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Expression of toll-like receptors in chicken cell lines of macrophages and hepatocytes following infection with *Histomonas meleagridis*

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1. Introduction and aim of the study

Histomonas meleagridis is a protozoan parasite and member of the genus *Histomonas*, order *Tritrichomonadida* (Hess and McDougald 2020). The parasite is the etiological agent of the disease histomonosis (syn. blackhead disease, histomoniasis, or infectious typhlohepatitis) (Tyzzer et al. 1920). *H. meleagridis* is a pathogen with a raising economic impact since the ban of different curative and preventive drugs against the disease to ensure food safety (Liebhart et al. 2017). It affects gallinaceous birds, especially turkeys (*Meleagris gallopavo*) and chickens (*Gallus gallus*). In turkeys the disease can cause high mortality while in chickens it is usually less severe (McDougald 2005). Clinical changes in turkeys include yellow faeces, drowsiness, dropped wings and anorexia (Hess and McDougald 2020). The mortality can be up to 100% (McDougald 2005). In chickens, severe economic losses are mainly caused by an impaired performance (Hess et al. 2015). The pathological changes in both poultry species can include lesions in the caecum and the liver (Hess and McDougald 2020).

As a consequence of an increasing number of outbreaks of histomonosis in poultry flocks since the ban of chemotherapeutics in Europe and many countries worldwide, research on new strategies against the disease resulted in the development of an efficacious live-attenuated vaccine (Hess et al. 2008). Nevertheless, further research is required to implement vaccination in the field (Liebhart et al. 2017).

Several studies focused on the immunity of poultry against *H. meleagridis* (Mitra et al. 2018, Lagler et al. 2019). However, so far there is no information on the role of chicken toll-like receptors (chTLRs) as part of the innate immune system to an infection with *H. meleagridis*. Knowledge about the initial events during the innate immune response following the infection would be of mandatory to further understand the immunological mechanisms triggered by the parasite. Therefore, the aim of this study was to investigate chTLRs expression in a chicken macrophage-like cell line (HD11) (Beug et al. 1979) and a leghorn male hepatoma cell line (LMH) (Kawaguchi et al. 1987) as response to *H. meleagridis* stimulation to get a step ahead towards understanding further traits relevant for protection against histomonosis.

2. Literature survey

2.1. Historical facts about histomonosis

More than a century ago histomonosis was firstly described by Cushman (1893) in turkeys. Two years later Smith (1895) described the causative protozoan of the disease as "*Amoeba meleagridis*". In the following years fundamental research on the aetiology of the disease and the morphology of the protozoan was performed by Tyzzer (1919, 1920), who recognized the flagellate character and re-named it *"Histomonas meleagridis*". In the following decades several studies deciphered the morphology of the parasite and the pathogenesis of the disease which is given below in detail.

2.2. *Histomonas meleagridis*

H. meleagridis is a flagellated amboid protozoan (Hess and McDougald 2020) and member of the genus *Histomonas*, in the family *Dientamoebidae* of the order *Trichomonadida*. It is an unicellular parasite with 3-12 µm in size. The cell organelles are typical for trichomonads, composed of an axosytle, a pelta, a costa and parabasal bodies (Liebhart et al. 2017). Instead of mitochondria the parasite utilizes hydrogenosomes for energy metabolism (Hess and McDougald 2020). The parasite is pleomorphic and in general two forms are known: i) the tissue form without flagella and ii) the flagellated caecal lumen form. The tissue form is present in penetrated organs of the host. The shape can be variable but mostly it appears round to ovoid. The caecal lumen form propagates in the caecum and has a round or amoeboid shape (Tyzzer 1919, 1920). Usually this form possesses a single flagellum, except during early cell division two of these organelles may occur (Honigberg and Benett 1971). For culturing purposes histomonads need different essential conditions and components supporting their growth. Concerning environmental conditions, a neutral pH and an anaerobic milieu are most suitable. Furthermore, serum, starch sources and nutritious culture media are necessary (Hauck et al. 2010). In the last mentioned review, the presence of bacteria was also described as an indispensable need, highlighting *Escherichia coli* (*E. coli*) as a bacteria of great importance enabling the growth of histomonads *in vivo* and *in vitro*.

2.3. Histomonosis

2.3.1. Etiology and host range

Histomonosis is defined as the disease caused by *H. meleagridis*. The most common synonym is blackhead disease based on an initial case report (Smith 1895). It affects mainly gallinaceous birds, including chickens, turkeys, quails and a variety of gamebirds (McDougald 2005). Histomonosis was also reported to infect other orders of *Aves*, for example ostriches and rheas which mostly suffer from a milder form of the disease (Dhillon 1983, Borst et al. 1985). Ducks may act as asymptomatic carriers (Hess and McDougald 2020).

2.3.2. Infection and pathology

The infection of host birds can either be directly from bird to bird or via the worm *Heterakis gallinarum* (Hess and McDougald 2020). *H. gallinarum* belongs to the phylum *Nematoda* mainly parasitizing in the caeca of gallinaceous birds (Eckert et al. 2012). Embryonated eggs of the ceacal worm can release histomonads in the intestine of infected host birds (Hess and McDougald 2020). The direct infection was experimentally shown by Hu et al. (2004) who hypothesized that the uptake of histomonads occurs via the cloaca. However, later on the successful oral infection of turkeys without the vector was demonstrated (Liebhart et al. 2009).

Regardless of the way of infection, histomonads replicate in the lumen and mucosa of the caecum, causing ulceration and inflammation of the caecal walls. Further pathological manifestations can be thickening of the caecal walls, bleeding in the mucosa, fibrinous masses in the caecal lumen and peritonitis. Within 2-3 days the parasites may infiltrate into the blood vessels and migrate to the liver via the hepatic-portal system (Hess and McDougald 2020). As a consequence, inflammation and tissue destruction can occur in caecum and liver. Anyhow, necrotic areas in the liver are commonly observed in turkeys suffering from histomonosis, while in chickens these lesions may be less severe (Hess et al. 2015).

Grabensteiner et al. (2006) demonstrated that *H. meleagridis* DNA also occurs in other organs including duodenum, jejunum, ileum, spleen, heart, lungs, brain and bursa of Fabricius, despite the absence of macroscopic lesion.

2.3.3. Clinical signs

Clinical manifestation of histomonosis shows some variability among chickens and turkeys. Turkeys can suffer more severe from the disease with a mortality up to 100 % (Hess et al. 2015). Signs of histomonosis include ruffled feathers, drooped wings, apathy and sulphur-coloured diarrhoea in later stages when the liver function is severely constrained. The cyanotic head which leads to the synonym “blackhead” is rarely seen in diseased turkeys. In chickens the outcome of histomonosis is milder: a decrease in weight gain leads to a loss of flock uniformity (Hess and McDougald 2020). In layers a substantial decrease in egg production can occur as shown by experimental infection (Liebhart et al. 2013b).

2.3.4. Therapy and prophylaxis

In the past, chemical substances such as arsenics or nitroheterocycles have been available to prevent or treat histomonosis. Later on, benzimidazole, nithiazide and quinolines showed a prophylactic effective against the disease whereas nitrothiazoles were effective for prevention and therapy and carbamates were applied for therapy. However, in most industrial countries no anti-histomonal drug is available for poultry to ensure food safety. The aminoglycoside antibiotic paromomycin showed a positive effect by prophylactic application and can be applied for poultry. However, antibiotics must not be administered prophylactically. To sum up, currently the most effective intervention to prevent outbreaks of histomonosis in poultry flocks is a strict adherence of biosecurity (Liebhart et al. 2017).

2.4. Immune response against *H. meleagridis*

Modulations of the adaptive and innate immune reaction of the host by pathogens are mostly responsible for the outcome of infectious diseases. The fact that clinical signs of

histomonosis occur less severe in chickens in contrast to turkeys can be linked with the host defence, indicating a substantial difference between these two species (Mitra et al. 2018).

2.4.1. Previous research

There is only few information available about the immune response of turkeys and chickens following an infection with *H. meleagridis*.

In older studies, Tyzzer (1934, 1936) and Lund (1959) used an apathogenic strain of the parasite to induce a protective immunity in turkeys and chickens against virulent histomonads but could not prevent all vaccinated birds from the disease. Clarkson (1963) studied the immunological responses of drug-treated turkeys that recovered from histomonosis and noticed a protective effect in those birds. Furthermore, the same author demonstrated that passive immunization against *H. meleagridis* did not protect turkeys against a challenge. Decades later, the development of a protective immunity after infection and treatment was verified by Bleyen et al. (2009) who also confirmed that passive immunization cannot protect turkeys from histomonosis. Other experiments applying active immunization using killed vaccines failed to protect turkeys against a challenge (Hess et al. 2008, Bleyen et al. 2009). The most promising experimental approach was to apply *in vitro* attenuated histomonads as vaccine against the disease that prevented clinical signs and mortality in turkeys and chickens (Hess et al. 2008, Liebhart et al. 2011).

