chicken IFN-γ specific monoclonal antibodies (IgG1: 7E12, 7E3, 2B7; IgG2a: 12F7) was investigated in intracellular cytokine staining of turkey splenocytes. For this, 5×10^5 splenocytes per well were isolated from turkeys and seeded in 96-well round-bottom microtiter plates (Greiner Bio-One, Kremsmünster, Austria) in 200 μL RPMI 1640 (PAN Biotech GmbH, Aidenbach, Germany) supplemented with stable glutamine, 10% heat inactivated FCS (Sigma-Aldrich), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (PAN Biotech GmbH). Following overnight incubation at 41 °C and 5% CO_2, PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) in the presence of Brefeldin A (1 μg/mL; BD GolgiPlug™, BD Biosciences) or Brefeldin A only as control was added for additional 4 h. After two washing steps with PBS (470×*g* for 4 min at 4 °C; also used for all subsequent washing steps), lymphocytes were stained with a mAb for CD4 (clone: 2–35, mouse IgG2b, Bio-Rad Laboratories, Hercules, CA, USA) and a biotinylated mAb for CD8α (clone: 3–298, mouse IgG2b, Southern Biotech) which were added for 20 min at 4 °C. Cells were washed with PBS prior to the addition of Streptavidin eFluor™ 450 (Thermo Fisher Scientific) as well as the Fixable Viability Dye eFluor® 780, (Thermo Fisher Scientific) (20 min, 4 °C). PBS +2% FCS was used for washing and followed by fixation/permeabilization of cells using the BD Cytofix/Cytoperm™ (BD Biosciences) kit according to manufacturer's instructions. Accordingly, cells were stained with the above-mentioned mAbs specific for IFN-γ in serial dilutions (50 ng–3 ng) and incubated overnight at 4 °C. Before the isotype specific secondary antibody (goat anti-mouse IgG1-RPE or goat anti-mouse IgG2a-RPE; Southern Biotech, 30 min, 4 °C) was added, cells were washed twice with the BD Perm/Wash™ Buffer (BD Biosciences). The most promising mAb candidate 7E12 was tested further in a dilution range of 6 ng–0.75 ng. This labelling was also combined with a rat anti-human CD3ε-Alexa Fluor® 647 mAb (cone: CD3-12, isotype: IgG1, Bio-Rad) for 30 min at 4 °C. To block free binding sites of the secondary antibody, ChromePure mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added prior to the anti-CD3ε mAb. After two washes, cells were resuspended in 200 μL BD Perm/Wash™ Buffer (BD Biosciences) and analyzed by flow cytometry (FCM) using 5 mL tubes.

2.9. In vitro stimulation of cells and intracellular cytokine staining

Splenocytes and mononuclear cells isolated from liver were cultivated in 96-well round-bottom microtiter plates (Greiner Bio-One). Per FCM sample six wells (chicken) and twelve wells (turkey) of 5×10^5 cells/well in 100 μL RPMI 1640 (PAN Biotech GmbH) supplemented with stable glutamine, 10% heat inactivated FCS (Gibco™, Thermo Fisher Scientific), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (PAN Biotech GmbH) were used. For *in vitro* re-stimulation, 100 μL of either virulent *H. meleagridis* (5×10^4/mL) with *E. coli* (chicken: 4×10^5 CFU/mL, turkey: 5.9×10^5 CFU/mL), attenuated *H. meleagridis* (5×10^4/mL) with *E. coli* (chicken: 4×10^4 CFU/mL, turkey: 7.6×10^4 CFU/mL), *E. coli*-only (chicken: 4×10^5 CFU/mL, turkey: 5.9×10^5 CFU/mL) or culture medium as a negative control was added to the cells. For the *E. coli*-only stimulation, a concentration, which was equivalent to the higher *E. coli* concentration of the corresponding *H. meleagridis* with *E. coli* preparations, was chosen. Cell cultures were incubated overnight in a humidified incubator at 41 °C and 5% CO_2. In addition, to inhibit cytokine secretion Brefeldin A (BD GolgiPlug™, BD Biosciences) at a concentration of 1 μg/mL was added for the final 4 h of incubation.

Following *in vitro* stimulation, cells were harvested and transferred for FCM staining to round-bottom 96-well plates (Greiner Bio-One) and washed twice with PBS + 2% FCS at 470×*g* for 4 min at 4 °C (used for all following washing steps). For chicken cells, the surface staining panel included mouse anti-chicken CD4-FITC (clone: 2–35, isotype: IgG2b, Bio-Rad Laboratories), mouse anti-chicken CD8α-APC (clone: CT-8, isotype: IgG1, Southern Biotech) and biotinylated TCRγδ (clone: TCR-1, isotype: IgG1, Southern Biotech) mAbs which were incubated for 20 min at 4 °C. In a secondary staining step Streptavidin eFluor™ 450 (Thermo Fisher Scientific) as well as Fixable Viability Dye eFluor® 780, (Thermo Fisher Scientific) was applied (20 min, 4 °C). For subsequent intracellular cytokine staining, cells were fixated and permeabilized with the BD Cytofix/Cytoperm™ (BD Biosciences) kit. For detection of IFN-γ, cells were incubated overnight at 4 °C with a mouse anti-chicken mAb (clone: 12F7, isotype: IgG2a). Subsequently, cells were washed with Perm/Wash™ Buffer (BD Biosciences) and stained with goat anti-mouse IgG2a-RPE (Southern Biotech) for 30 min at 4 °C. For lymphocytes isolated from turkeys, cells were stained on the surface with mouse anti-chicken CD4-FITC (clone: 2–35, isotype: IgG2b, Bio-Rad Laboratories) and biotinylated mouse anti-chicken CD8α (clone: 3–298, isotype: IgG2b, Southern Biotech) mAbs which were added for 20 min at 4 °C. The secondary staining step included Streptavidin eFluor™ 450 (Thermo Fisher Scientific) as well as Fixable Viability Dye eFluor® 780, (Thermo Fisher Scientific) (20 min, 4 °C). For subsequent intracellular staining, cells were fixated and permeabilized with the BD Cytofix/Cytoperm™ (BD Biosciences) kit. Detection of IFN-γ was achieved by incubating cells with a mouse anti-chicken mAb (clone: 7E12, isotype: IgG1) and incubated overnight at 4 °C. After washing with the Perm/

Wash™ Buffer (BD Biosciences), an incubation with goat anti-mouse IgG1-RPE (Southern Biotech) was performed for 30 min at 4 °C. Following two washing steps and a blocking step with ChromePure mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) (20 min, 4 °C) cells were stained with a rat anti-human CD3ε-Alexa Fluor® 647 mAb (clone: CD3-12, isotype: IgG1, Bio-Rad) (30 min, 4 °C). Cells were resuspended in 230 µL (chicken) or 150 µL (turkey) Perm/Wash™ Buffer (BD Biosciences) solution and transferred to 5 mL tubes for subsequent FCM-analysis.

2.10. Cell analysis by flow cytometry

Flow cytometry analysis of 5×10^5–1×10^6 splenocytes and intrahepatic lymphocytes (identified by light scatter properties) was acquired on a CytoFLEX LX flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with five lasers (355, 405, 488, 561 and 638 nm). Data were analyzed by the FlowJo™ software (Version 10.5.3, Tree Star, Ashland, OR, USA). The gating strategy for lymphocytes isolated from chickens and turkeys is illustrated in Supplementary Fig. 1A. For all analyzed lymphocyte subsets, IFN-γ gates were set individually per bird within the unstimulated medium sample and applied to the corresponding *H. meleagridis* and *E. coli* stimulated samples as depicted in Supplementary Fig. 1B.

2.11. Processing of results and statistical analysis

To calculate frequencies of IFN-γ-producing *H. meleagridis*-specific T cells, a subtraction of the percentages of IFN-γ-producing lymphocytes obtained in *E. coli*-only stimulated samples from percentages in *H. meleagridis/E. coli* co-stimulated samples was performed. In those cases where the subtraction produced a negative value, *H. meleagridis/E. coli* co-stimulated samples were set to zero. To test for significant differences of IFN-γ-producing lymphocyte frequencies between the two necropsy time points (two wpi and five wpi) the Wilcoxon matched-pairs signed rank test was applied. To compare IFN-γ-producing cell subsets of birds from the infected, vaccinated and vaccinated/infected groups with birds from the control groups an unpaired Mann-Whitney test was applied. *P*-values are indicated as follows: n.s. (not significant, *P*-value > 0.05), * (*P*-value ≤ 0.05), ** (*P*-value < 0.01). GraphPad Prism software 8.3.0 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses and preparation of diagrams.

3. Results

3.1. Clinical signs, lesion scores and H. meleagridis detection by IHC, ELISA and re-isolation

Table 1 summarizes lesion scores in the cecum and liver including detection of *H. meleagridis* by IHC, *H. meleagridis*-specific antibodies by ELISA and parasite re-isolation from cloacal swabs for chickens and turkeys. A detailed overview on the before-mentioned parameters for all sampled time points is given in Supplementary Table 1. Location of *H. meleagridis* in the ceca by IHC is exemplary shown in Supplementary Fig. 2.

Table 1
Lesion scoring and *H. meleagridis* detection by IHC, ELISA and re-isolation of chickens and turkeys.

Inoculation group	Bird number	Days post-infection	Cecum				Liver				ELISA[c]		Cloacal swab[d]	
			LS[a]		IHC[b]		LS		IHC					
			C[e]	T[f]	C	T	C	T	C	T	C	T	C	T
Control	1	14	0	0	–	–	0	0	–	–	–	–	–	–
	2	15	0	0	–	–	0	0	–	–	–	–	–	–
	3	16	0	0	–	–	0	0	–	–	–	–	–	–
	4	35	0	0	–	–	0	0	–	–	–	–	–	–
	5	36	0	0	–	–	0	0	–	–	–	–	–	–
	6	37	0	0	–	–	0	0	–	–	–	–	–	–
Vaccinated	7	14	0	0	+	–	0	0	–	–	–	–	+	–
	8	15	0	0	–	–	0	0	–	–	–	–	–	–
	9	16	0	0	+	–	0	0	–	–	–	–	–	–
	10	35	0	0	+	+	0	0	–	–	–	+	+	+
	11	36	0	0	+	+	0	0	–	–	–	+	+	+
	12	37	0	0	–	+	0	0	–	–	–	+	+	+
Infected	13	14	4	4	+	–	0	0	–	–	–	+	–	+
	14	15	0	3	–	+	0	0	–	–	–	+	–	+
	15	16	4	4	+	+	2	0	–	–	–	+	–	+
	16	35	2	n.a.[g]	+	n.a.	0	n.a.	–	n.a.	–	n.a.	+	n.a.
	17	36	2	n.a.	+	n.a.	0	n.a.	–	n.a.	–	n.a.	+	n.a.
	18	37	2	n.a.	–	n.a.	0	n.a.	–	n.a.	–	n.a.	–	n.a.
Vaccinated/Infected	19	14	3	4	+	+	0	0	–	–	–	–	+	+
	20	15	1	2	+	+	0	0	–	–	–	+	+	+
	21	16	4	4	+	+	0	0	–	+	–	+	–	+
	22	35	2	3	+	+	0	0	–	–	–	+	+	+
	23	36	1	3	–	+	0	0	–	–	–	+	+	+
	24	37	1	1	+	+	0	0	–	–	+	+	+	+

[a] Lesion scoring (LS) system from 0 to 4 was applied; Cecum: 0 = no pathological changes; 1 = sporadic inflammation and/or mild thickening of the wall of one cecum; 2 = sporadic inflammation and/or mild thickening of the wall of both ceca; 3 = inflammation of both ceca and thickening of the intestinal wall with liquid fibrin or sporadic fibrinous coagula in the lumen. If only one cecum was affected, then lesion score 2 was applied; 4 = severe inflammation and necrosis in both ceca with compact fibrinous masses in the lumen of the ceca. If only one cecum was affected, then lesion score 3 was applied. Liver: 0 = no pathological changes; 1 = a few single punctiform necrosis up to 1 mm; 2 = single punctiform necrosis disseminated throughout the organ up to 1 mm or a few single punctiform necrosis more than 1 mm; 3 = single punctiform necrosis, disseminated throughout the organ more than 1 mm or some large areas of necrosis; 4 = confluent necrosis throughout the organ.
[b] Detection of the parasite in cecum and liver was performed by immunohistochemistry.
[c] Detection of *H. meleagridis*-specific antibodies by ELISA; - indicates O.D. values below threshold of positivity; + indicates O.D. values above threshold of positivity.
[d] Detection of *H. meleagridis* by re-isolation of viable cells from cloacal swab samples.
[e] C indicates chicken.
[f] T indicates turkey.
[g] n.a. indicates not applicable; The infected group of turkeys consisted only of 3 birds, which were sacrificed two wpi.

For chickens, clinical signs could not be detected in any bird independent of the group. Three turkeys of the vaccinated/infected group (VIT) showed slight depression reflected by dropped head and half-closed eyes during the second week after inoculation. Two turkeys recovered fully within two days while the remaining turkey was sacrificed according to the experimental plan at two wpi. No clinical signs were observed in the remaining turkeys including birds from the vaccinated (VT) and infected (IT) groups (data not shown).

Lesion scoring during necropsy for both, chickens and turkeys, revealed for the cecum a maximum lesion score (LS) of 4 in some birds of the infected (median LS: IC = 4; IT = 4) and vaccinated/infected (median LS: VIC = 3; VIT = 4) group at two wpi. The cecal LS decreased five wpi for the IC group (median LS: 2) and for the VIC/VIT groups (median LS: VIC = 1; VIT = 3). Lesions in the liver were only found in one chicken from the IC group (LS 2) while none of the turkeys showed any changes in the liver (median LS for all groups 0). Ceca and livers from control (CC and CT) and vaccinated birds (VC and VT) did not present any lesions.

Detection of *H. meleagridis* cells by IHC was performed in cecum and liver. Four chickens from the VC and IC group and five chickens from the VIC group were found positive in their cecum by IHC, whereas the liver of all chickens was tested negative. For turkeys, parasites in the cecum could be successfully detected in three birds of the VT group, two birds of the IT group and all birds from the VIT group. One turkey liver from the VIT group resulted positive by IHC. Birds from the CC and CT group were confirmed to be not infected by IHC. Localization of the parasite in the cecum is illustrated in Supplementary Fig. 2 by one representative bird per group. Ceca from the VC/VT group and some birds from the IC and VIC group showed a non-infiltrative presence of the parasite in the cecal lumen. For the remaining birds from the IC and VIC group as well as all birds from the IT and VIT group parasites could be found within the cecal mucosa (infiltrative). Localization of the parasites are given in detail in Supplementary Table 1.

Circulating *H. meleagridis*-specific antibodies in sera could only be found in one chicken of the VIC group at two wpi and remained positive until termination of the experiment. For turkeys, three birds of the VT group (five wpi), all birds from IT group (two wpi) and five birds from the VIT group (two wpi) showed antibody titers above the threshold. All birds of the control groups (CC and CT) stayed negative at all sampled time points. Results for each time point are indicated in Supplementary Table 1.

Viable parasite cells could be re-isolated from cloacal swabs in four chickens of the VC group, two chickens of the IC group and five chickens of the VIC group at various time points. In turkeys, *H. meleagridis* cells could be re-isolated in three birds from the VT group as well as all turkeys from IT and VIT groups. Birds from the control group (CC, CT) were negative at all sampled time points. Results for each time point are listed in Supplementary Table 1.

3.2. Testing of mAbs for cross-reactivity with Turkey IFN-γ in intracellular cytokine staining

To the best of our knowledge, turkey-specific anti–IFN–γ monoclonal antibodies are currently missing. Hence, mAbs specific for chicken IFN-γ, which had been previously successfully established for use in ICS with chicken lymphocytes (Lagler et al., 2019), were investigated for cross-reactivity with turkey IFN-γ. The amino acid sequences of IFN-γ from chicken and turkey were aligned and revealed an identity of 97% (Supplementary Fig. 3A). Among several tested mAbs (Lambrecht et al., 2004), clone 7E12 with a mouse IgG1 isotype gave the highest reactivity (data not shown) and was used for further experiments. Supplementary Fig. 3B shows data of a titration experiment of this mAb with splenocytes isolated from a turkey, which were stimulated with PMA/ionomycin (bottom row) or left untreated (top row). A serial dilution (6 ng–0.75 ng, this range was selected based on findings of a previous experiment) of the mAb was performed to identify the optimal quantity. A distinct population of IFN-γ-producing lymphocytes upon PMA/ionomycin stimulation ranging from 0.71% to 0.95% (Supplementary Fig. 3B, bottom row) within live lymphocytes was identified. The highest percentage of IFN-γ-producing cells was reached at a quantity of 1.5 ng. This amount of mAb was used for subsequent experiments with *H. meleagridis* re-stimulated lymphocytes.