Powell et al. (2009) investigated the expression of the cytokines and chemokines IL-1β, IL-6, CXCLi2, IFN-γ, IL-4, IL-13 and IL-10 in chickens and reported a distinct caecal innate immune response during infection, accompanied with a confinement of the parasite to the caecum. In comparison turkeys failed to mount such an effective response allowing more histomonads migrating to the liver which lead to an uncontrolled immune response determined by the up-regulation of IL-1β, CXCLi2, IFN-γ, IL-13, IL-4 and IL-10 and additionally $CD4^+$, $CD8\alpha^+$, $CD28^+$ and $CD44^+$ cells.

Windisch and Hess (2010) measured an increase of local antibodies in different parts of the intestine of chickens following infection. IgA increased significantly compared to control birds, leading to the speculation that the detected local mucosal IgA play an important role in the resistance against the disease.

Mitra et al. (2017) investigated the cellular immune response of turkeys and chickens after vaccination and/or infection with *H. meleagridis*. An increase of B cells and T-cell subsets in

peripheral blood samples already within the first few days after infection could be detected in challenged, vaccinated turkeys, demonstrating a distinct immune response. In the caeca of non-vaccinated but infected turkeys a decrease of T cells was detectable within the first week after infection while these changes appeared later in vaccinated, infected turkeys. In general, non-vaccinated but infected turkeys showed more pronounced changes in the distribution of B cells and T-cell subsets in the caecum compared to non-vaccinated but infected chickens. Chickens however showed a general increase of monocytes/macrophages.
The role of cytokine expressing cells was later on investigated by Kidane et al. (2018) who detected a higher amount of interferon gamma (IFN-γ) mRNA positive cells in caeca of control chickens than of control turkeys, leading to the assumption of IFN-γ acting as a protective signature cytokine against histomonosis. This was supported by an increase of IFN-γ positive cells in the caeca of vaccinated turkeys that were protected against histomonosis.
Most recently, Lagler et al. (2019) detected an increase of histomonads-specific IFN-γ-producing cells in the spleen of infected chicken. The rise of these $CD4^+$ T cells was investigated two and five weeks post infection. In comparison, specific T-cell subsets derived from the liver did not show different IFN-γ producing levels compared to control birds.

Overall, the mentioned studies on the immune response against histomonosis mainly focused on the adapted immunity. In contrast substantial data on the innate immunity is lacking, especially the expression of TLRs of host cells due to *H. meleagridis* has not been investigated so far.

2.4.2. Toll-like receptors

2.4.2.1. Definition

TLRs are receptors of the innate immunity that interact with cell-associated components, called pathogen-associated molecular patterns (PAMP). The definition “PAMP” covers conserved components of pathogens. The ability of the immune system to recognize and react to these ligands forms the basis for an appropriate innate immune response and also induces the development of a specific acquired immunity. All identified TLRs are type 1 integral membrane glycoproteins, their extracellular part is a ligand binding domain

composed of leucine rich repeats and the intracellular one a toll/interleukin 1 receptor domain (Akira and Takeda 2004). The PAMP bind to the extracellular leucine rich repeats and there the recognition is achieved. As a reaction to ligand binding, the intracellular toll/interleukin 1 receptor domain activates signalling resulting in an inflammatory reaction and a rise of inflammatory cytokines (West et al. 2006).

2.4.2.2. Research history on TLRs

The discovery of the human TLRs more than 20 years ago (Medzhitov and Janeway 1997) was soon followed by the identification of 13 mammalian TLRs and further in reptiles, fish, amphibians and birds (Juul-Madsen et al. 2012). Lynn et al. (2003) firstly described homologs to mammalian TLRs in expressed sequence tags of chickens, leading to the suggestion of significant similarities between the chicken and mammalian TLR systems.
As first step in the discovery of TLRs, toll was initially described as a regulatory protein essential for insects, especially *Drosophila melanogaster*, a genus of flies, for dorsoventral polarity during embryogenesis (Hashimoto et al. 1988). Further studies identified the real role of toll, up-regulating the innate immune system of these adult flies as a response to a fungal infection (Lemaitre et al. 1996). The term “toll-like receptor” derives from the similarity to this *Drosophila* toll protein.

2.4.2.3. Role during infection

TLRs are one of the most important receptors of the innate immune system. These specialized receptors recognize a broad range of invading pathogens through the pathogen individually presented PAMPs (Juul-Madsen et al. 2012). So, for different TLRs different PAMPs act as specific ligands. In general, features of these ligands are physiological absent in the host, structurally conserved and important for the survival of the pathogen (Keestra et al. 2013).
The recognition of a PAMP leads to an activation of the TLR resulting in the production of pro-inflammatory cytokines, chemokines and the activation of immune cells. This host response plays a critical role in the clearance and reduction of pathogens (West et al. 2006).
TLR expression is widespread in chicken tissues which is an important characteristic because it has an influence on detecting the invading pathogens (Iqbal et al. 2005a).

2.4.2.4. Chicken TLRs

Until now, ten avian TLRs are known, including TLR 1a, 1b, 2a, 2b, 3, 4, 5, 7, 15 and 21. TLR 2a, 2b, 3, 4, 5 and 7 have orthologues in mammals, until now TLR 1a, 1b and 15 are only found in chickens whereas TLR 21 has orthologues in amphibian and fish (Temperley et al. 2008).
The functionality of the respective chicken TLRs and their unique properties are described below and summarized in **Tab. 1**.
The term “expression” used in the following sections is based on detection of the respective TLRs at mRNA level.

2.4.2.4.1. TLR 1

Through gene duplication chicken express two forms of TLR 1, named TLR 1a and TLR 1b (Temperley et al. 2008). Previously, Yilmaz et al. (2005) already suggested the existence of a second TLR 1 gene and identified a high sequence identity between the two TLR1 candidates.
It appears that these two forms of TLRs can only be located in the avian genome (Temperley et al. 2008). ChTLR 1 binds lipoprotein and peptidoglycan of Gram-positive bacteria. The expression pattern in different cell types and tissues is broadly allocated with comparatively high levels in the kidney and spleen as well as in B cells and heterophils (Iqbal et al. 2005a). The expression in heterophils is in agreement with the work of Farnell et al. (2003) who also showed the constitutively expression of TLR 1 in heterophils. After stimulation with bacterial TLR agonists heterophils show a significant expression leading to up-regulation of the pro-inflammatory cytokines IL-1β and IL-6 and the chemokines CXCLi2, CXCLi1 and CCLi4 (Kogut et al. 2006).

2.4.2.4.2. TLR 2

Resulting from gene duplication, chickens also express two isoforms of TLR 2, termed TLR 2a and TLR 2b, sharing a high sequence homology (Fukui et al. 2001). Those two genes are both orthologs of the mammalian single TLR 2 (Temperley et al. 2008).
TLR 2 shows the greatest variety of ligands of all the TLRs: lipoprotein and peptidoglycan as components of the cell wall from Gram-positive bacteria, zymosan from fungal cell walls and lipoarabinomannan as mycobacterial cell wall component (Juul-Madsen et al. 2012). Further microorganisms, including additionally viruses and parasites, can activate this receptor (St. Paul et al. 2013).
Iqbal et al. (2005a) detected a more restricted expression of both chTLR 2 isoforms in tissues compared to the other TLRs with highest levels found in spleen, caecal tonsils and liver. TLR 2a shows lower expression in tissues in comparison to the other isoform TLR 2b. This characteristic can also be found in immune cell subsets, where TLR 2b gives signal for all investigated cell types, with highest levels in $CD8^+$ cells and B-cell fractions compared to TLR 2a which is less expressed, showing the highest signals in heterophils. A significant up-regulation of the pro-inflammatory cytokines IL-1β and IL-6 and the chemokines CXCLi2, CXCLi1 and CCLi4 follows to an activation of TLR 2b in heterophils (Kogut et al. 2006).

2.4.2.4.3. TLR 3

ChTLR 3 is a direct orthologue of mammalian TLR 3 (Temperley et al. 2008) and is localized in the endosome (St. Paul et al. 2013).
Double stranded RNA (dsRNA) is a compound with immunostimulatory facilities in vertebrates, long known before the discovery of TLRs (Juul-Madsen et al. 2012). Some viruses show dsRNA as a product of their replication cycle which is recognized by TLR 3 (Iqbal et al. 2005a, St. Paul et al. 2013).
A wide range of tissues and cells express chTLR 3. Especially in all parts of the intestine, liver and kidney very high levels are detectable in tissues. TCR1 and $CD8^+$ fractions gave the strongest RT-PCR signal from the investigated immune cell subsets (Iqbal et al. 2005a). Kogut et al. (2005) detected a constitutive expression of chTLR 3 in avian heterophils.
Type 1 and type 2 IFN production follows stimulation of this TLR in a variety of cell types (Matsumoto et al. 2004).

2.4.2.4.4. TLR 4

ChTLR 4 are true orthologues to those TLR 4 found in mammals (Temperley et al. 2008).
Lipopolysaccharide (LPS), structural cell walls components of all Gram-negative bacteria, act as potential immune system activators in general, along with their recognition by TLR 4 (Iqbal et al. 2005a).
ChTLR 4 mRNA is detectable in a broad range of tissues, including the liver, spleen and caecal tonsils. Heterophils and macrophages represent the immune cell subsets with the highest chTLR 4 expression levels (Iqbal et al. 2005a).
Stimulation of this TLR in humans and mice induces a signalling cascade resulting in the expression of pro-inflammatory cytokines, as IL-1β, and chemokines, as IL-8 (Takeuchi and Akira 2010). Kogut et al. (2005) demonstrated the same response in chicken heterophils by *in vitro* stimulation.