3.3. T-cell response following H. meleagridis vaccination and/or infection

Following the methodology established in a previous study (Lagler et al., 2019), the frequencies of IFN-γ-producing T-cell subsets from *H. meleagridis* vaccinated, infected and vaccinated/infected chickens and turkeys were investigated. Birds were sacrificed at two different time points (two wpi and five wpi) for comparing an early with a later, potentially memory, immune T-cell response. Statistical analysis using the Wilcoxon matched-pairs signed rank test showed that results for IFN-γ-producing T-cell subsets from both time points did not significantly differ. Therefore, Figs. 2–4 show *E. coli* corrected results of IFN-γ^+ T-cell frequencies from both necropsies (two and five wpi) grouped together per inoculated group. Vaccinated, infected and vaccinated/infected groups were statistically analyzed by the unpaired Mann-Whitney test against the control group.

3.3.1. IFN-γ production of $CD4^+$ T cells from spleen and liver

H. meleagridis-specific IFN-γ-producing $CD4^+$ T cells were identified by ICS in combination with markers for CD4 in chickens and a combination of CD3ε and CD4 in turkeys. The applied gating hierarchy is presented in Supplementary Fig. 1A and the gates applied for the identification of IFN-γ^+ cells are illustrated in Supplementary Fig. 1B for both species. Results for *E. coli* corrected *H. meleagridis*-specific IFN-γ-producing $CD4^+$ T cells are shown in Fig. 2 and corresponding representative flow cytometry raw data in Supplementary Fig. 4. For chickens, IFN-γ-producing $CD4^+$ T-cell frequencies in spleens were significantly higher upon re-stimulation with the attenuated *H. meleagridis* antigen in the VIC group ($P \leq 0.05$) and with the virulent *H. meleagridis* antigen in the IC group ($P < 0.01$; Fig. 2A, scatter plots on the left) compared to controls (CC). Turkeys showed a significant increase of IFN-γ^+CD4^+ T-cell splenocytes in the VIT group upon both re-stimulation variants ($P \leq 0.05$; Fig. 2A, scatter plots on the right) in comparison to the CT group. For livers, significant differences were only determined in turkeys of the VIT group after re-stimulation with attenuated ($P < 0.01$) or virulent ($P \leq 0.05$) histomonads (Fig. 2B, scatter plots on the right).

3.3.2. IFN-γ production of $CD8\alpha^+$ T cells from spleen and liver

H. meleagridis-specific IFN-γ-producing $CD8\alpha^+$ T cells were identified by ICS with markers for CD8α in chickens and a combination of CD3ε and CD8α in turkeys. The applied gating hierarchy is presented in Supplementary Fig. 1A. Results for *E. coli* corrected *H. meleagridis*-specific IFN-γ-producing $CD8\alpha^+$ T cells are shown in Fig. 3 and corresponding representative flow cytometry raw data in Supplementary Fig. 5. Significant differences for IFN-γ-producing $CD8\alpha^+$ T cells between vaccinated/infected and control birds were only found in turkeys. IFN-γ-producing $CD8\alpha^+$ T cells from turkey spleens of the VIT group ($P \leq 0.05$) differed significantly upon re-stimulation with virulent *H. meleagridis* antigen (Fig. 3A, scatter plot on the right, bottom). In addition, turkey-derived $CD8\alpha^+$ T cells from the VIT group also showed significant differences in the liver following re-stimulation with attenuated ($P < 0.01$) and virulent histomonads ($P \leq 0.05$) compared to controls (Fig. 3B, scatter plots on the right).

3.3.3. IFN-γ production of $TCR\gamma\delta^+/CD3\varepsilon^+CD4^-CD8\alpha^-$ T cells from spleen and liver

In chickens, IFN-γ-producing $TCR\gamma\delta^+$ T cells were identified by ICS in combination with a marker for TCRγδ while in turkeys IFN-γ-producing $CD3\varepsilon^+CD4^-CD8\alpha^-$ T cells were identified by ICS in combination with markers for CD3ε, CD4 and CD8α. This labelling strategy was chosen

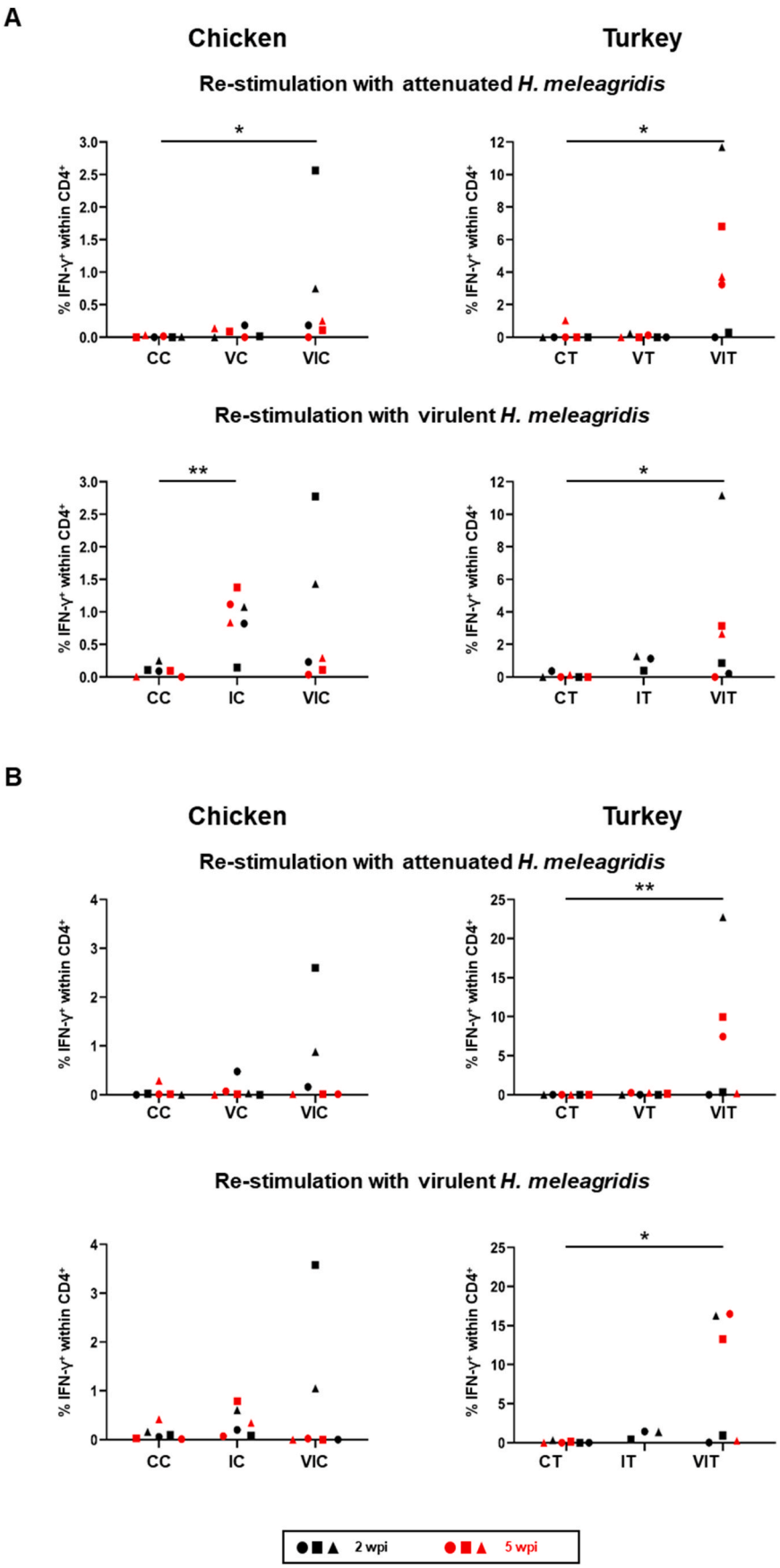

Fig. 2. Frequencies of IFN-γ-producing CD4$^+$ T cells isolated from spleen and liver of chickens and turkeys following re-stimulation with *H. meleagridis*. Scatter plots show *E. coli* corrected frequencies of IFN-γ-producing CD4$^+$ T cells from chickens (left column) and turkeys (right column) isolated from spleen (A) and liver (B) following stimulation with attenuated (top row) and virulent (bottom row) *H. meleagridis* antigen. Plots with IFN-γ-producing cell frequencies stimulated with attenuated *H. meleagridis* are depicted for the control group (chicken: CC, turkey: CT), vaccinated group (chicken: VC, turkey: VT) and vaccinated/infected group (chicken: VIC, turkey: VIT). Plots with IFN-γ-producing cell frequencies stimulated with virulent *H. meleagridis* are depicted for the infected group (chicken: IC, turkey: IT), CC/CT and VIC/VIT. Black and red symbols represent birds euthanized two wpi and five wpi, respectively. Asterisks indicate different *p*-values: $*P \leq 0.05$ and $**P < 0.01$.

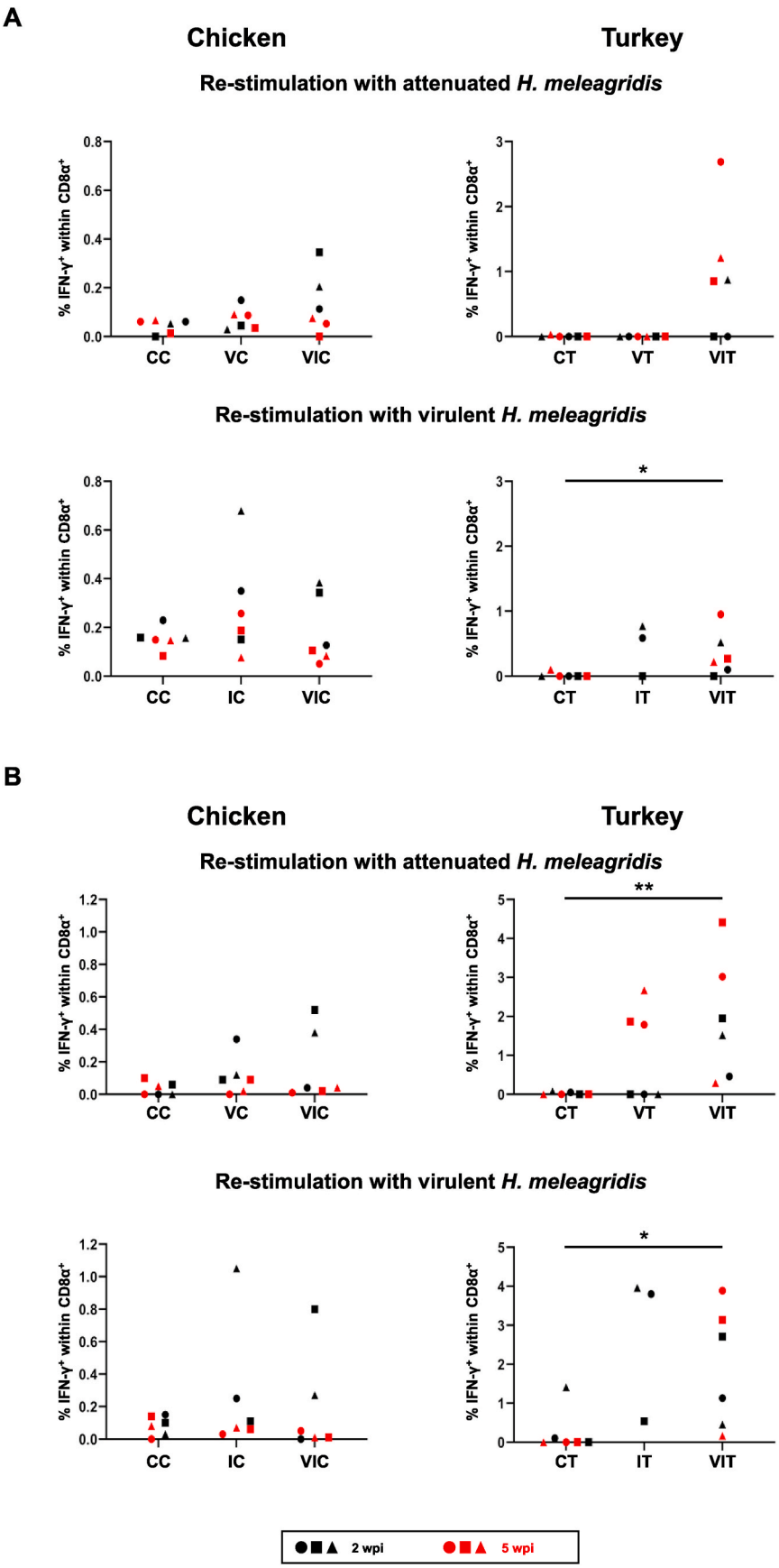

Fig. 3. Frequencies of IFN-γ-producing CD8α$^+$ T cells isolated from spleen and liver of chickens and turkeys following re-stimulation with *H. meleagridis*. Scatter plots show *E. coli* corrected frequencies of IFN-γ-producing CD8α$^+$ T cells from chickens (left column) and turkeys (right column) isolated from spleen (A) and liver (B) following stimulation with attenuated (top row) and virulent (bottom row) *H. meleagridis* antigen. Plots with IFN-γ-producing cell frequencies stimulated with attenuated *H. meleagridis* are depicted for the control group (chicken: CC, turkey: CT), vaccinated group (chicken: VC, turkey: VT) and vaccinated/infected group (chicken: VIC, turkey: VIT). Plots with IFN-γ-producing cell frequencies stimulated with virulent *H. meleagridis* are depicted for the infected group (chicken: IC, turkey: IT), CC/CT and VIC/VIT. Black and red symbols represent birds euthanized two wpi and five wpi, respectively. Asterisks indicate different *p*-values: $^*P \leq 0.05$ and $^{**}P < 0.01$.

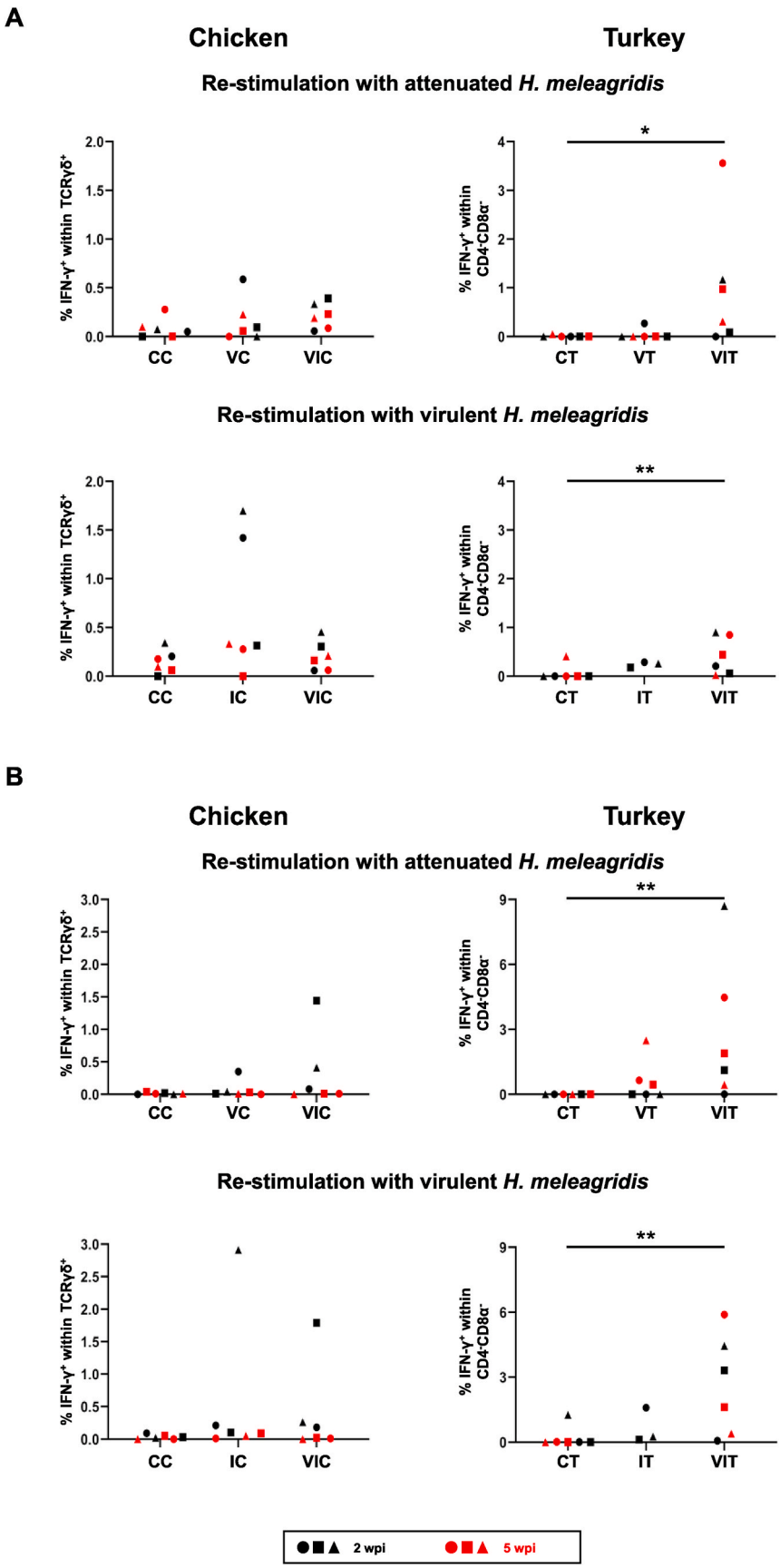

Fig. 4. Frequencies of IFN-γ-producing TCRγδ$^+$/CD3ε$^+$CD4$^-$CD8α$^-$ T cells isolated from spleen and liver following re-stimulation with *H. meleagridis*. Scatter plots show *E. coli* corrected frequencies of IFN-γ-producing TCRγδ$^+$ (chicken, left column) and CD3ε$^+$CD4$^-$CD8α$^-$ (turkey, right column) T cells isolated from spleen (A) and liver (B) following stimulation with attenuated (top row) and virulent (bottom row) *H. meleagridis* antigen. Plots with IFN-γ-producing cell frequencies stimulated with attenuated *H. meleagridis* are depicted for the control group (chicken: CC, turkey: CT), vaccinated group (chicken: VC, turkey: VT) and vaccinated/infected group (chicken: VIC, turkey: VIT). Plots with IFN-γ-producing cell frequencies stimulated with virulent *H. meleagridis* are depicted for the infected group (chicken: IC, turkey: IT), CC/CT and VIC/VIT. Black and red symbols represent birds euthanized two wpi and five wpi, respectively. Asterisks indicate different *p*-values: $^*P \leq 0.05$ and $^{**}P < 0.01$.

because of a lack of TCRγδ-specific antibodies for turkeys. The gating hierarchy applied for this is presented in Supplementary Fig. 1A. Results for *E. coli* corrected *H. meleagridis*-specific IFN-γ-producing TCRγδ$^+$ T cells (for chickens) and CD3ε$^+$CD4$^-$CD8α$^-$ T cells (for turkeys) are shown in Fig. 4 and corresponding representative flow cytometry raw data in Supplementary Fig. 6. Significant differences for IFN-γ-producing cells with these two phenotypes between vaccinated/infected and control birds were only found in turkeys. IFN-γ-producing CD3ε$^+$CD4$^-$CD8α$^-$ T cells derived from spleens in turkeys were significantly enhanced for the VIT group stimulated with the attenuated ($P \leq 0.05$) and virulent ($P < 0.01$) *H. meleagridis* antigen (Fig. 4A, scatter plots on the right). Also, in the livers of turkeys IFN-γ-producing cells of the VIT group differed significantly from the control group upon both stimulation variants ($P < 0.01$; Fig. 4B, scatter plots on the right).

3.3.4. Summary of significant differences of IFN-γ-producing cell frequencies in spleen and liver from H. meleagridis inoculated chickens and turkeys

For a complete overview on the magnitude of IFN-γ T-cell responses across species, organs and treatment groups, obtained significant differences are summarized in Fig. 5. Within the spleen, significant differences of IFN-γ-producing T-cell frequencies in comparison to the control group were found in infected chickens and vaccinated/infected birds of both species (Fig. 5A). In chickens, significant differences for the IFN-γ$^+$CD4$^+$ T-cell subset were found for the IC group upon virulent *H. meleagridis* antigen re-stimulation ($P < 0.01$). Moreover, IFN-γ-producing CD4$^+$ T cells differed significantly in the VIC group upon attenuated *H. meleagridis* antigen re-stimulation ($P \leq 0.05$) and reached almost significance upon virulent re-stimulation ($P = 0.065$; Fig. 5A, left box). In turkeys, significant differences of IFN-γ-producing cell

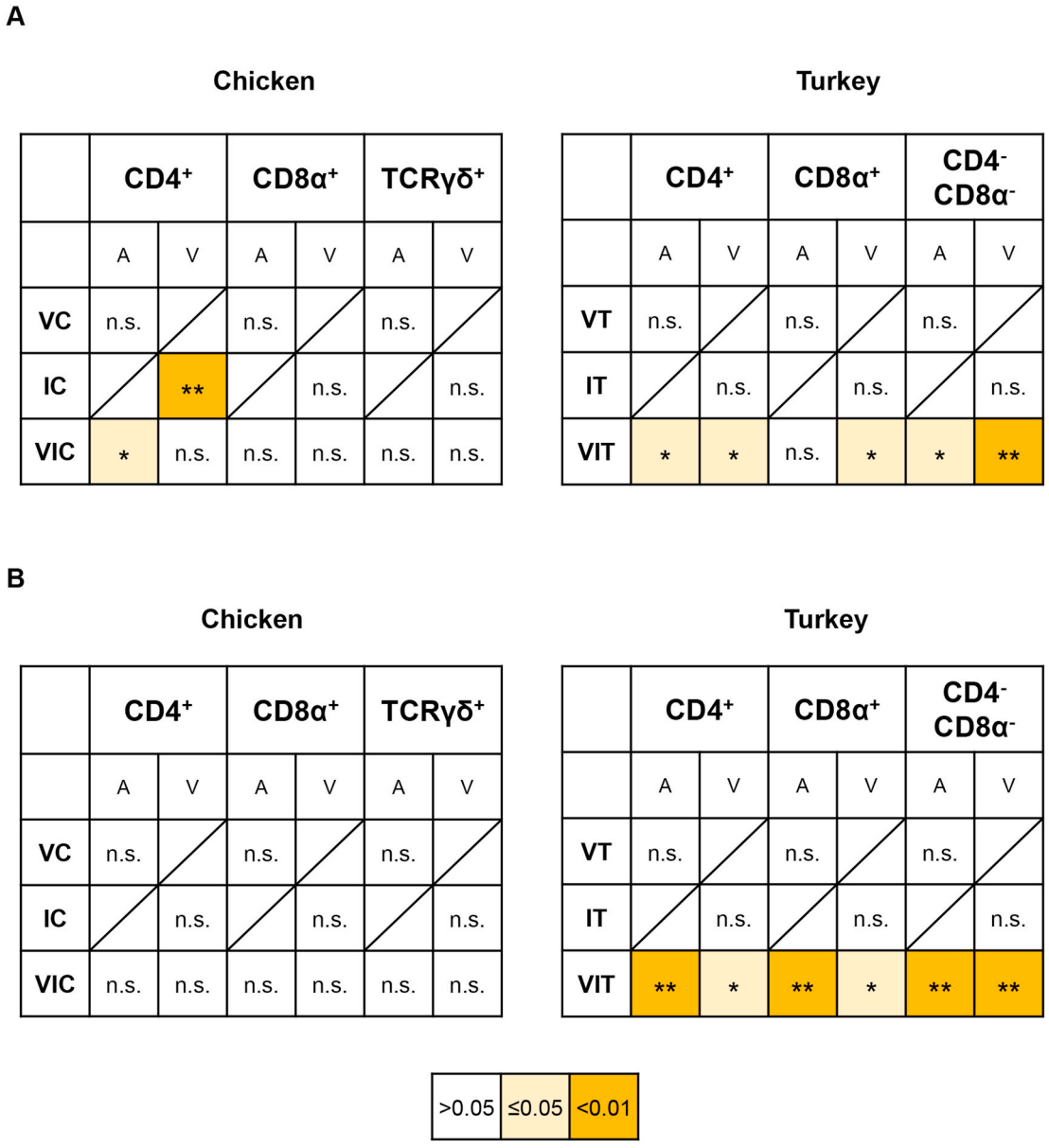

Fig. 5. Differences of IFN-γ-producing lymphocyte subsets isolated from spleen and liver of chickens and turkeys following stimulation with *H. meleagridis*. Summary of significant differences of *E. coli* corrected IFN-γ-producing cell frequencies within the CD4$^+$, CD8α$^+$ and TCRγδ$^+$/CD3ε$^+$CD4$^-$CD8α$^-$ T-cell subsets from the vaccinated group (chicken: VC, turkey: VT), infected group (chicken: IC, turkey: IT) and vaccinated/infected group (chicken: VIC, turkey: VIT) in comparison to the corresponding control group (chicken: CC, turkey: CT) using the non-parametric unpaired Mann-Whitney test. (A) Significant differences in spleens. (B) Significant differences in livers. Columns labeled with "A" and "V" indicate results following re-stimulation with attenuated and virulent histomonads, respectively. Asterisks indicate different *p*-values: *$P \leq 0.05$ and **$P < 0.01$. Non-significance is depicted as n.s. ($P > 0.05$).

frequencies were detected among all three analyzed T-cell subsets in the VIT group. IFN-γ$^+$CD4$^+$ T-cell percentages differed significantly following both stimulation variants ($P \leq 0.05$). In addition, IFN-γ-producing CD8α$^+$ T cells re-stimulated with virulent histomonads showed significant differences compared to controls ($P \leq 0.05$) while re-stimulation with attenuated parasites almost met a p-value of 0.05 ($P = 0.06$). Frequencies of IFN-γ-producing CD3ε$^+$CD4$^-$CD8α$^-$ splenocytes were also found to be significantly elevated in comparison to the control group upon attenuated ($P \leq 0.05$) and virulent ($P < 0.01$) histomonads re-stimulation (Fig. 5A, right box).

In the liver, significant differences between treated and control birds were found only in turkeys belonging to the VIT group. All three analyzed T-cell subsets showed significant differences following attenuated (all subsets: $P < 0.01$) as well as virulent (CD4$^+$ and CD8α$^+$ T-cell subset: $P \leq 0.05$; CD3ε$^+$CD4$^-$CD8α$^-$ T-cell subset: $P < 0.01$) *H. meleagridis* antigen re-stimulation (Fig. 5B, right box). Chickens from the IC group reached almost significance compared to controls within the IFN-γ$^+$TCRγδ$^+$ T-cell subset ($P = 0.058$; Fig. 5B, left box).

4. Discussion

The *H. meleagridis*-specific T-cell immune response following vaccination and/or infection of chickens and turkeys has not been studied in detail yet. Therefore, in the present study, IFN-γ production of major T-cell subsets isolated from spleen and liver of both species was investigated.

Alongside to the T-cell immune response several histomonosis relevant clinical and pathological parameters were analyzed and were largely in accordance with already published data. However, previously mainly xenic cultures of *H. meleagridis* were used in experimental studies while investigations with a monoxenic strain are still limited and only focused on infected or vaccinated turkeys, but not chickens as reviewed recently (Liebhart et al., 2017).

In chickens, no clinical signs or mortalities were detected in any bird of all inoculated groups. Additionally, lesions in the cecum were found in birds of the IC and VIC group and in the liver of one bird of the IC group, which is consistent with other studies using the same strain of *H. meleagridis* with co-cultivated cecal bacteria (Zahoor et al., 2011; Liebhart et al., 2013; Mitra et al., 2017). In accordance with previous studies, parasites detected by IHC were present in the cecum and intermittent cloacal shedding was found for all inoculated groups but not all birds were found positive (Liebhart et al., 2011; Zahoor et al., 2011). Screening of sera by ELISA revealed *H. meleagridis*-specific antibodies only in one chicken of the VIC group that is in contrast to other studies in which positive antibody titers in sera from vaccinated and/or infected chickens were found more frequently (Windisch and Hess, 2009, 2010; Liebhart et al., 2013). This discrepancy might suggest that inoculation with either xenic or monoxenic histomonads cultures leads to a delayed or weakened humoral immune response in chickens.

In turkeys, no clinical signs except in three birds of the VIT group were found and no birds died due to histomonosis. Lesions in the cecum with maximum scores were detected in the IT and VIT group while no lesions were found in the livers. Parasites could be detected by IHC in ceca of birds from all inoculated groups and in the liver of one bird of the VIT group, which is largely in agreement with our previous data (Singh et al., 2008; Sulejmanovic et al., 2016). Turkeys of the inoculated groups shed the parasites intermittently and *H. meleagridis*-specific antibodies from sera were frequently detected as seen earlier using xenic and monoxenic histomonads for infection (Windisch and Hess, 2009; Liebhart et al., 2010; Ganas et al., 2012). Since parasite detection by cloacal re-isolation, IHC and specific antibodies was only shown at five wpi in the VT group compared to the other inoculated groups it can be hypothesized that the attenuated compared to the virulent monoxenic *H. meleagridis* strain requires more time to establish in turkeys.

Due to the extracellular occurrence of *H. meleagridis*, a type-2 immune response towards this parasite has been hypothesized in the past (Powell et al., 2009; Schwarz et al., 2011). However, previous investigations by our group on IL-13 mRNA-producing lymphocytes revealed very low frequencies of such cells in spleen and liver of chickens and these cells did not increase after *H. meleagridis* infection (Lagler et al., 2019). Additionally, no elevated IL-13 mRNA$^+$ T-cell levels were detected in turkeys vaccinated with attenuated *H. meleagridis* (own non-published findings). Hence, for the current study we focused on IFN-γ-producing T cells.

Analysis of the antigen-specific T-cell response in chickens elucidated significant differences in splenic IFN-γ-producing CD4$^+$ T cells of the IC group. Two birds of the same group euthanized two wpi showed increased IFN-γ$^+$TCRγδ$^+$ cell frequencies in the spleen as well. Both findings, and the lack of IFN-γ production by intrahepatic lymphocytes (IHL), are consistent with recently published data from our group (Lagler et al., 2019). A negligible involvement of IFN-γ-producing liver lymphocytes might be expected as Powell et al. (2009) already suggested that an early pro-inflammatory cytokine production in cecal tonsils limits the spread of histomonads to the liver in chickens. Of note, the chicken of the IC group showing lesions in the liver exhibited increased IFN-γ$^+$ cell frequencies within the CD8α$^+$ and TCRγδ$^+$ but not the CD4$^+$ T-cell subset. Besides the IC group, two birds of the VIC group euthanized two wpi showed consistently high levels of IFN-γ-producing CD4$^+$ and CD8α$^+$ T cells of spleen and liver. As no such trend was seen in the VC group, it can be speculated that this effect is induced *in vivo* by the virulent *H. meleagridis* strain rather than the attenuated strain. In general, *H. meleagridis*-specific splenic CD4$^+$ T cells in the VIC group showed weaker responses than CD4$^+$ T cells from the IC group. This relatively weak systemic T-cell immune response might be explained by a regress of responsive T cells from the spleen to the local site of infection, the cecum. A similar phenomenon was suggested for *Eimeria*-specific T cells migrating from the spleen to the site of infection in infected chicken (Rothwell et al., 2000). Due to low lymphocyte yields isolated from cecal tissue in combination with substantial cell death during *in vitro* cultivation, IFN-γ detection for intraepithelial lymphocytes by ICS could not be performed in this study but might be of interest in future investigations.

Similar to infected chickens, infected turkeys showed increased levels of IFN-γ$^+$CD4$^+$ T-cell frequencies in the spleen but this did not reach significance due to the low number of IT (n = 3). Unlike chickens, birds from the VIT group showed an involvement of IFN-γ-producing CD4$^+$, CD8α$^+$ and CD3ε$^+$CD4$^-$CD8α$^-$ T-cell subsets from both organs: spleen and liver. This emphasizes again a general difference in the host-pathogen interaction comparing infection within these two species. It might be speculated that even the attenuated *H. meleagridis* strain, which was used for vaccination, induces a stronger immune response in turkeys than in chickens. However, in our experimental system this difference was only revealed after a challenge infection, suggesting that the challenge had a booster effect on the T-cell response - most probably locally and systemically. Another explanation might be that in chickens the mucosal immune response triggered by vaccination is sufficient to fight a challenge infection locally, whereas in turkeys after the challenge virulent histomonads still manage to breach this barrier, resulting in the observed T-cell responses in liver and spleen.