2.4.2.4.5. TLR 5

ChTLR 5 and the TLR 5 found in other vertebrates share homologous genes (Temperley et al. 2008).
The agonist for TLR 5 is flagellin, constituting the major part of bacterial flagella (Juul-Madsen et al. 2012). It is mainly found on Gram-positive and Gram-negative bacteria (Iqbal et al. 2005a).
The distribution pattern of chTLR 5 is broad, moderate levels are detectable in tissues such as intestine, lung and spleen. Greater differences can be seen in immune cell subsets, where expression was highest in heterophils (Iqbal et al. 2005a).
Stimulation of chicken heterophils with flagellin resulted in the up-regulation of the pro-inflammatory cytokines IL-1β and IL-6 and the chemokines CXCLi2, CXCLi1 and CCLi4 (Kogut et al. 2005).

2.4.2.4.6. TLR 7

ChTLR 7 is a true ortholog of TLR 7 found in mammals (Temperley et al. 2008). Its localization is in the endosome (St. Paul et al. 2013). There, TLR 7 senses single-stranded RNA (ssRNA), a common feature of RNA viruses (Diebold et al. 2004).

The expression of chTLR 7 in tissues, compared to other chTLRs, is lower. Lymphoid-associated tissues, especially bursa and spleen give the highest signals, but expression can also be detected in non-primary-lymphoid tissues as skin, lung and small intestine (Iqbal et al. 2005a, Brownlie et al. 2009). Concerning specific immune cells, chTLR 7 shows the highest expression levels in B cells but is also detectable in T cells. Additionally, TLR 7 expression can be found in chicken thrombocytes (Iqbal et al. 2005a, St. Paul et al. 2012b).
The activation of this TLR results in a type 1 IFN production and an increase of degranulation and oxidative burst in chicken heterophils (Kogut et al. 2005, St. Paul et al. 2013).

2.4.2.4.7. TLR 15

This TLR, absent in fish and mammals, was first identified after a *Salmonella* infection of chickens, leading to the assumption to be avian specific (Roach et al. 2005, Higgs et al. 2006).
ChTLR 15 does not bind a specific agonist, as known so far, but proteases from fungal or bacterial origin, resulting in proteolytic activity at the cell surface, activate this TLR (de Zoete et al. 2011). This kind of TLR activation mechanism is novel and unique to chickens (St. Paul et al. 2013).
ChTLR 15 is expressed in lymphoid and non-lymphoid tissues, including bursa, spleen, bone marrow, small intestine, skin and lung (Higgs et al. 2006, Brownlie et al. 2009). So far, there is no information on the expression of chTLR 15 in immune cells.
Cleavage of the receptor and TLR signalling is following an activation (de Zoete et al. 2011).

2.4.2.4.8. TLR 21

chTLR 21 has no mammalian orthologues but shares homologous genes with amphibians and fish (Temperley et al. 2008). It is an intracellular receptor localized in the endoplasmic reticulum of the cells, activated through recognition of microbial DNA (Brownlie et al. 2009).
Expression of chTLR 21 is detectable in a broad range of tissues (Juul-Madsen et al. 2012). The highest levels are found in bursa, spleen and small intestine. In skin, lung, kidneys, liver and brain further expression can be detected. Several immune cell subsets, including macrophages and B cells, are also positive for chTLR 21.
The activation is followed by an induction of downstream signalling (Brownlie et al. 2009).

Tab. 1: Overview on investigations on avian TLRs and their functionality.

chTLR	LIGAND	TISSUE EXPRESSION	CELL EXPRESSION	REFERENCES
TLR 1a	lipopeptides, peptidoglycan	spleen, kidney	B cells, heterophils	Iqbal et al. 2005a
TLR 1b	lipopeptides, peptidoglycan	spleen, kidney	B cells, heterophils	Iqbal et al. 2005a
TLR 2a	lipoprotein, peptidoglycan, zymosan, etc.	spleen, caecal tonsills, liver	heterophils	Iqbal et al. 2005a Juul-Madsen et al. 2012
TLR 2b	lipoprotein, peptidoglycan, zymosan, etc.	spleen, caecal tonsills, liver	CD8+, B cells	Iqbal et al. 2005a Juul-Madsen et al. 2012
TLR 3	dsRNA	intestine, liver, kidney	TCR 1, CD8+ fractions, heterophils	Iqbal et al. 2005a Kogut et al. 2005
TLR 4	LPS	spleen, liver	macrophages, heterophils	Iqbal et al. 2005a
TLR 5	Flagellin	intestine, spleen, lung	heterophils	Iqbal et al. 2005a Juul-Madsen et al. 2012
TLR 7	ssRNA	spleen, bursa, skin, lung, small intestine	B and T cells, thrombocytes	Diebold et al. 2004 Iqbal et al. 2005a Brownlie et al. 2009 St. Paul et al. 2012b
TLR 15	Protease	spleen, bursa, bone marrow, small intestine, skin, lung		Higgs et al. 2006 Brownlie et al. 2009 de Zoete et al. 2011
TLR 21	DNA	spleen, bursa, small intestine, skin, lung, kidneys, liver, brain	B cells, macrophages	Brownlie et al. 2009

2.4.2.5. Expression of TLRs in cell culture

Distribution patterns of TLRs in tissue and cells are important characteristics of the receptor functionality, influencing the ability to detect invading pathogens.

Cell cultures, as an extracorporeal cultivation of cells in culture medium enable a practicable detection method of the expression patterns of TLRs in both infected and non-infected cultured cells of different origin. In human cell lines various expression profiles of TLRs are detectable without an exposure to pathogens or their derived products (Rehli et al. 2002, Abdi et al. 2013). A similar conclusion resulted from infection studies with cultures from human immune cells. Most noticeable, Muzio et al. (2000) examined the TLR expression and regulation in human leukocytes, concluding an ubiquitous expression of TLR 1, a restriction

of TLR 2,4 and 5 and a selective expression of TLR 3. Furthermore, Zarember and Godowski (2002) confirmed the great variety of TLRs expressed by human leukocytes.

2.4.2.5.1. TLR expression in HD11 and LMH cell lines

In this thesis the expression of chTLRs in two established chicken cell lines, HD11, a macrophage-like cell line obtained by retroviral transformation (Beug et al. 1979) and LMH, a chicken liver hepatocellular carcinoma cell line (Kawaguchi et al. 1987), both infected with cultivated *H. meleagridis*, was determined.
Iqbal et al. (2005a) reported the detection of high expression levels of TLR1/6/10, TLR 2a, TLR 4 and especially TLR 7 in uninfected HD11 cell lines and mentioned that cell culture systems including HD11 have valuable proven tools for further studies of pathogen interactions and TLR repertoires.
Han et al. (2010) infected HD11 cell lines with baculovirus and showed an up-regulation of TLR 21.
Ciraci and Lamont (2011) performed infection studies with HD11 cell lines, including infections with *E. coli*- and *Salmonella enteritidis*-derived LPS. As immune response TLR 15 was significantly up-regulated. This result accompanies with St. Paul et al. (2013) as TLR 15 showed a significant increase in response to stimulation with *E. coli* and *Salmonella enteritidis* in different tissue samples including chicken spleen.
Qi et al. (2017) detected a more rapid up-regulation of TLR 4 in HD11 cell line following avian H9N2 influenza virus as single infection.

As so far known, the only study investigating TLRs expression in LMH cell lines was by Weder (2012) who demonstrated the general expression of TLR 3, 7 and 21 in naive LMH cells. After an infection with Gallid Herpesvirus-1 in the same work, TLR 3 and 7 expressions were decreased.

Overall, there is no information about the expression of TLRs in chicken cell cultures against extracellular parasites. Therefore, in the present work *H. meleagridis* was selected as pathogen to investigate the TLR expression profile of cells derived from macrophages (HD11 cells) as well as liver (LMH cells). *In vivo*, histomonads have a direct contact with both cell

types by infiltrating the caecum (macrophages) and the liver (hepatocytes and macrophages), therefore both cell cultures are highly suitable to be used as *in vitro* models.