While CD4$^+$ T cells are well characterized as one of the main IFN-γ sources during the adaptive immune response against many pathogens, functional mechanisms of CD8$^+$ T cells in the context of an infection with an extracellular pathogen are less clear. For Eimeria, another protozoan parasite in chickens, authors hypothesized that IFN-γ-producing CD8$^+$ T cells are playing a major role in protection by activating macrophages to kill the parasite (Breed et al., 1997). Although the former mentioned pathogen is of intracellular occurrence, CD8$^+$ T cells could act in a similar manner during *H. meleagridis* infections. In addition, for an extracellular parasitic worm in humans, *Echinococcus multilocularis*, IL-10 production by CD8$^+$ T cells was observed (Kilwinski et al., 1999). This suggests that by hitherto unknown routes even extracellular parasites might be capable of triggering CD8$^+$ T-cell

responses.

In turkeys, there are currently no mAbs available for the identification of γδ T cells. Nevertheless, based on observations in the chicken, it could be assumed that γδ T cells can be divided into three different subsets according to their CD8 expression: CD8α^{hi+}β^{+}, CD8αα^{hi+}, CD8α^{-}βα^{-} (Tregaskes et al., 1995; Berndt et al., 2006). Previous studies on *Salmonella* Typhimurium infected chickens identified IFN-γ mRNA in γδ T cells expressing CD8α^{hi+}β^{+} and CD8αα^{hi+} (Pieper et al., 2011). Hence, it can be speculated that with the phenotyping applied for turkey T cells in our study both subsets, CD8α^{+} and CD3ε^{+}CD4^{-}CD8α^{-}, harbor γδ T cells of which some respond to *H. meleagridis* by IFN-γ production. Indeed, for another primarily extracellular pathogen, *Borrelia burgdorferi*, γδ T cells seem to be involved in promoting the adaptive immunity by stimulating dendritic cells (Shi et al., 2011). Next to γδ T cells, the CD3ε^{+}CD4^{-}CD8α^{-} T-cell subset might comprise putative iNKT cells that are also known to play a role in various parasitic infections, including protozoal ones (Yang et al., 2016). CD4/CD8 double negative IFN-γ-producing iNKT intrahepatic lymphocytes seem to inhibit parasite growth in the liver of mice during a malaria infection (Miller et al., 2014). Clearly, a more precise phenotyping of the IFN-γ-producing CD3ε^{+}CD4^{-}CD8α^{-} T cells observed in our study would be desirable but is hampered by the limitations of available reagents to study T cells in turkeys.

In summary, this study provides, for the first time, a comparative analysis of the *H. meleagridis*-specific T-cell immune response based on IFN-γ production in chickens and turkeys. Our data indicate the involvement of different IFN-γ-producing T-cell subsets, in particular in turkeys. This highlights the differences in the immune response towards this parasite in the two species. Moreover, the identification of IFN-γ-producing *H. meleagridis*-specific T-cells in chickens and turkeys suggests an important role for type-1 dominated immune responses towards this parasite, despite its extracellular life cycle.

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Authors' contributions

Conceived the study and designed the experiments: WG, DL. Performed necropsy including sample collection: JL, TM, PW, DL. Preparation of cultures for inoculation and *in vitro* stimulation: TH, JL. Performed *in vitro* stimulation and flow cytometry experiments: JL, SS, MS. Performed ELISA experiments: BG, JL. Performed IHC experiments: PW. Analyzed and interpreted the data: JL, MH, WG, DL. Wrote the manuscript: JL, WG, DL. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103949.

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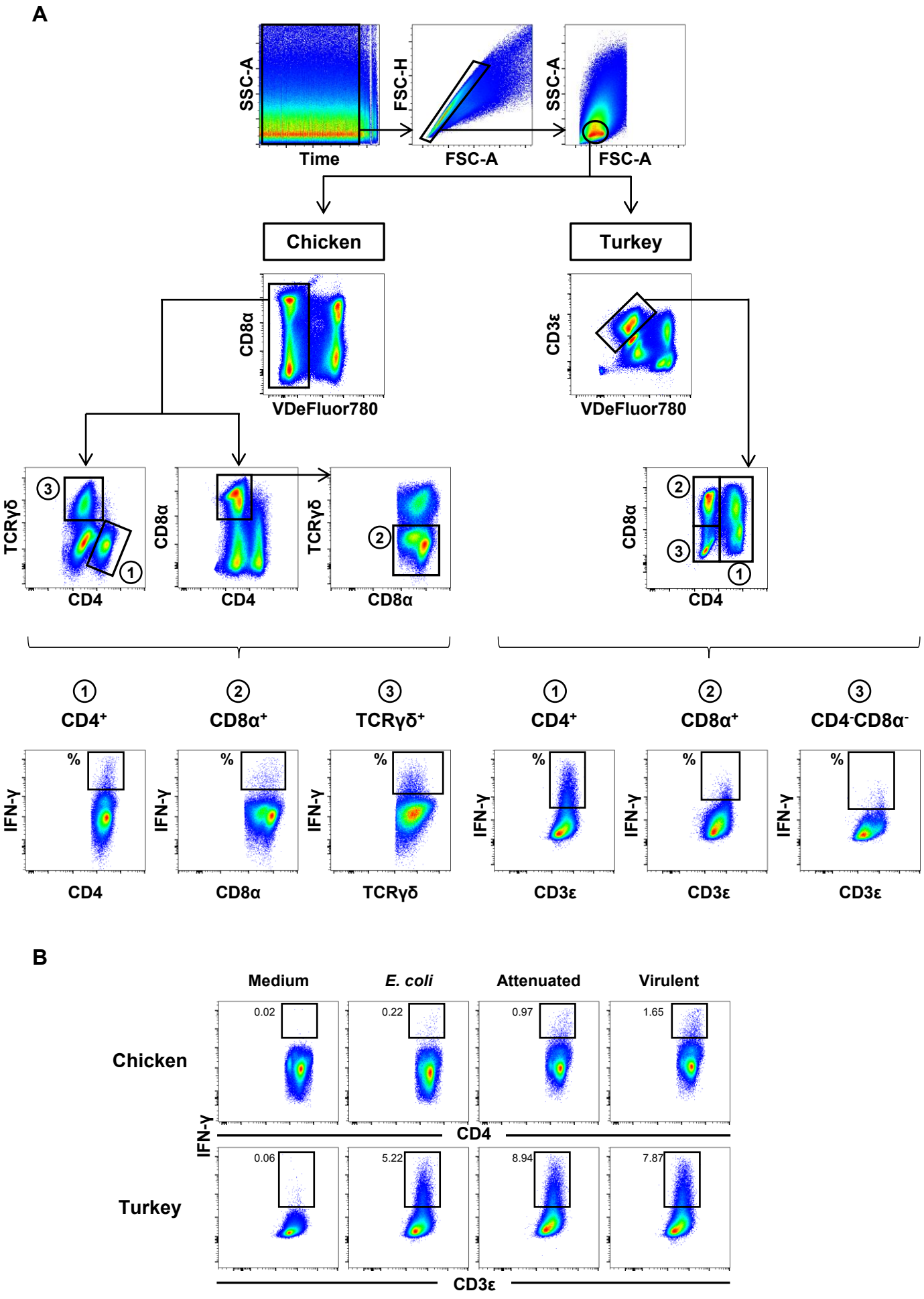
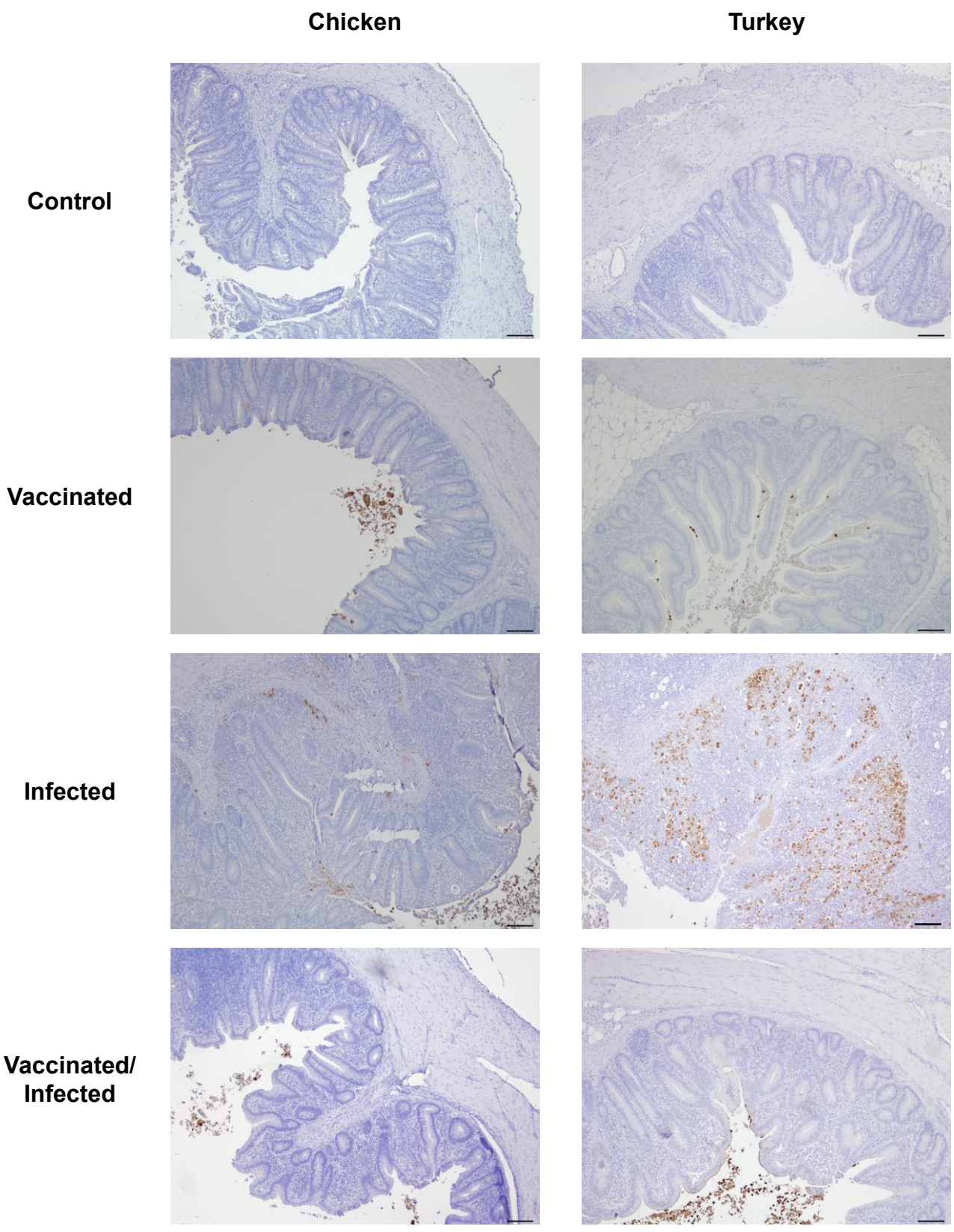
Supplementary Figure 1



Supplementary Fig. 1. Flow cytometry gating hierarchy for lymphocytes from spleen and liver of chickens and turkeys. (A) To identify lymphocytes from chickens and turkeys the following gates were applied in consecutive order: a time gate, a doublet discrimination gate (FSC-A/FSC-H) and a lymphocyte gate (FSC-A/SSC-A). Dead cells were excluded by using the Fixable Viability Dye eFluor® 780. For chickens, frequencies of IFN-γ^+ cells within total $CD4^+$ (1) and total $TCR\gamma\delta^+$ (3) (gating applied within CD4/TCRγδ plot) as well as $CD8\alpha^+$ (2) (within $CD4^-CD8\alpha^{high}TCR\gamma\delta^-$ cells) were determined by sub-gating. For turkeys, live $CD3^+$ cells were gated and IFN-γ^+ cells within total $CD4^+$ (1), $CD8\alpha^+$ (2), and $CD4^-CD8\alpha^-$ (3) cells were determined by sub-gating within a CD4/CD8α plot. (B) Gates for the quantification of IFN-γ across all T-cell subsets were defined per bird based on the non-stimulated control samples and applied consistently to *E. coli* and *H. meleagridis* stimulated samples. Splenocytes of one representative chicken sample (top row) and one turkey sample (bottom row) from the vaccinated/infected group are displayed.

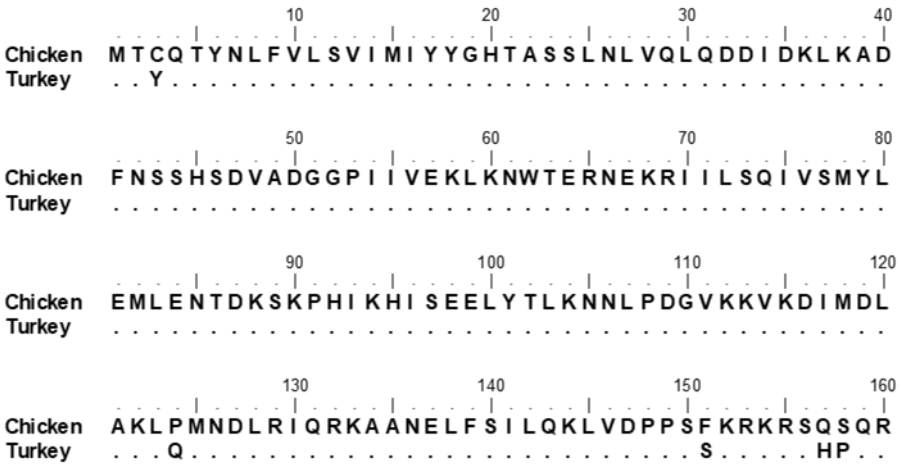
Supplementary Figure 2



Supplementary Fig. 2. Localization of *H. meleagridis* in the ceca of chickens and turkeys by immunohistochemistry. *H. meleagridis* in the ceca of chickens (left column) and turkeys (right column) were labeled by immunohistochemistry. Representative pictures were selected from birds of the control (CC and CT), vaccinated (VC and VT), infected (IC and IT) and vaccinated/infected groups (VIC and VIT). Presence of *H. meleagridis* cells is indicated by brown staining in the cecal lumen (non-infiltrative) or within the cecal mucosa (infiltrative). Bar = 100 μm.