3. Material and Methods

3.1. Cell lines

3.1.1. Chicken macrophage-like cell line HD11

The HD11 cell line used in this study was kindly provided from Prof. Dr. med. vet. Thomas Göbel (Department of Animal Physiology, Ludwig-Maximilians-University Munich). The cells were cultured in tissue culture flasks (Sarstedt™, Nümbrecht, Germany) in media (RPMI 1640 Medium Supplement, Gibco™ Invitrogen, Lofer, Austria) supplemented with 10 % fetal calve serum (Gibco™ Invitrogen) and 2,5 % antibiotics (Penicillin 40000 IU/ml + Streptomycin 40 mg/ml, Sigma-Aldrich, Austria) at 37 °C in a 5 % CO_2 humidified air incubator. The incubation lasted for six days with a passage after three days according to Peng et al. (2018). During this time the growth was verified by a phase contrast microscope (Nikon®, Tokio, Japan). At the end of the incubation, the media was discarded and subsequently 10 ml RPMI media (Gibco™ Invitrogen) supplemented with 10 % fetal calve serum (Gibco™ Invitrogen) and 2,5 % antibiotics (Penicillin 40000 IU/ml + Streptomycin 40 mg/ml, Sigma-Aldrich) was immediately added to the cells. For detachment of the cells from the surface a 25 cm cell scraper (Sarstedt™)) was used. The suspension was centrifuged for five minutes with 1300 repeats per minute (rpm) at 22 °C and afterwards the supernatant was discarded. The cells were then re-suspended in 38 ml of RPMI media (Gibco™ Invitrogen). Subsequently 1 ml of the cell suspension and 2 ml of RPMI media (Gibco™ Invitrogen) were added in each well of a Cellstar® six well plates (Greiner Bio-One, Kremsmünster, Austria). The plates were then incubated for 36 hours at 37 °C in a 5% CO_2 humidified air incubator. Cell numbers of live cells were determined by using cellometer K2 fluorescent viability cell counter system according to the manufacturer's instructions (Nexcelom Bioscience, LLC, Lawrence, MA, USA). For both cell lines $1x10^3$ cells were seeded and after 36 hours of incubation the cell number was determined to be to $1x10^8$/well by cell counting as described above.

3.1.2. Chicken liver hepatocellular carcinoma cell line LMH

The LMH cell line was obtained from ATCC® (Wesel, Germany). The protocol for culturing of LMH was consistent as described above for HD11 with minor modifications. At the end of the six-day incubation, the media was discarded and 10 ml of PBS media (phosphate buffered saline, Gibco™ Invitrogen) was added very gently to wash the cells. Subsequently PBS (Gibco™ Invitrogen) was removed and 3 ml of Trypsin (Gibco™ Invitrogen) was added to detach the cells for 2-3 minutes at 37 °C in a 5 % CO_2 humidified air incubator. Then 7 ml of RPMI media (Gibco™ Invitrogen) was added before the suspension was transferred to a 50 ml falcon tube (Sarstedt™). The next steps of the protocol were consistent with the protocol for HD11.

3.2. Preparation of *H. meleagridis*

The clonal culture *H. meleagridis*/Turkey/Austria/2922-C6/04 co-cultivated with *Escherichia coli* (DH5α) was kept *in vitro* for 21 passages to obtain virulent histomonads according to a previously established protocol (Ganas et al. 2012). Viable *H. meleagridis* cells were stained and counted with the Cellometer® ViaStainTM AOPI staining solution (Nexcelom Bioscience) and an Olympus BX53 microscope (Olympus Europa SE & Co. KG, Hamburg, Germany) equipped with a X-Cite® Series 120Q fluorescence lamp (Excelitas Technologies Corp., Waltham, MA, USA) (**Fig. 1**) to calculate the required cell numbers for preparation of the infection and vaccination inoculum. Accordingly, a *H. meleagridis*+*E. coli* culture (HMEC) with a final concentration of $1x10^6$ histomonads/ml of culture media (500ml RPMI media (Gibco™ Invitrogen) supplemented with 15% FCS (Gibco™ Invitrogen) and 0,66 mg/ml rice starch (Sigma-Aldrich) was adjusted.

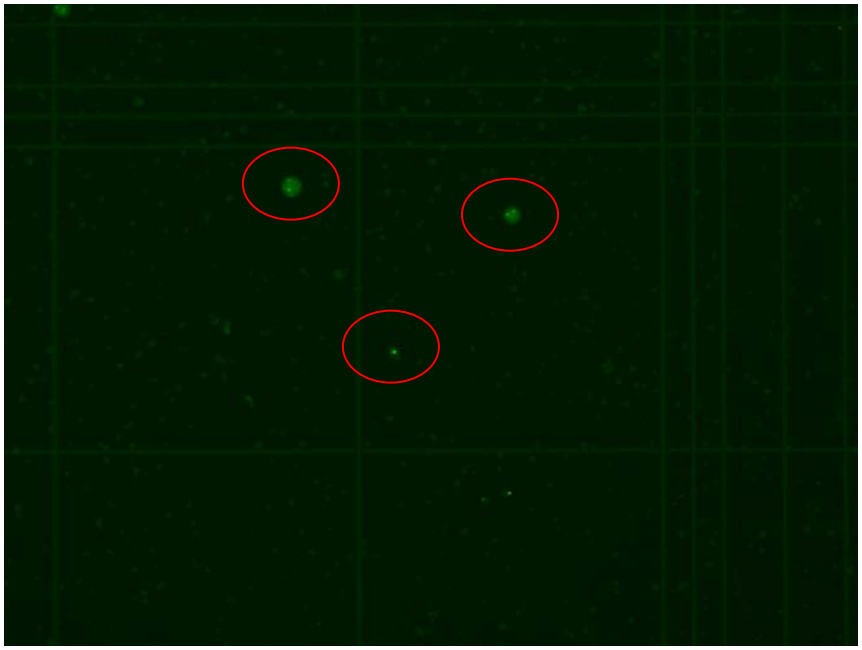

Fig. 1: Living histomonads indicated by green fluorescence using the Cellometer® ViaStainTM AOPI staining solution (Nexcelom Bioscience) and a fluorescence microscope (Olympus Europa SE & Co. KG).

3.3. Preparation of the *E. coli*

Since *E. coli* was mandatory to cultivate *H. meleagridis* the effect of the bacteria had to be determined separately in the cell cultures. In this process a conditioned but sterile culture medium was established. For that two 50 ml tubes (Sarstedt™) were filled with HMEC suspension and centrifuged with 1350 rpm for 5 minutes to separate histomonads from bacteria. The supernatant with bacteria was transferred into new tubes before and an additional centrifugation step with 4000 rpm for 10 minutes was performed. The supernatant was then passed through a 0.45 µm and additionally through a 0.22 µm filter. To verify that no bacteria were present in the filtered medium, it was streaked out on coliform agar plates. Following an incubation period of 24 and 48 hours at 37 °C in a 5 % CO_2 humidified air incubator the non-growth of bacteria was visually examined.

The growth of DH5α in HMEC and an only *E. coli* DH5α culture was determined over two passages for four days by bacterial plating on coliform agar plates that were incubated for 24 hours at 37 °C in a 5% CO_2 humidified air incubator according to Ganas et al. (2012).The obtained number of colonies was multiplied by the used dilution factor 10^7 to get the CFU

(colony forming units) per ml. Based on this CFU/ml growth curves (**Fig. 2 and 3**) were obtained to assess the necessary culturing period for DH5α until an approximately equivalent CFU/ml level for the *E. coli* DH5α in HMEC and the only *E. coli* culture. Our results revealed an incubation period of 72 hours for *E. coli* to get a comparable amount of bacteria as contained in the HMEC suspension. Specifically, the *E. coli* culture reached $1.7x10^8$ CFU DH5α/ml and the HMEC $2.9x10^8$ CFU DH5α/ml. This procedure ensured that almost the same concentration of *E. coli* was used for infection of HD11 or LMH cells as it was applied in the suspension with histomonads and that EC contained the same conditioned media as HMEC.

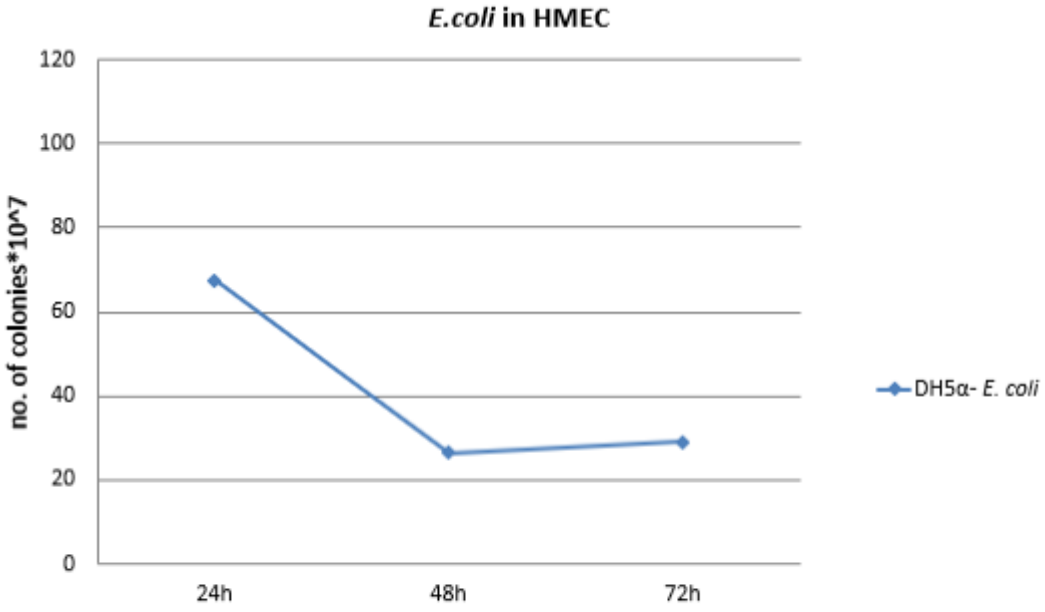

Fig. 2: *E. coli* growth curve in the *H. meleagridis* culture. The number of colonies was determined once per time step.