Supplementary Figure 3

A



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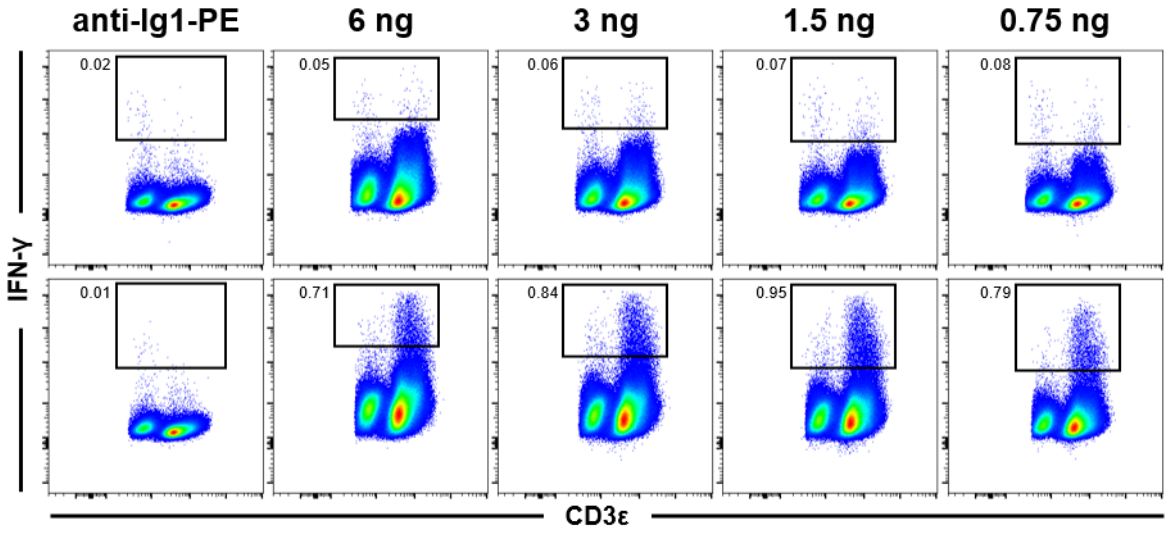
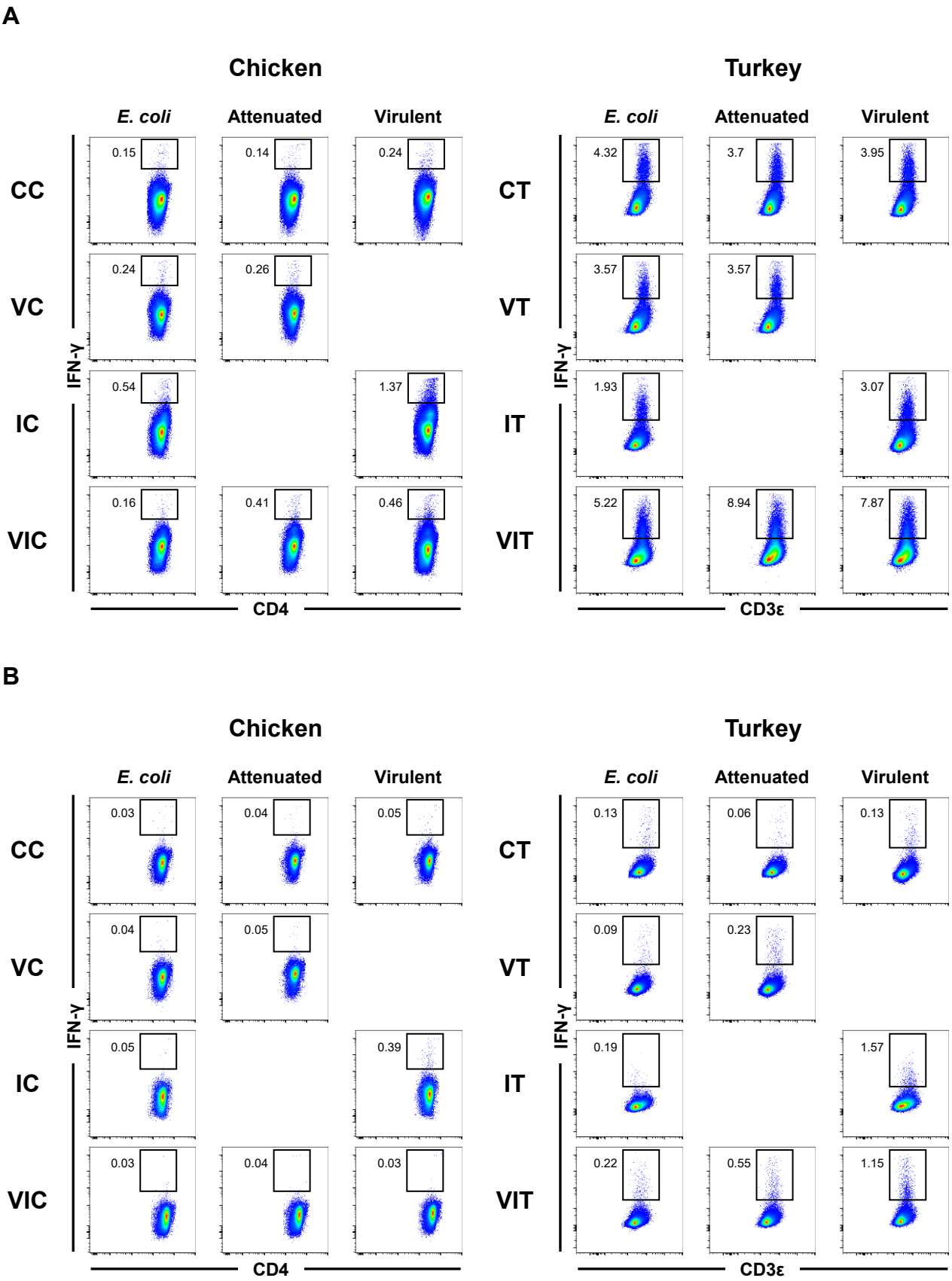


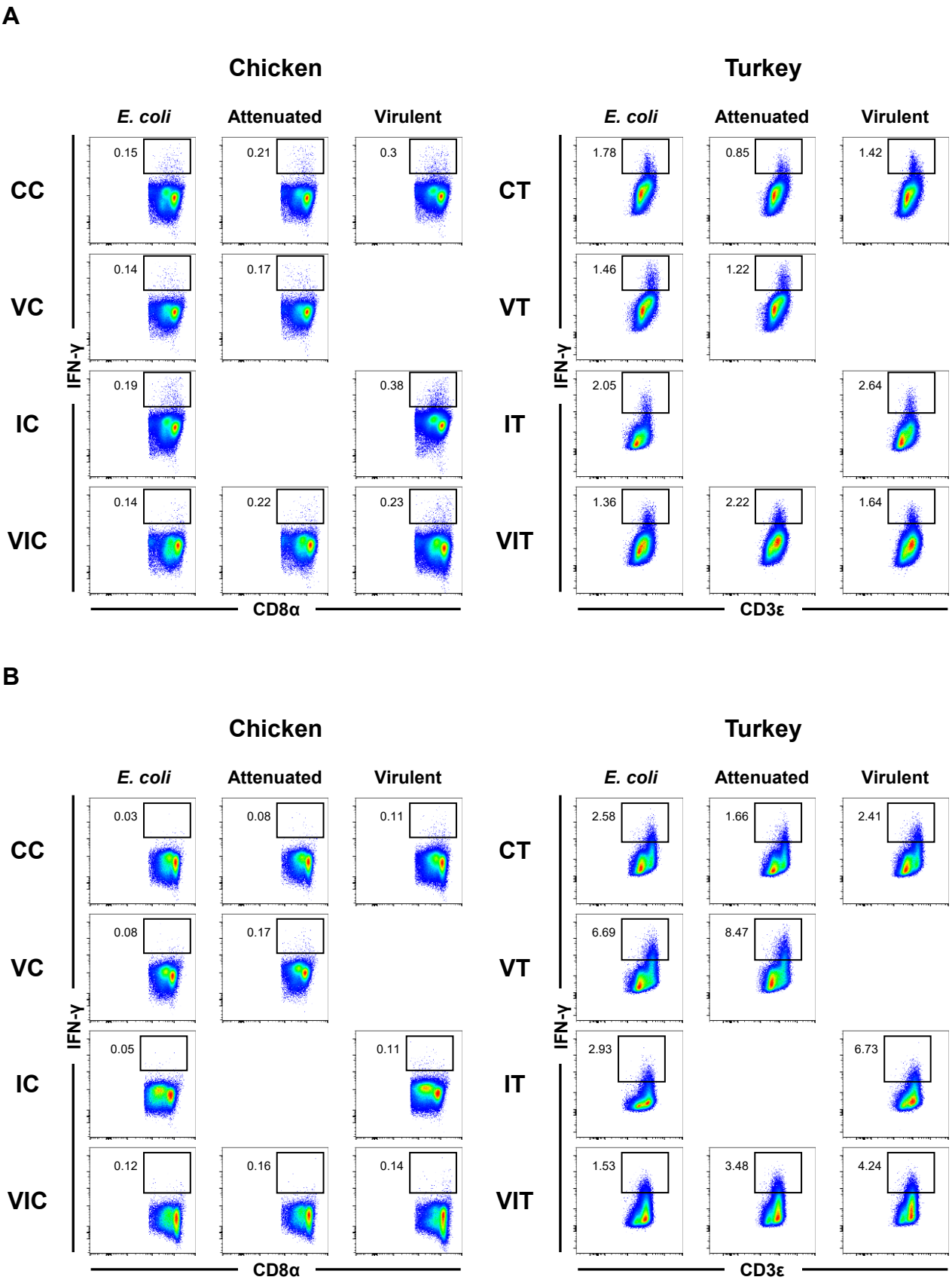
Supplementary Fig. 3. Cross-reactivity of an anti-chicken IFN-γ monoclonal antibody for intracellular cytokine staining in the turkey. (A) Amino acid sequence alignment of IFN-γ (accession numbers for protein sequences are given in parentheses): chicken = *Gallus gallus* (NP_990480); turkey = *Meleagris gallopavo* (XP_003202096). Identical amino acid residues are denoted as a dot. (B) The monoclonal antibody 7E12 (mouse IgG1) was tested for cross-reactivity on PMA/ionomycin stimulated splenocytes isolated from a turkey (bottom row) and compared to non-stimulated splenocytes (top row). A serial dilution from 6 ng–0.75 ng was performed to identify the optimal quantity. Fluorescence labelling of the IFN-γ mAb was performed by staining with a goat-*anti*-mouse IgG1 RPE-conjugated antibody. $CD3\varepsilon^+$ T cells were pre-gated as depicted in Supplementary Fig. 1A.

Supplementary Figure 4



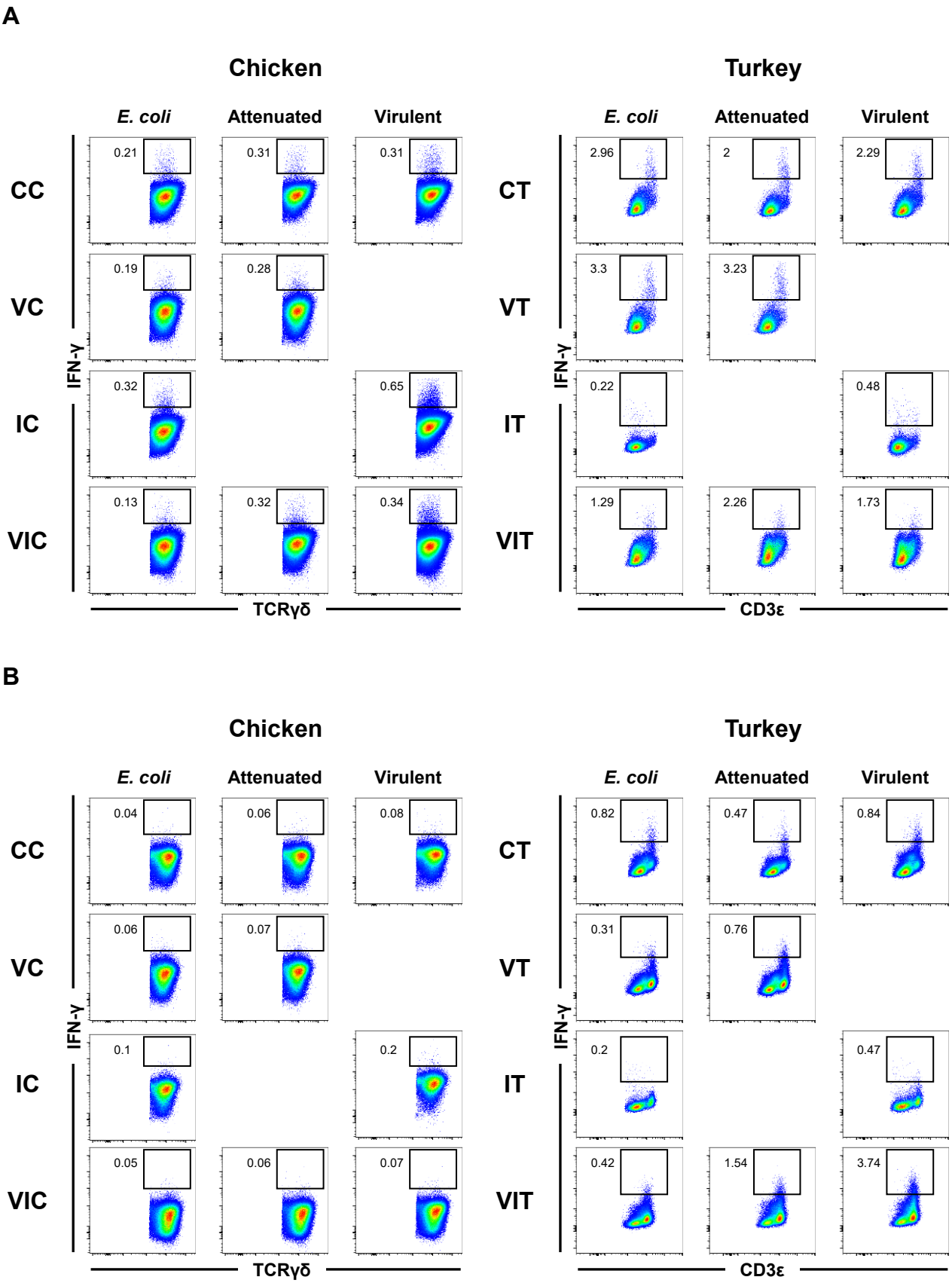
Supplementary Fig. 4. Representative flow cytometry data of IFN-γ-producing $CD4^+$ T cells isolated from spleen and liver of chickens and turkeys following re-stimulation with *H. meleagridis*. Pseudocolor plots depict IFN-γ versus CD4 (chicken) or CD3ε (turkey) expression in $CD4^+$ pre-gated (chicken) or $CD3\varepsilon^+CD4^+$ pre-gated (turkey) (not depicted) splenocytes (A) and intrahepatic lymphocytes (B). Plots with *E. coli* stimulated samples are shown for the control group (chicken: CC, turkey: CT), vaccinated group (chicken: VC, turkey: VT), infected group (chicken: IC, turkey: IT) and vaccinated/infected group (chicken: VIC, turkey: VIT). Plots with attenuated *H. meleagridis* stimulated samples are shown for CC/CT, VC/VT and VIC/VIT. Plots with virulent *H. meleagridis* stimulated samples are shown for CC/CT, IC/IT and VIC/VIT. Approximately (A) 150 000 (chicken) or 200 000 (turkey) and (B) 50 000 (chicken) or 170 000 (turkey) $CD4^+$ cells are shown in each plot. Numbers indicate percentages of IFN-γ^+CD4^+ cells within total $CD4^+$ (chicken) or $CD3\varepsilon^+CD4^+$ T cells (turkey).

Supplementary Figure 5



Supplementary Fig. 5. Representative flow cytometry data of IFN-γ-producing $CD8\alpha^+$ T cells isolated from spleen and liver of chickens and turkeys following re-stimulation with *H. meleagridis*. Pseudocolor plots depict IFN-γ versus CD8α (chicken) or CD3ε (turkey) expression in $CD8\alpha^+$ pre-gated (chicken) or $CD3\varepsilon^+CD8\alpha^+$ pre-gated (turkey) (not depicted) splenocytes (A) and intrahepatic lymphocytes (B). Plots with *E. coli* stimulated samples are shown for the control group (chicken: CC, turkey: CT), vaccinated group (chicken: VC, turkey: VT), infected group (chicken: IC, turkey: IT) and vaccinated/infected group (chicken: VIC, turkey: VIT). Plots with attenuated *H. meleagridis* stimulated samples are shown for CC/CT, VC/VT and VIC/VIT. Plots with virulent *H. meleagridis* stimulated samples are shown for CC/CT, IC/IT and VIC/VIT. Approximately (A) 200 000 (chicken) or 150 000 (turkey) and (B) 100 000 (both species) $CD8\alpha^+$ cells are shown in each plot. Numbers indicate percentages of $IFN\text{-}\gamma^+CD8\alpha^+$ cells within total $CD8\alpha^+$ (chicken) or $CD3\varepsilon^+CD8\alpha^+$ T cells (turkey).

Supplementary Figure 6



Supplementary Fig. 6. Representative flow cytometry data of IFN-γ-producing TCRγδ$^+$ / CD3ε$^+$CD4$^-$CD8α$^-$ T cells isolated from spleen and liver of chickens and turkeys following re-stimulation with *H. meleagridis*. Pseudocolor plots depict IFN-γ versus TCRγδ (chicken) or CD3ε (turkey) expression in TCRγδ$^+$ (chicken) or CD3ε$^+$CD4$^-$CD8α$^-$ pre-gated (turkey) (not depicted) splenocytes (A) and intrahepatic lymphocytes (B). Plots with *E. coli* stimulated samples are shown for the control group (chicken: CC, turkey: CT), vaccinated group (chicken: VC, turkey: VT), infected group (chicken: IC, turkey: IT) and vaccinated/infected group (chicken: VIC, turkey: VIT). Plots with attenuated *H. meleagridis* stimulated samples are shown for CC/CT, VC/VT and VIC/VIT. Plots with virulent *H. meleagridis* stimulated samples are shown for CC/CT, IC/IT and VIC/VIT. Approximately (A) 200 000 (chicken) or 50 000 (turkey) and (B) 180 000 (chicken) or 80 000 (turkey) TCRγδ$^+$/CD3ε$^+$CD4$^-$CD8α$^-$ cells are shown in each plot. Numbers indicate percentages of IFN-γ$^+$TCRγδ$^+$/IFN-γ$^+$CD3ε$^+$CD4$^-$CD8α$^-$ cells within total TCRγδ$^+$/CD3ε$^+$CD4$^-$CD8α$^-$ T cells.

Supplementary Table 1. Overview on lesion score, *H. meleagridis* -specific immunohistochemistry, circulating antibodies and parasite re-isolation of chickens and turkeys.