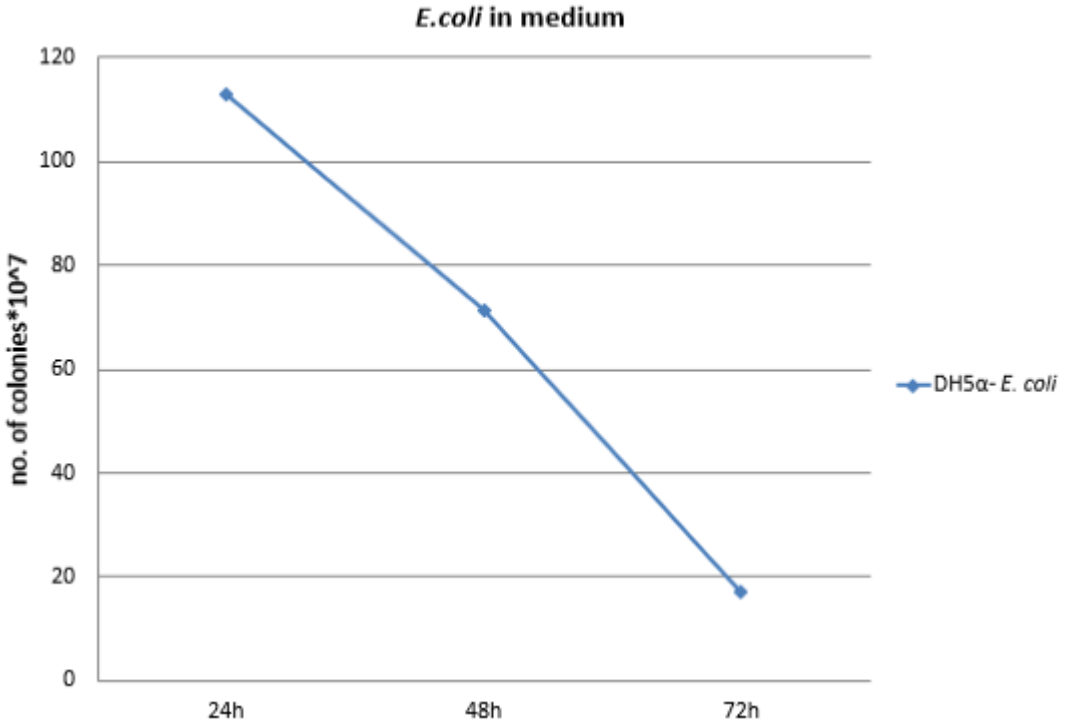

Fig. 3: *E. coli* growth curve in the *E. coli* culture without *H. meleagridis*. The number of colonies was determined once per time step.

3.4. Setup of the cell infection

After the mentioned preparation of HD11/LMH cell suspensions and their transfer into Cellstar® six well plates (Greiner Bio-One), the media in the wells was discarded and supplemented with 3 ml/well of HMEC (corresponded to $3x10^6$ histomonads/well and $8.7x10^8$ DH5α CFU/well) or EC (corresponded to $5.1x10^8$ DH5α CFU/well) in duplicate as illustrated in **Fig. 4** (HD11) and **Fig. 5** (LMH). This resulted in a cells/histomonads ratio of $10^8/3x10^6$ per well. Additionally, the negative controls without HMEC or EC in duplicate complemented the remaining wells on the Cellstar® six well plate. Overall, from every time step two biological duplicates of every group (HD11/LMH+HMEC, HD11/LMH+EC and HD11/LMH negative control) were investigated.

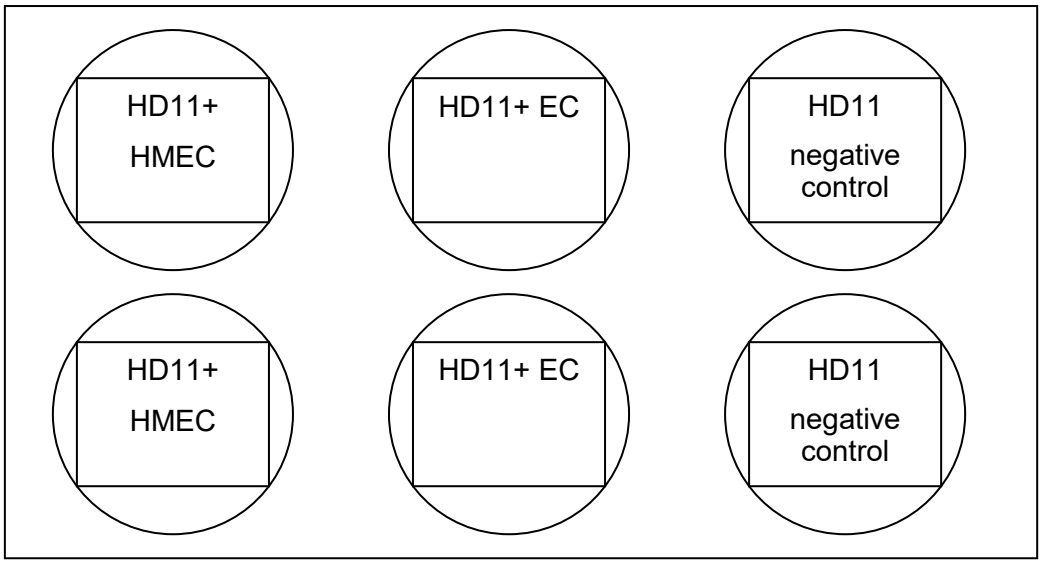

Fig. 4: Draft of the Cellstar® six well plates for infecting HD11 cells with *H. meleagridis*+*E. coli* (HMEC), only *E. coli* (EC) or without any infection (negative control) for each time point.

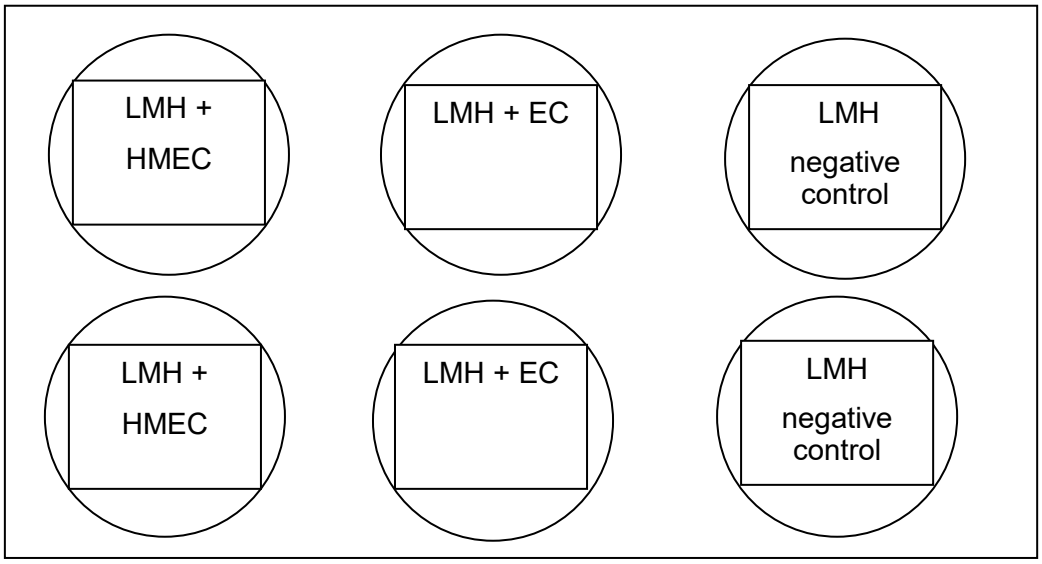

Fig. 5: Draft of the Cellstar® six well plates for infecting LMH cells with *H. meleagridis*+*E. coli* (HMEC), only *E. coli* (EC) or without any infection (negative control) for each time point.

The cell cultures were then incubated for varying incubation periods (30 minutes, 2 hours, 4 hours, 6 hours, 12 hours and 24 hours) at 37 °C in a 5 % CO2 humidified air incubator.

For harvesting, the cells were detached using a 16 cm cell scraper (Sarstedt™) and mixed thoroughly with the media. After transferring the cell suspension of each well in a 15 ml tube (Sarstedt™), a centrifugation step with 4000 rpm for 10 minutes followed. The supernatants were discarded and the remaining cell pellets were vortexed. Finally, 600 µl of TRI Reagent (Zymo Research, California, USA) were added to each suspension. Subsequently the final samples were immediately put into liquid nitrogen before they were stored at -80 °C.
Bacterial growth was determined from culture material of both groups (HMEC and EC) using coliform agar plates which were incubated for 24 hours at 37°C in a 5% CO_2 humidified air incubator. To obtain the bacterial amount the colonies were counted and the CFU/ml was calculated (**Add. Tab. 3**).