Species	Inoculation group	Bird number	Lesion score[1] Cecum	Lesion score[1] Liver	Immunohistochemistry[2] Cecum	Immunohistochemistry[2] Liver	*H. meleagridis*-specific antibodies by ELISA[3] Experiment week 2 OD value	+/-	Experiment week 3 OD value	+/-	Experiment week 4 OD value	+/-	Experiment week 5 OD value	+/-	Experiment week 6 OD value	+/-	Experiment week 7 OD value	+/-	Experiment week 8 OD value	+/-	Experiment week 9 OD value	+/-	Experiment week 10 OD value	+/-	*H. meleagridis* detection by re-isolation from cloacal swab samples[4] Experiment week 1	Experiment week 2	Experiment week 3	Experiment week 4	Experiment week 5	Experiment week 6	Experiment week 7	Experiment week 8	Experiment week 9	Experiment week 10
	Control	1	0	0	-	-	0,096	-	0,063	-	0,053	-	0,098	-	0,104	-	0,133	-							- - -	- - -	- - -	- - -	- - -	- - -	-			
	Control	2	0	0	-	-	0,157	-	0,062	-	0,094	-	0,076	-	0,11	-	0,097	-							- - -	- - -	- - -	- - -	- - -	- - -	-			
	Control	3	0	0	-	-	0,195	-	0,084	-	0,08	-	0,097	-	0,184	-	0,12	-							- - -	- - -	- - -	- - -	- - -	- - -	- -			
	Control	4	0	0	-	-	0,095	-	0,106	-	0,083	-	0,077	-	0,168	-	0,115	-	0,105	-	0,168	-	0,124	-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	-
	Control	5	0	0	-	-	0,282	-	0,068	-	0,112	-	0,114	-	0,079	-	0,088	-	0,098	-	0,189	-	0,14	-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- -
	Control	6	0	0	-	-	0,106	-	0,106	-	0,064	-	0,074	-	0,07	-	0,087	-	0,079	-	0,095	-	0,097	-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- n.d. -
	Vaccinated	7	0	0	+ (non-infiltrative)	-	n.d.[5]		n.d.		n.d.		0,129	-	0,114	-	0,125	-							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	+ - -	-			
	Vaccinated	8	0	0	-	-	n.d.		n.d.		n.d.		0,163	-	0,204	-	0,096	-							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	-			
	Vaccinated	9	0	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,123	-	0,197	-	0,136	-							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- -			
	Vaccinated	10	0	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,082	-	0,122	-	0,14	-	0,279	-	0,361	-	0,284	-	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- - -	- - +	- - -	-
	Vaccinated	11	0	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,091	-	0,143	-	0,174	-	0,187	-	0,294	-	0,49	-	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- - -	- - -	- - -	- -
Chicken	Vaccinated	12	0	0	-	-	n.d.		n.d.		n.d.		0,119	-	0,161	-	0,179	-	0,162	-	0,22	-	0,215	-	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- - -	- - -	+ + -	- n.d. -
	Infected	13	4	0	+ (infiltrative)	-	n.d.		n.d.		n.d.		0,085	-	0,245	-	0,375	-											- - -	- - -	-			
	Infected	14	0	0	-	-	n.d.		n.d.		n.d.		0,083	-	0,2	-	0,116	-											- - -	- - -	-			
	Infected	15	4	2	+ (infiltrative)	-	n.d.		n.d.		n.d.		0,121	-	0,177	-	0,277	-											- - -	- - -	- -			
	Infected	16	2	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,198	-	0,19	-	0,188	-	0,198	-	0,206	-	0,319	-	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- - -	- - +	- - -	+
	Infected	17	2	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,14	-	0,197	-	0,195	-	0,143	-	0,326	-	0,292	-	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - +	- - -	- - -	- - +	- - -	- -
	Infected	18	2	0	-	-	n.d.		n.d.		n.d.		0,086	-	0,192	-	0,189	-	0,152	-	0,222	-	0,163	-	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- - -	- - -	- - -	- n.d. -
	Vaccinated/Infected	19	3	0	+ (non-infiltrative)	-	0,209	-	0,052	-	0,122	-	0,125	-	0,19	-	0,143	-							- - -	- - -	- - -	- + -	+ - +	+ + +	-			
	Vaccinated/Infected	20	1	0	+ (infiltrative)	-	0,117	-	0,162	-	0,072	-	0,139	-	0,101	-	0,507	-							- - -	- - -	- - -	- - -	- - -	- - -	+			
	Vaccinated/Infected	21	4	0	+ (infiltrative)	-	0,117	-	0,063	-	0,079	-	0,078	-	0,106	-	0,186	-							- - -	- - -	- - -	- - -	- - -	- - -	- -			
	Vaccinated/Infected	22	2	0	+ (infiltrative)	-	0,119	-	0,065	-	0,072	-	0,093	-	0,122	-	0,153	-	0,218	-	0,304	-	0,317	-	- - -	- - -	- - -	- - -	- - -	+ - -	- - +	- - -	- - -	-
	Vaccinated/Infected	23	1	0	-	-	0,098	-	0,056	-	0,07	-	0,138	-	0,132	-	0,102	-	0,169	-	0,175	-	0,158	-	- - -	- - -	- - +	- - -	+ - -	- - -	- - -	- - -	- - -	- -
	Vaccinated/Infected	24	1	0	+ (non-infiltrative)	-	0,106	-	0,065	-	0,108	-	0,147	-	0,146	-	0,693	+	0,584	+	0,82	+	0,712	+	- - -	- - -	- - -	- - -	- - +	- - -	- - -	- - +	- - -	- n.d. -
	Control	1	0	0	-	-	0,14	-	0,12	-	0,089	-	0,098	-	0,103	-	0,228	-							- - -	- - -	- - -	- - -	- - -	- - -	-			
	Control	2	0	0	-	-	0,303	-	0,145	-	0,103	-	0,161	-	0,123	-	0,167	-							- - -	- - -	- - -	- - -	- - -	- - -	-			
	Control	3	0	0	-	-	0,141	-	0,096	-	0,091	-	0,091	-	0,13	-	0,25	-							- - -	- - -	- - -	- - -	- - -	- - -	- -			
	Control	4	0	0	-	-	0,16	-	0,165	-	0,15	-	0,126	-	0,116	-	0,136	-	0,161	-	0,154	-	0,204	-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	-
	Control	5	0	0	-	-	0,242	-	0,142	-	0,098	-	0,105	-	0,111	-	0,201	-	0,107	-	0,201	-	0,188	-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- -
	Control	6	0	0	-	-	0,319	-	0,115	-	0,091	-	0,168	-	0,104	-	0,149	-	0,21	-	0,167	-	0,199	-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- n.d. -
	Vaccinated	7	0	0	-	-	n.d.		n.d.		n.d.		0,095	-	0,156	-	0,168	-							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	-			
	Vaccinated	8	0	0	-	-	n.d.		n.d.		n.d.		0,111	-	0,187	-	0,237	-							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	-			
	Vaccinated	9	0	0	-	-	n.d.		n.d.		n.d.		0,111	-	0,22	-	0,241	-							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- -			
	Vaccinated	10	0	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,305	-	0,194	-	0,18	-	0,173	-	0,197	-	0,722	+	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- - -	+ + +	+ + -	+
Turkey	Vaccinated	11	0	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,091	-	0,154	-	0,197	-	0,138	-	0,399	+	0,475	+	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- + -	- - +	+ + -	- + -	- -
	Vaccinated	12	0	0	+ (non-infiltrative)	-	n.d.		n.d.		n.d.		0,138	-	0,136	-	0,158	-	0,084	-	0,196	-	0,525	+	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	- - -	- + +	+ + +	+ + +	+ n.d. -
	Infected	13	4	0	-	-	n.d.		n.d.		n.d.		0,134	-	0,398	+	0,655	+							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	+ - +	-			
	Infected	14	3	0	+ (infiltrative)	-	n.d.		n.d.		n.d.		0,142	-	0,158	-	0,509	+							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - +	+ - +	-			
	Infected	15	4	0	+ (infiltrative)	-	n.d.		n.d.		n.d.		0,128	-	0,129	-	0,505	+							n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.	- - -	+ - -	- -			
	Vaccinated/Infected	19	4	0	+ (infiltrative)	-	0,182	-	0,1	-	0,102	-	0,163	-	0,193	-	0,242	-							- - -	- - -	- - -	- - +	- - +	- - -	+			
	Vaccinated/Infected	20	2	0	+ (infiltrative)	-	0,214	-	0,185	-	0,26	-	0,36	+	0,675	+	1,273	+							- - -	- - -	- - +	+ + -	- - +	+ + -	+			
	Vaccinated/Infected	21	4	0	+ (infiltrative)	+	0,172	-	0,088	-	0,146	-	0,48	+	0,554	+	1,147	+							- - -	- - -	- - -	- -	+ + +	- - -	- +			
	Vaccinated/Infected	22	3	0	+ (infiltrative)	-	0,123	-	0,086	-	0,097	-	0,183	-	0,344	-	0,811	+	0,422	+	0,728	+	0,81	+	- - -	- - -	- + -	+ - -	- - +	- - +	- + +	+ + -	+ + -	+
	Vaccinated/Infected	23	3	0	+ (infiltrative)	-	0,152	-	0,102	-	0,204	-	0,404	+	0,815	+	0,724	+	0,726	+	0,409	+	0,401	+	- - -	- - -	- - -	- - -	- - +	+ + +	+ - -	+ + +	- + +	+ +
	Vaccinated/Infected	24	1	0	+ (infiltrative)	-	0,315	-	0,174	-	0,128	-	0,212	-	0,616	+	0,64	+	0,821	+	0,375	+	0,871	+	- - -	- - -	- - -	- - +	- - -	+ - -	+ + +	+ + +	+ + -	+ n.d. +

[1] Lesion scoring (LS) system from 0 to 4 was applied; Cecum: 0 = no pathological changes; 1 = sporadic inflammation and/or mild thickening of the wall of one cecum; 2 = sporadic inflammation and/or mild thickening of the wall of both ceca; 3 = inflammation of both ceca and thick ening of the intestinal wall with liquid fibrin or sporadic fibrinous coagula in the lumen. If only one cecum was affected, then lesion score 2 was applie d; 4 = severe inflammation and necrosis in both ceca with compact fibrinous masses in the lumen of the ceca. If only one cecum was affected, then lesion sco re 3 was applied. Liver: 0 = no pathological changes; 1 = a few single punctiform necrosis up to 1 mm; 2 = single punctiform necrosis disseminated through out the organ up to 1 mm or a few single punctiform necrosis more than 1mm; 3 = single punctiform necrosis, disseminated throughout the organ more than 1 mm or some large area necrosis; 4 = confluent necrosis throughout the organ.

[2] Detection of the parasite in cecum and liver was performed by immunohistochemistry.

[3] Detection of *H. meleagridis*-specific antibodies by ELISA; - indicates O.D. values below threshold of positivity; + indicates O.D. values above threshold of positivity.

[4] Detection of *H. meleagridis* by re-isolation of viable cells from cloacal swab samples.

[5] n.d. indicates not done.

4. Discussion

4.1. Experimental design for reproducing histomonosis

One of the most remarkable features of histomonosis is the vastly different severity of the disease comparing chickens to turkeys. While chickens mainly show mild or no clinical signs with full recovery, this disease is far more severe and often fatal in turkeys (Tyzzer, 1934). Previously, it was shown that an *in vitro* clonal culture of an attenuated *H. meleagridis* strain provides protection against histomonosis (Hess et al., 2008). Later on, several studies analyzed its pathogenesis compared to the virulent strain (Liebhart et al., 2017). Parameters for monitoring the progression of histomonosis include clinical signs, characteristic lesions in the cecum and liver, detection of the parasite by IHC, specific serum antibodies and re-isolation from cloacal swabs. In previous studies, it was shown that the humoral immune response by production of serum antibodies was not protective against histomonosis. Turkeys were passively immunized by an intraperitoneal inoculation with pooled, neutralizing antisera containing *H. meleagridis*-specific antibodies from prior infected donor turkeys. Successful transfer of antisera was confirmed by heightened serum antibody titers compared to non-inoculated controls but passive immunization did not lead to protection of birds following a histomonad challenge infection (Clarkson, 1963; Bleyen et al., 2009). Hence, cellular immune responses were concluded to play key roles in protection. Studies on the immune response against this parasite are scarce and especially the *H. meleagridis*-specific T-cell immune response including involved T-cell subsets and their cytokine production was not investigated.

In this project, an initial pilot experiment with a reduced number of chickens and turkeys was performed in order to establish and evaluate assays for detection of cytokine-producing T cells. IFN-γ (type-1 cytokine) and IL-13 (type-2 cytokine) were analyzed using the intracellular cytokine staining and the PrimeFlow^{TM} RNA assay, respectively. For the pilot experiment in the chicken, birds were infected with the virulent strain of the monoxenic *H. meleagridis* culture. A more pronounced immune response was to be expected upon infection compared to vaccination giving the best conditions for sufficiently evaluating assay establishment. On the contrary, turkeys were vaccinated with an attenuated monoxenic *H. meleagridis* culture. This approach was applied in order to evaluate both euthanasia time points (two and five weeks p.i.) since earlier studies showed high mortality rates for infected turkeys within two weeks p.i.

Inoculated birds of both species were compared to naïve controls. On a weekly basis, sera for detection of *H. meleagridis*-specific antibodies and cloacal swabs for monitoring parasite

shedding were collected in addition to daily clinical examination. Birds were euthanized at two different time points (two and five weeks p.i.) to compare early to late phases of histomonosis. Cecum and liver of sacrificed birds were screened for pathological changes according to an earlier established lesion scoring system (Windisch and Hess, 2010; Zahoor et al., 2011). Apart from that, samples from both organs were investigated by IHC for detecting parasite presence according to the protocol of Singh et al. (2008). To study the T-cell immune response, lymphocytes from spleens and livers were isolated and analyzed for cytokine production within $CD4^+$, $CD8\beta^+$ and non-CD4/CD8 T cells by flow cytometry. To get a comprehensive overview, the spleen was selected to study the systemic immune response while the liver as one of the main affected organs was chosen for studying the local immune response. On that note, it has to be mentioned that the cecum as the primary site of infection would be more insightful for investigating the local responses. Obstacles in culturing sufficient amounts of intraepithelial lymphocytes from cecal tissue could not be overcome during this project but might be of interest in future experiments. Findings acquired during the pilot experiment with chickens were published in Veterinary Research (Lagler et al., 2019). Data obtained from the turkey pilot experiment were not published but will be summarized briefly within this chapter of the PhD-thesis. In the subsequently performed main experiments, the focus was set on studying IFN-γ-producing T cells only as the pilot experiments revealed a negligible role of IL-13-producing T cells. The staining panel was expanded in order to identify all major T-cell subsets ($CD4^+$, $CD8\alpha^+$ and γδ T cells). In addition, the immune response following inoculation with both monoxenic strains, attenuated and virulent, was investigated in order to elucidate vaccine induced antigen-specific T-cell subsets following a challenge infection. Hence, chickens (C) and turkeys (T) were either vaccinated with attenuated *H. meleagridis* (VC and VT), infected with virulent *H. meleagridis* (IC and IT) or vaccinated/infected with both strains (VIC and VIT) in comparison to naïve control birds (CC and CT). Results from the main experiment of chickens and turkeys were combined in one manuscript, which has been published in Developmental and Comparative Immunology (Lagler et al., 2020).

4.2. Pathology, parasite detection and antibody development

Corroborating other studies, chickens from the pilot and the main experiment including all inoculation groups did not develop any clinical signs or mortalities. Cecal lesions up to score 4 were observed in birds of the IC and VIC group but not within the VC group, which is largely in agreement with previous data (Zahoor et al., 2011; Liebhart et al., 2013; Mitra et al., 2017). However, Mitra et al. (2017) detected mild to moderate lesions in the cecum of birds from the

VC group inoculated with the xenic strain. In chickens, lesions in the liver are in general less common than in the ceca, though one bird from the pilot experiment and one bird from the main experiment of the IC group developed a score of 2. Studies using the xenic parasite culture showed a slight tendency to higher liver lesion scores in chickens of the IC group as well as the VIC group in comparison to the monoxenic strain applied in the studies of this project (Zahoor et al., 2011; Liebhart et al., 2013; Mitra et al., 2017). Histomonads detected by IHC were present in the ceca of birds from all inoculation groups, but not each bird was found positive. In coherence with earlier studies, in cases with lower lesions scores a positive detection by IHC was not always possible (Liebhart et al., 2011; Zahoor et al., 2011). Positive titers for *H. meleagridis*-specific antibodies were found in three birds of the IC group (pilot experiment) and in one bird of the VIC group (main experiment). This is in contrast to other publications using xenic cultures, in which positive antibody titers in sera were first observed for birds of the VC group at 8 weeks post vaccination, the IC group at 2 weeks post infection and the VIC group at 1 week post challenge (Liebhart et al., 2013). It can be concluded that the xenic parasite culture independent from number of passages might induce an earlier and stronger induction of antibody production in the serum compared to the monoxenic culture. This might be explained by a potentially better growth environment for the parasite under *in vitro* conditions provided by a diverse bacterial flora of the xenic culture, which in turn might also positively influence the parasites' proliferation in the host. On this note it should be highlighted that serum antibodies are playing a minor role in the immune response since they do not provide efficient protection in turkeys against histomonosis (Clarkson, 1963; Bleyen et al., 2009). As known from other investigations on histomonad shedding via the feces, an intermittent excretion of parasites can be observed (Zahoor et al., 2011). In our work, parasites could be successfully re-isolated from two infected birds (pilot experiment) and from birds of all inoculation groups (main experiment) but not at each sampled time point. Initial vaccination resulted in overall higher rates of positive fecal parasite detection upon challenge infection (five birds of VIC group) in comparison to infection alone (two birds of IC group). Birds from the non-inoculated control group were negative for all investigated histomonads related parameters.

Similar to a previous study applying the same monoxenic parasite culture, infected turkeys of the main experiment did not exhibit clinical signs and did not die within the study period of 2 weeks p.i. Ganas et al. (2012) investigated the progression of histomonosis of infected turkeys beyond 2 weeks p.i. and could show mortalities starting at 3 weeks p.i. leading to 100%

mortality at 4 weeks p.i. Even though for our studies a 60-fold higher parasite load (6 x 10^5) for inoculation was used compared to the previous study (1 x 10^4) no increase in the severity of the disease was observed in our experiment comparing the same study period (Ganas et al., 2012). On the contrary, Mitra et al. (2017) studying turkeys infected with the xenic culture showed that clinical signs including diarrhea, depression and ruffled feathers started to develop around 7 days p.i. Within 14 days p.i. all birds of the IT group died or had to be euthanized due to severe symptoms. In comparison to our studies, the same amount of histomonad cells (6 x 10^5) applied orally and via the cloacal route at four weeks of life was used for infection (Mitra et al., 2017). When infecting turkeys only cloacally with lower doses (1 x 10^4) of the xenic strain at the same age fatal cases were detected delayed starting at 3 weeks p.i. and reaching 100% mortality at 4 weeks p.i. (Liebhart et al., 2010). These findings highlight in difference to infection with the monoxenic strain that doses and application routes potentially do effect the progression of mortality when using the xenic strain for infection. In our work, none of the birds from the VT group (pilot and main experiment) showed any signs while three birds of the VIT group (main experiment) exhibited mild clinical signs such as half-closed eyes and slight depression. However, these birds of the VIT group recovered fully within three days and no fatalities were occurring. These findings are coherent with earlier results, where turkeys from the VT and VIT group also showed no clinical signs of histomonosis (Liebhart et al., 2010; Mitra et al., 2017). Cecal lesions reaching maximum scores were detected in birds of the IT and VIT groups (main experiment), which is concordant with other studies (Mitra et al., 2017). However, within the same studies lesions in the cecum were found for some birds of the VT group, which were not detected in our work (main and pilot experiment). Lesions in livers were not found in any inoculation group (main and pilot experiment). Contrariwise, turkeys infected with the virulent xenic parasite culture developed severe liver lesions whereas attenuated histomonads caused only mild pathological changes in the same organ following a high inoculation dosage (6 x 10^5 histomonads per bird) (Mitra et al., 2017). Consistent with previous findings is the detection of parasites in the cecum by IHC in turkeys from all inoculation groups, but not all individual turkeys tested positive (Singh et al., 2008; Sulejmanovic et al., 2013; Sulejmanovic et al., 2016). In addition, the liver of one bird from the VIT group was identified positive by IHC (main experiment). Coherent with other studies, serum antibodies specific to *H. meleagridis* were perceived within all turkey inoculation groups at various sampling time points (Windisch and Hess, 2009; Liebhart et al., 2010). As previously observed, birds from all groups showed parasite shedding, although for some birds re-isolation of parasites was not possible at any time point (Liebhart et al., 2010; Ganas et al., 2012; Mitra et

al., 2017). The attenuated vaccine strain required more time in the VT group (4 weeks p.i.) compared to the virulent challenge strain in the IT group (2 weeks p.i.) until for each sampled turkey parasite shedding could be detected. Prior vaccination led to even earlier parasite detection in the feces after challenge infection. All birds of the VIT group were detected positive for histomonads shedding within 1 week p.i. Of note, comparing the attenuated and virulent *H. meleagridis* strain a difference was discovered in parasite detection by IHC. In our work, turkeys of the VT group inoculated with the attenuated strain were only found positive in the cecum at five weeks p.i. (pilot and main experiment). Hence, it can be speculated that the attenuated in contrast to the virulent monoxenic strain needs considerably more time to establish properly in the host. Turkeys from the control group were found negative in terms of all above-mentioned parameters.

To summarize, comparing studies applying inoculations with xenic and monoxenic cultures independent of number of passages (virulent versus attenuated) histomonosis related parameters (clinical signs, liver and cecum lesions, parasite detection by IHC, serum antibodies, re-isolation of histomonads from cloacal swabs) are fairly similar in the early phase after infection. However, at later time points, they are slightly less pronounced for inoculations with the monoxenic strain. It was suggested that the xenic parasite culture resembles more closely the intestinal microbiota of the host and overall higher bacterial counts were found in xenic *in vitro* cultures compared to monoxenic cultures. This resulted in a better growth environment for the parasite and faster proliferation rates of *H. meleagridis* (Ganas et al., 2012).

4.3. Th1 and Th2 T cell differentiation in birds

For mammalian species, it has been shown that naïve $CD4^+$ T helper (Th) cells are differentiating into different cell types depending on the exposure to different pathogen types. This is mediated by cytokines present in the local microenvironment produced by antigen presenting cells or other activated innate immune cells. During the early phase of T-cell activation, the presence of such cytokines triggers the JAK-STAT signaling pathway, which in turn activates specific transcription factors. Intracellular pathogens such as viruses and intracellular bacteria typically drive polarization towards Th1 cells with the capability of IFN-γ production. The required cytokine milieu needed for type-1 differentiation of naïve T cells consists predominantly of IFN-γ and IL-12. The expression of the transcription factor T-bet is induced by IFN-γ activated STAT1, which in turn results in IFN-γ expression of the T cell. IL-12 promotes STAT4 expression leading to further up-regulation of T-bet expression. Via IFN-

γ production of Th1 cells a positive feedback loop leads to a continuous differentiation of naïve T cells into Th1 cells (Rengarajan et al., 2000; Yang et al., 2007; Annunziato et al., 2014). On the contrary, infections with helminth worms induce type-2 immune responses with production of IL-4, IL-5 and IL-13. Initially, IL-4 is required for STAT6 activation that drives the expression of the transcription factor GATA-3. This cascade induces IL-4 and IL-13 expression. These cytokines contribute to anti-helminth reactions and class switching of antibodies to IgE. Also, allergies are associated with Th2 immune responses via the involvement of eosinophils, basophils and mast cells (Chapoval et al., 2010; Lloyd and Snelgrove, 2018). However, in birds some Th2 related components such as basophiles or mast cells only occur at very low frequencies while functional eosinophils and IgE seems to be missing (Zhao et al., 2000; Kaiser et al., 2005). For the first time, Degen et al. (2005) described Th1 and Th2 differentiation of T cells in the chicken. Chickens were infected either with a viral pathogen (New Castle disease virus) or with a helminth parasite (*Ascaridia galli*). At several time points, spleen and ileal tissue were collected for RNA isolation to perform semi-quantitative RT-PCR to detect the signature type-1 cytokine IFN-γ and the type-2 cytokines, IL-4 and IL-13. The viral infection led to significant elevated IFN-γ mRNA levels in the spleen and slightly less pronounced in the ileal tissue. On the other hand, the helminth infection showed significant heightened IL-13 mRNA levels and to a lower extent IL-4 mRNA levels. This observation was more prominent in the ileal tissue than in the spleen. From this study, it can be concluded that Th1 and Th2 polarization seems to be evolutionary conserved between mammals and birds. However, IL-13 was suggested to be the predominant type-2 cytokine in birds, unlike IL-4 as seen in mammals.

With respect to the extracellular presence of the parasite *H. meleagridis*, a polarization towards Th2 differentiated T cells was hypothesized for chickens and turkeys. This hypothesis was further supported by a study from Powell et al. (2009b). These authors showed for infected chickens that during the early phase of infection numerous cytokine mRNA levels, including IFN-γ and IL-13, were up-regulated while at later stages only IL-13 mRNA levels remained heightened compared to non-infected animals. A similar phenomenon was found for another extracellular protozoan parasite, *Trypanosoma brucei*, where an early rise of IFN-γ-producing cells was detected followed by a concomitant increase of IL-4- and IL-5-producing cells at later stages of infection (Zhang and Tarleton, 1996). However, for most protozoans such as *Leishmania* or *Toxoplasma gondii*, all of which of intracellular occurrence, predominantly type-1 immune responses were developed (Silva-Barrios and Stäger, 2017). Hence, in this

project, Th1 and Th2 cytokines produced by T cells in the course of histomonosis were investigated upon establishment of suitable assays for detection.

4.3.1. Establishment of the intracellular cytokine staining assay for detection of IFN-γ in the chicken and turkey

For studying cytokines produced by antigen-specific T cells, the intracellular cytokine staining assay is a powerful tool. ICS assays, widely applied in human and mouse immunology, were only utilized in a limited number of studies in poultry so far. As a first approach, Ariaans et al. (2008) investigated chicken IFN-γ-producing cells applying the commercially not available antibody mAb80 in ICS assays. Lymphocytes isolated from blood and spleen were stimulated with either PMA/ionomycin or ConA. Significant but fairly low increments of IFN-γ^+CD4$^+$ and IFN-γ^+CD8α^+ cells were detected compared to the non-stimulated controls. PMA/ionomycin stimulated lymphocytes showed higher numbers of IFN-γ^+ cells within the CD8α^+ compared to the CD4$^+$ subset. Authors suggested a potential activation of IFN-γ-producing NK cells by this stimulation, which might have contributed to the heightened numbers of IFN-γ^+CD8α^+ cells. On the contrary, ConA stimulated samples did not differ in the frequency of IFN-γ^+CD4$^+$ and IFN-γ^+CD8α^+ subsets. In another study, the self-generated mAb EH9 was compared to the before-mentioned mAb80 clone for suitability of detecting chicken IFN-γ. Both mAbs were tested on PMA/ionomycin stimulated splenocytes and on an IFN-γ transfected CHO cell line. Similar IFN-γ^+ cell frequencies could be identified by ICS comparing both mAbs (Ruiz-Hernandez et al., 2015). In a study by Andersen et al. (2017) several antibodies specific to chicken IFN-γ were tested for their suitability in ICS assays as follows: capture and detection antibody of the ELISA kit Chicken IFN-γ CytoSet™ (Invitrogen), mAb80 and a polyclonal rabbit anti-chicken IFN-γ serum (BioRad). CHO cells transfected with chicken IFN-γ DNA were used for the screening of the before-mentioned antibodies. All tested antibodies could identify similar IFN-γ^+ cell frequencies with best results given by the capture antibody from the ELISA kit and the mAb80 clone.

Since the above-mentioned antibodies were not commercially available or deemed less suitable due to their polyclonality, for this work a panel of six anti-chicken mAbs specific to IFN-γ were investigated for their suitability in ICS assays as follows: clones: 2B7, 11G5, 7E3, 12F12 (all mouse IgG1), clone: 12F7 (mouse IgG2a) and clone: 12D4 (mouse IgG2b). The mAbs used in this study were provided by Sciensano (Belgium, Brussels) and previously successfully tested in ELISA experiments (Lambrecht et al., 2004). Freshly isolated splenocytes from chickens were stimulated with PMA/ionomycin and subjected to FCM analyses. Three mAbs

with IgG1 (2B7, 11G5, 7E3) and one mAb with IgG2a (12F7) isotype used in combination with isotype-specific fluorescent secondary antibodies identified similar percentages of IFN-γ^+ T cells. Most of these cells co-expressed CD4 (see Additional file 3, Lagler et al., 2019). For the pilot infection experiments in the chicken, IFN-γ clone 11G5 was used in the ICS assays. The ensuing main chicken experiments, which comprised an extended number of animals and an expanded staining panel, demanded the use of an IFN-γ clone with an IgG2a isotype (12F7). Due to a 97% amino acid sequence identity of IFN-γ between chickens and turkeys, the anti-chicken IFN-γ mAbs were screened for their suitability in ICS assays for the turkey as well. In detail, four IgG1 clones (2B7, 7E3, 7E12, 11G5) and one IgG2a clone (12F7) were analyzed by FCM using PMA/ionomycin stimulated splenocytes isolated from the turkey (own unpublished findings). Among all tested mAbs, clone 7E12 showed the best reactivity and was subsequently used for the main turkey experiments.

4.3.2. Establishment of PrimeFlow™ RNA assay for detection of IL-13 in the chicken and turkey

As no mAbs specific to chicken IL-13 suitable for ICS are available to date, IL-13 was investigated only on the mRNA level by qPCR so far (Powell et al., 2009b; Kidane et al., 2018). Hence, in this project a PrimeFlow™ RNA assay was developed allowing the identification of IL-13 mRNA on the single level by flow cytometry. A custom-made target probe specific to chicken IL-13 was purchased and scrutinized for its suitability to detect IL-13 mRNA$^+$ cells. Therefore, transfection of immortalized human epithelial 293 kidney cells with chicken IL-13 DNA was performed and cells were stained with the PrimeFlow™ RNA kit according to manufacturer's instructions. The specific target probe was able to stain a distinct IL-13 mRNA-expressing cell population within the transfected cells. In control samples, one without the addition of the label probe and one using cells transfected with an irrelevant plasmid, no positively labelled cell population could be identified (see Additional file 5, Lagler et al., 2019). Hence, the PrimeFlow™ RNA assay was considered as a reliable tool for studying this cytokine on the single cell level. This method was applied in the pilot experiments of the chicken and turkey.

4.3.3. Validation of established cytokine detection assays in *H. meleagridis* re-stimulation experiments

Only a few studies in the chicken analyzed the potential of antigen-specific re-stimulated T cells for cytokine production by flow cytometry. Further, all published studies addressed solely IFN-γ with no research being done on type-2 cytokines such as IL-13. Ruiz-Hernandez et al.

(2015) showed an increase in avian influenza virus re-stimulated IFN-γ-producing splenocytes co-cultured with chicken kidney cells from influenza virus vaccinated chickens (Ruiz-Hernandez et al., 2015). PBMC from Newcastle disease virus (NDV) vaccinated chickens were analyzed for their frequency and phenotype of IFN-γ^+ cells following NDV re-stimulation using the mAb80 antibody. This study demonstrated a significant increase of IFN-γ-producing cells among the $CD3^+CD8\alpha^+$ subset compared to naïve control birds. In addition, a large population of $CD3^-$ cells produced IFN-γ indicating potential involvement of NK cells, monocytes and thrombocytes (Andersen et al., 2017). Another protozoan parasite, *Eimeria tenella*, which includes intracellular stages in the reproduction cycle, was studied for IFN-γ production by ICS using a non-commercial mAb. Chickens orally immunized with an *E. tenella* live vaccine showed elevated levels of IFN-γ^+ cells within $CD4^+$ and $CD8^+$ T-cell subsets in the spleen (Huang et al., 2011).