The viability of the harvested HD11 and LMH cells was examined by using cellometer K2 fluorescent viability cell counter system (Nexcelom Bioscience) at the end of the experiment. The sowing of the same numbers of cells in every well at the beginning and the control of the *E. coli* growth during the experiment (**Add. Tab. 3**) was decided to be sufficient due to our purpose to only compare the relations of the TLR expression levels of the naive cells with those of the infected cells. Subsequently, a determination of the exact cell number at the end time point of the experiment was not performed. At the different time points the microscopical comparison of the wells served to ensure the optical integrity of the cells. In this way, a worse growth or a poorer viability of infected cells compared to naive cells was estimated by comparing their amount with those of the naive cells. No differences regarding the viability of naive cells were observed between start and the final time point of the experiment.

3.5. RNA Isolation and RT-qPCR

After thawing the frozen samples mentioned above, total RNA was isolated from the cells using Direct-zol™ RNA MiniPrep Plus (Zymo Research). The extraction was performed according to the manufacturer's instruction. The isolated RNA was eluted in 50 µl RNase-free water and stored in 1.5 ml Eppendorf tubes (Eppendorf). By using NanoDrop 2000 (ThermoFisher Scientific, Vienna, Austria) every sample was assessed for quantity, integrity

and purity. Additionally, an electrophoretic assessment to determine the RNA-quality was achieved by using the 2100 Bioanalyzer Instrument (Agilent Technologies, Waldbronn, Germany).

Depending on the detected RNA quantity the samples were diluted with RNAse free water to a final concentration of 20 ng/µl. Finally, the RNA samples were stored at -80 °C until further use.

The expression levels of the different TLRs were quantified by applying real-time qPCR using Brilliant III Ultra-Fast QRT-PCR master mix kit (Agilent Technologies, Waldbronn, Germany). Every sample was examined twice two get technical duplicates.

Primers and probes (Eurofins Genomics, Ebersberg, Germany) are given in **Tab. 2**. The designing of primers and probes for the TLRs and establishment of the RT-qPCR was done at our clinic in a previous study (unpublished work). The efficiency and slope of the applied RT-qPCR genes are given in additional table number 4 (**Add. Tab. 4**). Using the AriaMx real-time PCR system (Agilent Technologies) the amplification of the primary transcripts and the quantification of the specific products were analysed with the Agilent AriaMx1.0 software (Agilent Technologies).

The reference genes RPL13 (ribosomal protein L13) and TFRC (transferrin receptor protein 1) were selected according to the protocol of Mitra et al. (2016).

For RT-qPCR, 2 µl of mRNA was used with 10 µl Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 0.02 µM Dithiothreitol (Agilent Technologies), 1 µl RT/RNase Block (Agilent Technologies), forward and reverse primers and probes in a 20 µl final reaction volume. The primers and probes were used directly from the stock and the primer concentrations in nM are mentioned in **Tab. 2**. The probes were used in a concentration of 100 nM. The thermal cycle profile was: 1 cycle of RT at 50 °C for 10 min, followed by 95 °C for 3 min for a hot start, 40 cycles of amplification at 95 °C for 5 s and 60 °C for 30 s. All runs were performed in multiplex. The applied combinations were TLR 1a+TLR 2a+TLR 5+TLR 15, TLR 1b+TLR 2b+TLR 3+TLR 4, TLR 7+TLR 21 and TFRC+RPL 13. These primer combinations have already been determined for a previous project of the Clinic for Poultry and Fish Medicine. Hence, the establishment of these multiplex combinations was based on previous singleplex runs with noticed efficiency slopes. Then the values of the performed multiplex PCRs were confirmed to be in the same range and did not cross each other's standard curves. Specifically, the cycle of quantification (Cq) difference between singleplex and multiplex was below 5%, a range that should not be exceeded according to the manufacturer's instruction (Stratagene e.V. 2020). A detectable expression of all mentioned

TLRs was measured in chicken tissue samples including spleen in comparison to the negative controls.

To identify genomic DNA contamination and overall PCR contamination one no-RT control run for every sample and every gene and between 2 and 3 non-template controls per PCR run were performed.

Overall, the RT-qPCR investigation was performed according to the MIQE guidelines (Bustin et al. 2009).

Tab. 2: Primers and probes used for RT-qPCR.

GENE	ACCESSION NUMBER FOR CHICKEN	PRIMER AND PROBE SEQUENCES (5´-3´) F-forward primer; R-reverse primer; P-probe	PRIMER CONCEN-TRATION (nM)	COLOUR
chTLR 1a	NM_001007488.4	F: TGTCACTACGAGCTGTACTTTG R: CTCGCAGGGATAACATATGGAG P: TAGTCCTGATCTTGCTGGAGCCGA	400	FAM
chTLR 1b	DQ518918.1	F: CCATCACAAGTTGTTTAGC R: TCCAGGTAGGTTCTCTTG P: CCTGATCTTGCTGGAGCCGA	300	HEX
chTLR 2a	AB050005.2	F: CTGGCCCACAACAGGATAAA R: CCTCGTCTATGGAGCTGATTTG P:ACATGATCTGCAGCAGGCTGTGAA	500	HEX
chTLR 2b	AB046533.2	F: GATCCCCAAGAGGTTCTG R: CTGCTGTTGCTCTTCATC P: CTGCGGAAGATAATGAACACCAAGAC	300	FAM
chTLR 3	EF137861.1	F: GCATAAGAAGGAGCAGGAAGA R: GGAGTCTCGACTTTGCTCAATA P: TGGTGCAGGAGGTTTAAGGTGCAT	200	ROX
chTLR 4	KF697090.1	F: GAGGTTGTAGATTTGAGTG R: GAAGGTCCAAGTATAGCA P: CTCTCCTTCCTTACCTGCTGTTCC	400	CY5
chTLR 5	AJ626848.1	F: AGCCTACTAGTGTGGCTAAATG R: ACACTGGTACACCTGCTAATG P: ACCAATGTAACCCTAGCTGGCTCA	500	ROX
chTLR 7	NM_001011688.2	F: CCAGATGCCTGCTATGATGC R: TCAGCTGAATGCTCTGGGAA P: TGGCTTCCAGGACAGCCAGTCT	600	FAM
chTLR 15	NM_001037835.1	F: TCTGGTGCTAACTGGCTTATG R: CCTCTTCTTGTACTGCTTCCTC P: AGCCCATTCTCACATACCACAGCC	500	CY5
chTLR 21	NM_001030558.1	F: TCGCAACTGCATTGAGGATG R: ATGACAGATTGAGCGCGATG P: TTCCTGCAGTCGCCGGCCCT	500	CY5
TFRC	NM_205256.2	F: AGCTGTGGGTGCTACTGAA R: GGCAGAAATCTTGACATGG P: CTCTGCCATGCTGCATGCCA-BHQ1	400	ROX
RPL 13	NM_204999.1	F: GGAGGAGAAGAACTTCAAGGC R: CCAAAGAGACGAGCGTTTG P: CTTTGCCAGCCTGCGCATG-BHQ1	500	HEX

Using the Cq, the mean of the two technical replicates from every target at each timepoint was related to the mean expression of the gene over all time points for both cell lines. The resulting value was defined as ∆Cq. To calculate ΔΔCq, each ΔCq was normalized to the average ΔCq value of the reference genes RPL13 and TFRC to exclude variations

concerning technical aspects during RT qPCR and sampling. The resulting ΔΔCq values were applied for calculation using the formula $2^{(-\Delta\Delta Cq)}$ (Livak et al. 2011). The means of these $2^{(-\Delta\Delta Cq)}$ values were related to the negative control in order to obtain a comparison regarding a higher or lower TLR expression of the infected cells compared with the naive cells. This calculation was performed for all groups from both cell lines at every timepoint and the resulting values were used for the graphical representation in the results section. Additionally, the ΔΔCq values from EC were subtracted from these of HMEC to ensure expression levels of TLRs exclusively as reaction to the cultivated histomonads. These new values are named “HMEC (*E. coli* corrected)” and were calculated using the same method as mentioned above. Additionally, the respective standard deviations were calculated. The data analysis was performed using Microsoft Excel (Microsoft, Redmond, Washington, USA) and these values were additionally separately represented graphically in the results section.

The application of a statistical data analysis was renounced because of the implementation of this experiment as a preliminary study due to a too low number of biological duplicates (n=2).

4. Results

4.1. RT-qPCR

The expression levels of the ten chTLRs were investigated in RNA samples of the investigated cell lines after different incubation periods. 30 minutes, 2 and 4 hours post infection (hpi) were defined as early, 6, 12 and 24 hpi as late time points. The expression of uninfected cells was adjusted to 1 as described above to enable a direct comparison between the samples. Mean values of infected cells in the range of the uninfected cells' standard deviation are not mentioned as aberrant changes in the description below. The calculation of a statistical significance was dispensed because of the lack of substantial data due to the nature of a preliminary study.

Values resulting from only one biological sample are identifiable by the lack of a standard deviation in the respective figure. Possible reasons for the absence of signals are considered in the discussion.

All non-template control runs resulted negative. The no-RT control was most of the time negative, although for few samples it was above 37 Cq. If the Cq for no-RT control was less than 37 then the sample was again cleaned with DNAse I treatment to remove the genomic DNA contamination.

4.1.1. HD11 cell line

Results of the expression of the respective TLRs of HD11 cells are given in detail below. Raw data on the TLR-expression of all groups is attached at the end of this work (**Add. Tab. 1**). The microscopic examination of naive and infected HD11 cells at the end of the experiment revealed no obvious losses.

4.1.1.1. TLR 1a

The naive cell line showed a continuous expression of TLR 1a-RNA at each measured time point (**Add. Tab. 1**).

In the HD11+EC group, expression of TLR 1a-RNA was lower than in the uninfected cells at the last two time points.
In the HD11+HMEC group, TLR 1a expression was above those of the naive cells in all late time points. Exclusively at 2 hpi the expression in the HD11+HMEC cells showed a lower level (**Fig. 6**).

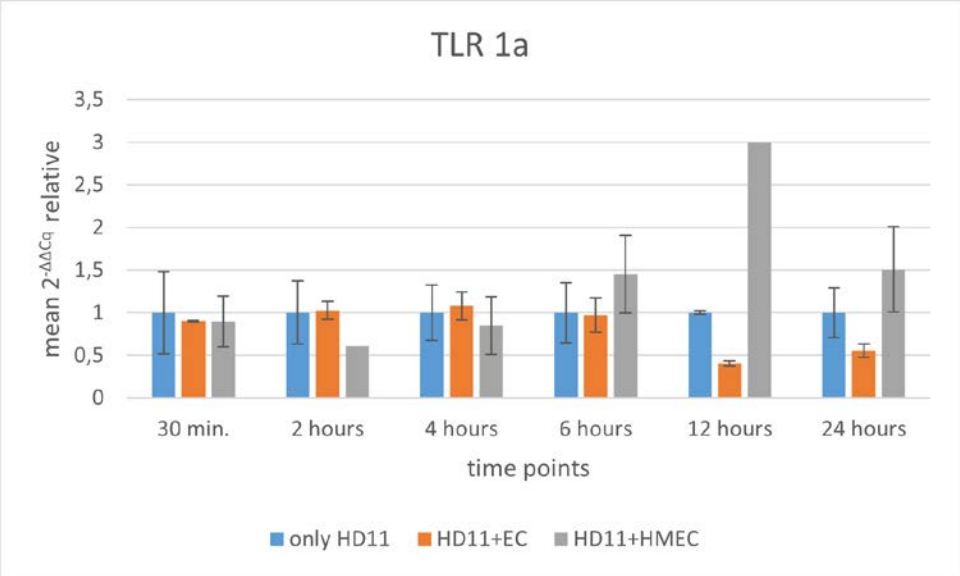

Fig. 6: Expression profiles of TLR 1a as average values of $2^{-\Delta\Delta Cq}$ with standard deviations in *E. coli* and *H. meleagridis* infected HD11 cells compared to naive cells (n=1, in duplicate). The expression of uninfected cells was adjusted to 1.

4.1.1.2. TLR 1b

Uninfected cells showed a constant expression of TLR 1b except at 12 hpi (**Add. Tab. 1**).
In only *E. coli* infected cells, TLR 1b varied between lower and higher expression compared to naive cells at all time points: at 30 min, 4, 6 and 24 hpi the RNA expression was lower, at 2 and 12 hpi higher than those of the uninfected cells. Similar to this, in the HD11+HMEC group variations were obvious: exclusively at 6 hpi a lower TLR expression, at 2, 12 and 24 hpi a higher one was detectable compared to naive cells (**Fig. 7**).

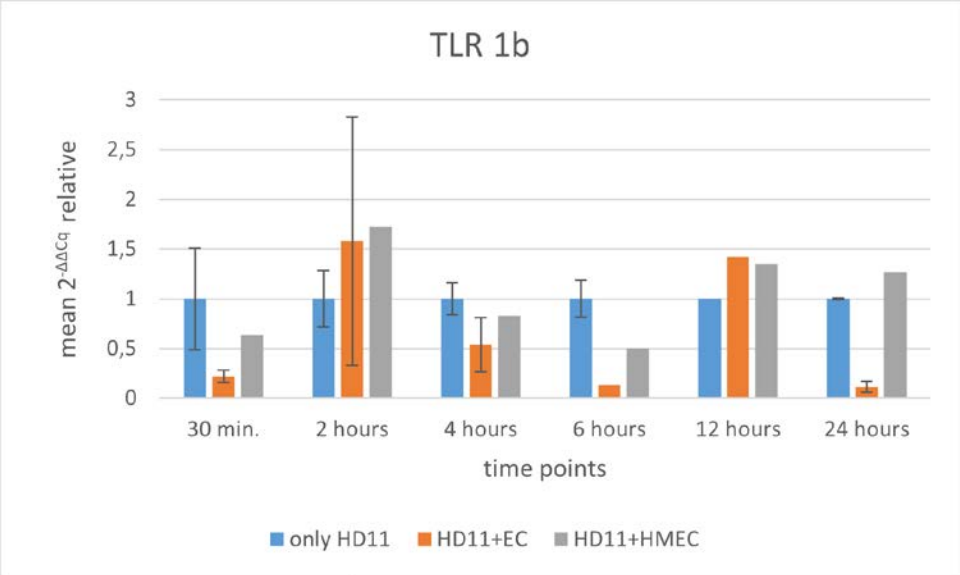

Fig. 7: Expression profiles of TLR 1b as average values of $2^{-\Delta\Delta Cq}$ with standard deviations in *E. coli* and *H. meleagridis* infected HD11 cells compared to naive cells (n=1, in duplicate). The expression of uninfected cells was adjusted to 1.

4.1.1.3. TLR 2a

No expression of TLR 2a-RNA could be detected at any time point in HD11 cells.

4.1.1.4. TLR 2b

The naive cell line showed a continuous expression of TLR 2b-RNA at each measured time point (**Add. Tab. 1**).

In EC infected cells TLR 2b expression was lower than those in uninfected cells at late time points showing a constant decrease of RNA level until 24 hpi. Exclusively at 30 min post infection a higher lever was detectable, followed by a lower level at 2 hpi compared to naive cells.

In the HD11+HMEC group, after 2 and 4 hpi TLR 2b-RNA expression was below those of uninfected cells, followed by a continuous higher expression level at late time points (**Fig. 8**).

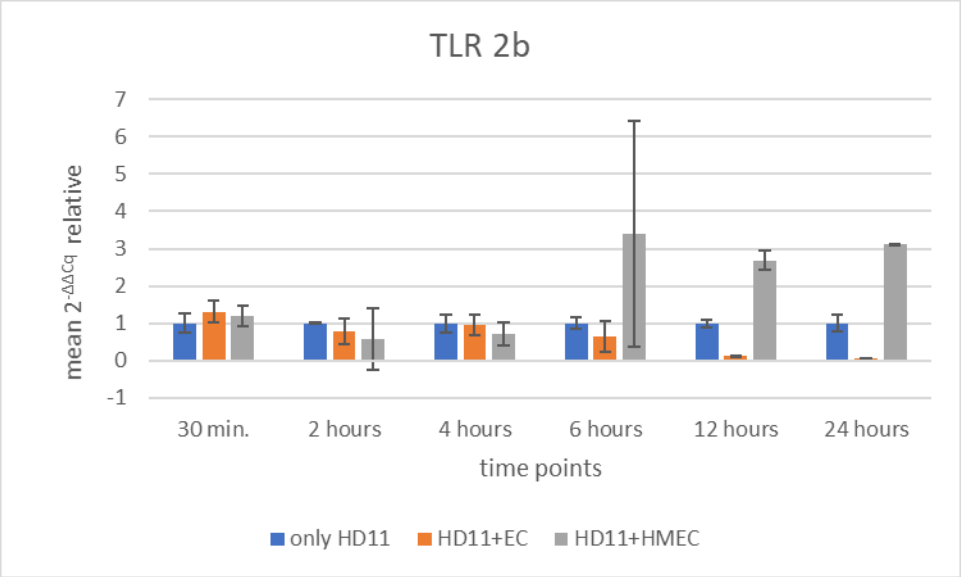

Fig. 8: Expression profiles of TLR 2b as average values of $2^{-\Delta\Delta Cq}$ with standard deviations in *E. coli* and *H. meleagridis* infected HD11 cells compared to naive cells (n=1, in duplicate). The expression of uninfected cells was adjusted to 1.

4.1.1.5. TLR 3

No expression of TLR 3-RNA could be detected at any time point in HD11 cells.

4.1.1.6. TLR 4

The naive cell line showed a continuous expression of TLR 4-RNA at each measured time point (**Add. Tab. 1**).
In EC infected cells TLR 4 showed higher expression levels at 30 min, 2 and 6 hpi, followed by lower levels at 12 hpi compared to uninfected cells.
In the HD11+HMEC group the initial higher expression at 30 min post infection resulted in lower levels of the TLR at 2 and 4 hpi and a continuous higher expression at late time points compared to uninfected cells (**Fig. 9**).