In this work, both assays, detection of IFN-γ by ICS and IL-13 by PrimeFlow™ RNA, were scrutinized in controlled infection/vaccination experiments. *Histomonads*-antigen re-stimulated splenocytes and intrahepatic lymphocytes (IHL) from *H. meleagridis* infected chickens were compared to control animals. For IFN-γ, significant increases could be found within the $CD4^+$ and the $CD4^-CD8\beta^-$ subset of the spleen from histomonads infected chickens. Based on the concept that γδ T cells in the chickens can be subdivided according to their CD8 expression ($CD8\alpha^{hi+}\beta^+$, $CD8\alpha\alpha^{hi+}$ and $CD8^-$) it can be assumed that the $CD4^-CD8\beta^-$ subset includes γδ T cells. Also, earlier studies reported that γδ T cells seem to be potent IFN-γ producers (Tregaskes et al., 1995; Berndt et al., 2006). No such increment was found for IL-13 mRNA-producing T cells in the spleen or liver. This finding is in coherence with another study showing extremely low frequencies of IL-13 mRNA-expressing cells in the spleen, liver and cecum of *H. meleagridis* infected chickens (Kidane et al., 2018). Next to antigen-specific re-stimulation, lymphocytes from spleen and liver were also stimulated with PMA/ionomycin in order to further validate IL-13 detection by the PrimeFlow™ RNA assay. Significant increases of IL-13 mRNA-producing splenocytes were found upon PMA/ionomycin stimulation compared to non-treated controls (see Figure 4, Lagler et al., 2019). Alongside to the chicken, the same experimental setup was applied in turkeys using the attenuated *H. meleagridis* strain for vaccination. Minor increases of IFN-γ^+ liver cells were detected within the $CD4^+$ and non-CD4 T cells of vaccinated compared to naïve turkeys. Similar to infected chickens, very low frequencies of IL-13 $mRNA^+$ lymphocytes from spleen or liver were found with no difference between treated and control turkeys. In addition, stimulation of lymphocytes with

PMA/ionomycin did not lead to significant differences of IL-13 mRNA-producing lymphocytes compared to non-stimulated controls (own unpublished findings).

Hence, the data of this project suggest that despite the extracellular occurrence of *H. meleagridis*, type-1 immune responses identified by IFN-γ production and mainly driven by $CD4^+$ T cells prevail over cells producing the type-2 cytokine IL-13. In accordance with earlier studies, an early rise of IFN-γ $mRNA^+$ cells in the cecum seems to be crucial in providing protection against histomonosis (Kidane et al., 2018). Hence, the focus of further analyses for studying the immune response towards *H. meleagridis* was set on IFN-γ.

4.4. *H. meleagridis*-specific T-cell response in the chicken and turkey

For the main experiment, IFN-γ production by all major T-cell subsets was investigated. IFN-γ^+CD4^+ Th1 cells were proven to play a key role in the immune response against *H. meleagridis* as indicated by findings from the pilot experiment. Putative CTLs producing IFN-γ were also investigated since an involvement was shown also for other intracellular protozoans (Jongert et al., 2010). However, how $CD8^+$ T cells recognize their antigen in regard to extracellular pathogens is not fully understood yet. Next to $CD4^+$ and $CD8^+$ T-cell subsets, IFN-γ-producing γδ T cells were studied as they are known to be potent cytokine producers in the chicken (Pieper et al., 2008). In order to identify differences in immunity against histomonosis with and without prior vaccination the following groups of chickens (C) and turkeys (T) were studied: vaccination (VC/VT) with the attenuated histomonad strain, infection (IC/IT) with the virulent histomonad strain and vaccinated/infected (VIC/VIT) with both strains were compared to non-infected control birds (CC/CT).

In agreement with the pilot infection experiment in the chicken, in the main experiment significant increases of IFN-γ^+ T cells were detected within the $CD4^+$ splenocytes from chickens of the IC group compared to control birds. In addition, for some chickens of the IC group a rise in IFN-$\gamma^+TCR\gamma\delta^+$ of the spleen was observed. Next to the IC group, some birds of the VIC group sacrificed two weeks p.i. showed heightened IFN-γ^+ T-cell levels within the $CD4^+$ and $CD8\alpha^+$ T-cell subsets of spleen and liver. As this trend was not seen for chickens of the VC group, it can be speculated that the virulent in contrast to the attenuated histomonad strain induced these responses. Overall, fairly weak systemic immune responses could be found upon vaccination and subsequent challenge infection compared to chickens infected only. It can be hypothesized that priming of the immune system by vaccination provided efficient protection from the challenge infection, which restricted the virulent parasite strain at the local

mucosal site and prevented it from spreading to systemic locations such as the spleen. Apart from that, another protozoan pathogen, *Eimeria tenella*, induced an emigration of responsive T cells from the spleen to the local site of infection (Rothwell et al., 2000). Our findings concerning an increment of IFN-γ^+ splenocytes within the IC and VIC group differ somewhat from a previously published study that could not detect any significant differences at two weeks p.i. in those inoculation groups (Kidane et al., 2018). Of note, at three weeks p.i. even a significant drop of IFN-γ mRNA$^+$ cells in the spleen from birds of the IC and VIC group was observed in the before-mentioned publication. A potential explanation for this discrepancy might be the different approaches for IFN-γ detection. While for this work IFN-γ, produced by *in vitro* re-stimulated putative memory and effector T cells, was identified on the protein level, Kidane et al. (2018) used the *in situ* hybridization technique to detect IFN-γ mRNA-expressing cells. Also as seen in the pilot experiments, no significant induction of local liver resident cytokine-producing lymphocytes could be found in any of the inoculated chicken groups. This finding is in coherence with previous observations, where restricted migration patterns of parasite cells into the liver due to an early pro-inflammatory cytokine up-regulation in the cecal tonsils was suggested for the chicken (Powell et al., 2009b). Also, in the study by Kidane et al. (2018) very low frequencies of IFN-γ mRNA$^+$ cells in the liver could be identified with no significant differences between inoculated chickens.

In difference to the chicken, an intense systemic immune response was mounted in turkeys of the VIT group. Significant rises of IFN-γ^+ T-cell frequencies in comparison to turkeys of the control group were detected within all major T-cell subsets of the spleen. Next to the spleen, significant elevated levels of IFN-γ-producing CD4$^+$, CD8α$^+$ and CD3ε$^+$CD4$^-$CD8α$^-$ T cells were found in the liver as well. It might be hypothesized that priming of the turkeys by the vaccination resulted in a limited protection by the local mucosal immune system. It cannot be excluded that during challenge infection some virulent parasite cells may be able to escape from the local site and reach systemic locations as well as the liver. This is also supported by positive detection of histomonads cells in the liver of one turkey of the VIT group. Since an involvement of these T-cell subsets was not found for turkeys of the VT group, the virulent challenge strain could also have a potential booster effect at the local and systemic site serving as another potential explanation for the strong responses in the VIT group. Besides, similar to chickens, turkeys of the IT group showed increased levels of IFN-γ^+CD4$^+$ T cells in the spleen. However, this finding did not reach significance. Our observations of the spleen are in contrast to the *in situ* hybridization study, which found decreased levels of IFN-γ mRNA$^+$ splenocytes

within the IT and VIT group at two weeks p.i. (Kidane et al., 2018). Regarding the liver, Kidane et al. (2018) detected overall extremely low frequencies of IFN-γ mRNA$^+$ cells in turkeys of the IT and VIT group, which is in coherence with our findings in case of the IT but not the VIT group.

Since tools for identifying T-cell subsets are extremely limited in turkeys, the direct identification of γδ T cells is currently not possible. Due to close genetic relationship of both species, the concept of classifying γδ T cells based on their CD8 expression in the chicken (CD8α^{hi+}β$^+$, CD8αα^{hi+}, CD8α$^-$β$^-$) may be applied to turkeys as well (Tregaskes et al., 1995). Based on this, it can be hypothesized that γδ T cells in the turkey are present within the CD3ε$^+$CD4$^-$CD8α$^+$ and CD3ε$^+$CD4$^-$CD8α$^-$ T-cell subsets.

The functional mechanisms of putative *H. meleagridis*-specific IFN-γ-producing CD8α$^+$ cytotoxic T cells are not fully elucidated in the context of extracellular parasites. Infections with other parasitic protozoans such as *Toxoplamsa gondii*, *Trypanosoma cruzi* or *Plasmodium* sp. sporozoites lead to an activation of CD8$^+$ T cells. IFN-γ-producing CD8$^+$ T cells are known to play key roles in immunity against the before-mentioned pathogens (Schofield et al., 1987; Tarleton, 1990; Jongert et al., 2010). However, those pathogens are of intracellular occurrence and antigen presentation takes place via the MHC class I molecule. Although specific intracellular pathways are not completely understood yet, the induction of CD8$^+$ T cells during a *H. meleagridis* infection might be explained by cross-presentation of histomonads antigens. Additionally, in protection against a coccidian parasite in chickens, *Eimeria tenella*, CD8$^+$ T cells are considered to be crucial in activation of macrophages (Breed et al., 1997). Also for some extracellular pathogens, an induction of antigen-specific CD8$^+$ T cells was reported. Mice infected with the helminth *Schistosoma mansoni* developed IFN-γ-producing CD8$^+$ T cells that helped to temper immunopathological events by counterbalancing IL-4 driven responses (Pedras-Vasconcelos and Pearce, 1996). IFN-γ-producing CD8$^+$ T cells are also known to be essential in immunity against the extracellular bacteria *Treponema pallidum* (Stary et al., 2010). Of note, the pilot infection experiment did not reveal an involvement of IFN-γ-producing CD8β$^+$ T cells in the chicken. However, it cannot be excluded that the *H. meleagridis*-reactive IFN-γ$^+$ cells within CD8α$^+$ T cells of the VIT group are negative for CD8β and hence do not represent cytotoxic T cells.

For a number of extracellular pathogens the involvement of γδ T cells in the immune response has been described. For clearance of the extracellular bacterium *Staphylococcus aureus* this T-

cell subset is of particular importance (Cheng et al., 2012). During the acute phase of a *Klebsiella pneumoniae* infection it was reported that γδ T cells, producing IFN-γ alongside to TNF-α, are essential in the immunity (Moore et al., 2000). Also, for protozoan infections such as malaria γδ T cells are responding with IFN-γ production at early blood stages of this disease (Inoue et al., 2013). Several aspects of γδ T cells including presentation and recognition of antigens as well as functional attributes are still not completely understood. In contrast to αβ T cells, mechanisms including presentation of non-peptide antigens facilitated via non-MHC pathways have been described for these "unconventional" T cells (Harly et al., 2015). Similar to the above addressed pathogens and potential ways of activation, IFN-γ-producing γδ T cells might act in similar manners regarding the immune response against *H. meleagridis*.

Another T lymphocyte subset with a CD4/CD8 double negative phenotype are iNKT cells which may be represented in the IFN-γ producing *H. meleagridis*-reactive subset identified in spleen and liver of the VIT group. This innate cell type is capable of producing vast amounts of cytokines in regards to bacterial, viral, fungal and protozoan infections (Brigl and Brenner, 2010). During the course of a cutaneous *Leishmania* infection in humans, this CD4/CD8 double negative T-cell subset was identified to be one of the strongest IFN-γ producers. About 75% harbored the TCRαβ lineage marker while the remaining 25% were TCRγδ$^+$. Those double negative T cells were highly activated with increased CD69 expression in diseased patients compared to healthy ones. The authors suggested that TCRαβ$^+$CD4$^-$CD8$^-$ T cells are responsible for macrophage activation and controlling of parasite burden while TCRγδ$^+$CD4$^-$ CD8$^-$ T cells exhibit more regulatory properties (Antonelli et al., 2006; Gois et al., 2018). For schistosomiasis, a murine helminth infection, activated IFN-γ-producing iNKT cells in the liver were suggested to play crucial roles in initiating and modulating the adaptive immune response (Mallevaey et al., 2006). From human and mouse immunology, it is known that iNKT cells respond to glycolipids of exogenous or endogenous origins such as bacteria, parasites, fungi as well as antigens derived from cancer, autoimmune and allergic diseases. Antigen recognition occurs in the context of the MHC-like molecule CD1d. CD1d is constitutively expressed by macrophages, dendritic cells, B cells and also on endothelial cells of sinusoids in the liver. Several ways of iNKT cell activation have been described including TCR-dependent and – independent ways, via NK cell receptor or expression of Toll like receptors (Chandra and Kronenberg, 2015). It is currently unknown whether iNKT cells do exist in avian species and what kind of antigen stimulates these cells, but from previous studies and the phenotype of *H.*

meleagridis reactive T cells identified in this work ($CD3\varepsilon^{+}CD4^{-}CD8\alpha^{-}$) it is conceivable that this cell subset might play a role in the immune response against histomonosis.

4.5. Conclusions

In conclusion, this work highlighted the difference in the *H. meleagridis* host-pathogen interaction between chickens and turkeys particularly upon vaccination and challenge infection. For the first time, the *H. meleagridis*-specific T-cell immune responses including their cytokine profiles were investigated in chickens and turkeys. Initial pilot experiments revealed a dominating type-1 immune response characterized by IFN-γ-producing $CD4^{+}$ T cells. Infected chickens with and without prior vaccination showed a rather weak systemic response including no induction of liver resident lymphocytes. This may suggest a strong activation of T cells at the local mucosal site of infection impeding histomonads to spread to the spleen or liver. On the contrary, vaccinated and subsequent challenge infected turkeys mounted a strong systemic immune response with an induction of IFN-γ-producing $CD4^{+}$, $CD8\alpha^{+}$ and $CD3^{+}CD4^{-}CD8\alpha^{-}$ splenic lymphocytes. This finding in addition to a re-activation of *H. meleagridis*-specific IFN-γ^{+} T cells in the liver led to the consideration that even with initial vaccination the mucosal immune response could not fully confine the infection to the cecum, even though no lesions could be observed in systemic organs.

4.6. Outlook

To acquire an insight into mucosal immune responses against *H. meleagridis*, analysis of cytokine-producing intraepithelial lymphocytes (IEL) isolated from the primarily affected organ, the cecum, would be desirable. Additionally, studying the immune response at time points earlier than investigated for this work might be informative. A more precise phenotyping, ideally with directly addressing γδ T cells in the turkey and markers for memory T-cell stages, would give an even deeper insight into the immune response against histomonosis as soon as suitable tools are available. Besides investigation of IFN-γ, a broader set of cytokines including pro-inflammatory as well as immunoregulatory cytokines (TNF-α, IL-17, IL-10, TGF-β) would be of interest. Due to a current lack of available mAbs to detect most of these cytokines by flow cytometry, RNA isolated from MACS sorted and *in vitro* histomonads-stimulated T-cell subsets could be subjected to RT-qPCR assays for screening cytokine RNA expression. Also, transcriptome analysis from sorted and antigen-specific re-stimulated single cells by scRNAseq might be useful for tissues, such as the cecum or cecal tonsils, with limited applicability in FCM-based assays requiring high cell yields. In addition,

this approach would overcome the necessity of currently not available mAbs for flow cytometric studies but might be of restrictive use due to incomplete annotation of all gene sequences of interest.

5. Summary

The aim of this PhD-project was to establish assays for detection of type-1 (IFN-γ) and type-2 (IL-13) cytokines to study the T-cell response towards *H. meleagridis* of chickens and turkeys. During the pilot experiments, intracellular cytokine staining (IFN-γ) and PrimeFlow^{TM} RNA (IL-13) assays could be successfully developed and further scrutinized in controlled infection/vaccination studies within the chicken and turkey, respectively. Clonal monoxenic histomonads cultures were applied for either infection using the virulent strain (low passage) or vaccination using the attenuated strain (high passage). Findings obtained from the pilot experiments in the chicken showed significant increases of *H. meleagridis*-specific IFN-γ-producing splenocytes within the $CD4^+$ and $CD4^-CD8\beta^-$ T-cell subset of infected chickens compared to naïve controls. For vaccinated turkeys, very slight increments of IFN-γ^+ liver cells were found within $CD4^+$ and non-CD4 T cells in comparison to non-treated control birds. On the contrary to IFN-γ, extremely low frequencies of IL-13 $mRNA^+$ cells were detected within spleens and livers of both species. As these observations suggested a dominating type-1 immune response towards *H. meleagridis,* the subsequent main experiments focused on IFN-γ detection employing an optimized staining panel for T-cell subsets. In addition, the T-cell immune responses of chickens and turkeys vaccinated and subsequent challenge infected were studied next to infected or vaccinated birds compared to non-inoculated control animals. While inoculation with the attenuated parasite strain seems to induce a rather weak systemic immune response in both species, the virulent strain leads to a strong systemic T-cell response in the spleen and in addition in the liver of vaccinated/infected turkeys. Significant increases of IFN-γ-producing splenocytes were detected within the $CD4^+$ T-cell subset isolated from infected chickens while no such differences were found in other T-cell subsets, inoculation groups or the liver. These findings suggest a strong involvement of responsive T cells at the local mucosal site of infection resulting in a retention of histomonads in the cecum. In contrast, vaccinated and infected turkeys showed an induction of IFN-γ-producing lymphocytes within all major T-cell subsets ($CD4^+$, $CD8\alpha^+$ and $CD3^+CD4^-CD8\alpha^-$) from spleen and liver, which indicates that immune responses at the mucosal site do not seem to be potent enough for restricting the infection to the ceca. To fully understand *H. meleagridis*-specific responses and potentially

identify correlates of protection, investigation of INF-γ-producing T cells at the mucosal site in addition to screening a broader panel of cytokines would be desirable as soon as suitable tools for detection are available.

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