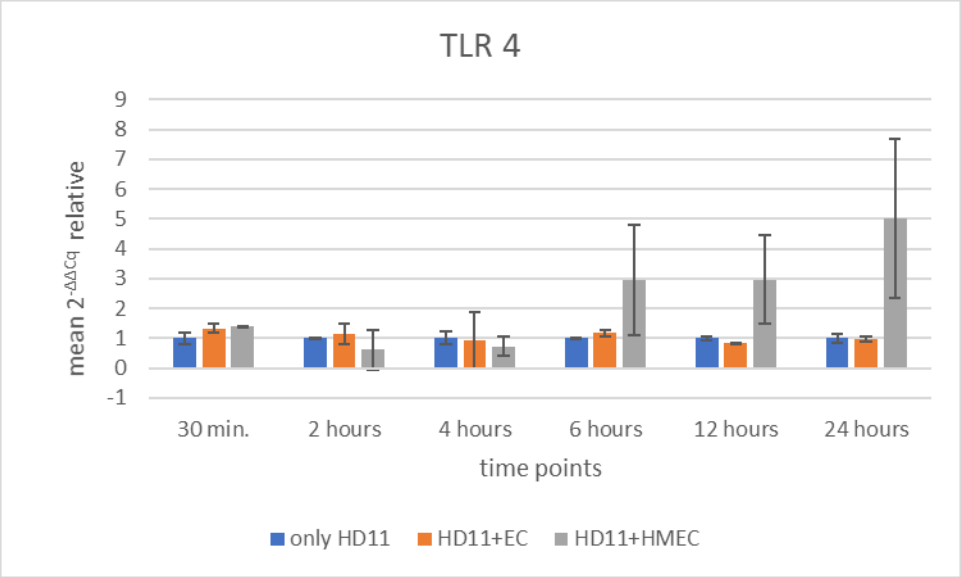

Fig. 9: Expression profiles of TLR 4 as average values of $2^{-\Delta\Delta Cq}$ with standard deviations in *E. coli* and *H. meleagridis* infected HD11 cells compared to naive cells (n=1, in duplicate). The expression of uninfected cells was adjusted to 1.

4.1.1.7. TLR 5

Naive cells showed an expression of this TLR after 30 min, 2 and 4 hours of incubation (**Add. Tab. 1**).

Expression of TLR 5-RNA could only be detected exclusively in infected cells, specifically in the HD11+EC group after 4 hpi. However, these expression values were within the range of the uninfected cells' standard deviation. No figure was created for this TLR due to the low number of values.

4.1.1.8. TLR 7

Naive cells expressed TLR 7 only after 30 minutes and 2 hours at a detectable level (**Add. Tab. 1**).

Expression of TLR 7-RNA could only be detected at 30 min and 2 hpi in EC infected HD11 cells. At these two time points the TLR expression was above those of the uninfected cells.

The lack of further comparisons and a figure is due to an absence of detectable signal for the remaining timepoints for the infected and the uninfected groups.

4.1.1.9. TLR 15

The naive cell line showed a continuous expression of TLR 15-RNA at each measured time point with the highest signal levels at early time points (**Add. Tab. 1**).
In the HD11+EC group TLR15 expression was higher compared to the uninfected cells at all time points but showing the highest levels at late time points.
In the HD11+HMEC group at 2 and 4 hpi very low levels of TLR 15-RNA were observed whereas at the remaining time points the expression was below the detection limit. (**Fig. 10**).

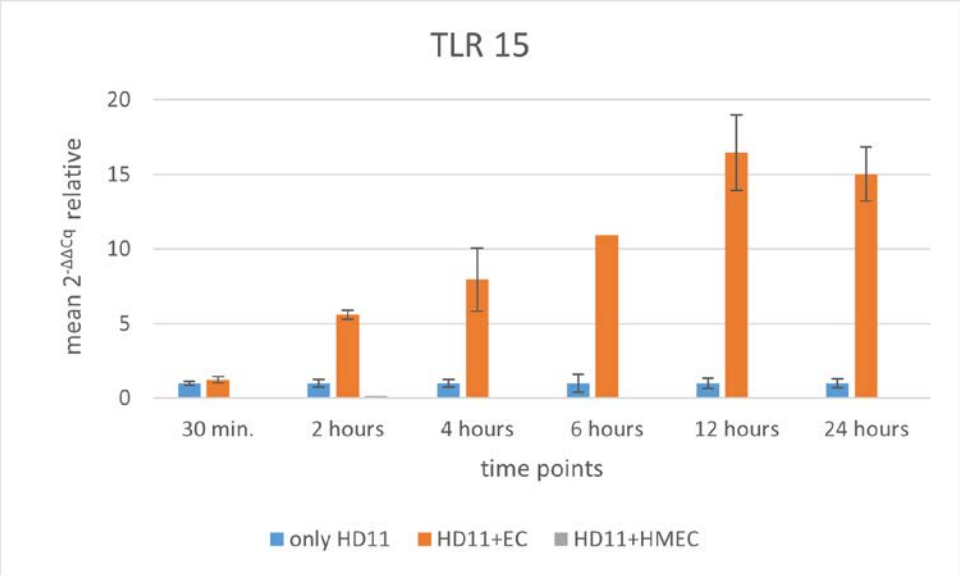

Fig. 10: Expression profiles of TLR 15 as average values of $2^{-\Delta\Delta Cq}$ with standard deviations in *E. coli* and *H. meleagridis* infected HD11 cells compared to naive cells (n=1, in duplicate). The expression of uninfected cells was adjusted to 1.

4.1.1.10. TLR 21

The naive cell line showed a continuous expression of TLR 21-RNA at each measured time point (**Add. Tab. 1**).

In the HM+EC infected cells TLR 21 expression was lower than those of the uninfected cells at 30 min, 12 and 24 hpi.

In the HD11+HMEC group, after an initial TLR21-RNA level below those of the naive cells at 30 min post infection followed higher levels at 2, 12 and 24 hpi (**Fig. 11**).

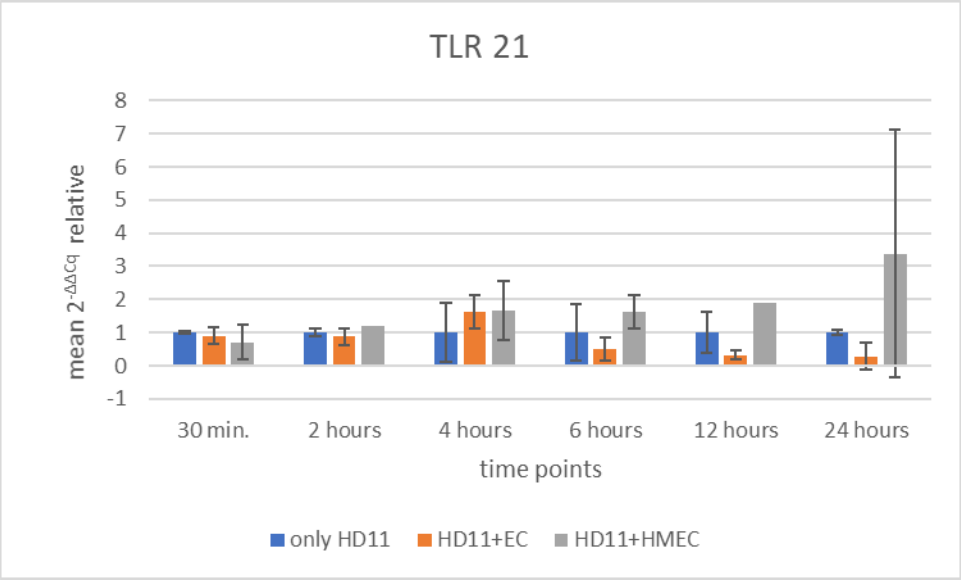

Fig. 11: Expression profiles of TLR 21 as average values of $2^{-\Delta\Delta Cq}$ with standard deviations in *E. coli* and *H. meleagridis* infected HD11 cells compared to naive cells (n=1, in duplicate). The expression of uninfected cells was adjusted to 1.

4.1.1.11. HMEC (*E. coli* corrected)

All the values obtained from naive cells resulted from two biological samples and each value of the infected cells was compared with the outcome of uninfected cells (**Fig.12**). Values of the uninfected cells were adjusted to 1. In the following text and figure (**Fig. 12**) only values outside the range of the respective uninfected cells' standard deviation are mentioned.

In HMEC (*E. coli* corrected) infected cells TLR 1a was higher expressed compared to uninfected cells at 6, 12 and 24 hpi (**Fig. 12**).

The lower expression of TLR 1b at the first two time points was followed by a higher one at 4 hpi compared to the control group.

TLR 2b showed a continuously lower expression at early time points. From 6 hpi on the expression of TLR 2b increased constantly resulting in higher levels compared to non-infected cells at late time points.

TLR 4 expression levels were higher after 30 min, 4, 6, 12 and 24 hours of incubation. After an unique expression level below those of the naive cells at 2 hpi, the expression of TLR 4 in infected cells increased constantly and reached the maximum at 24 hpi.

After 2 and 4 hours of incubation TLR 15 expression was lower in relation to those of the uninfected cells.

TLR 21-RNA was initially less expressed after 30 min of incubation in relation to the non-infected cells. Later at 6, 12 and 24 hpi an expression level above those of the naive cells was detected.

Overall, in HMEC (*E. coli* corrected) HD11 cells TLR 1a, 2b, 4 and 21 showed the most obvious changes with the highest expression levels at late time points. TLR 1b and 15 were noticed on earlier timepoints with lower expression variances compared to naive cells.

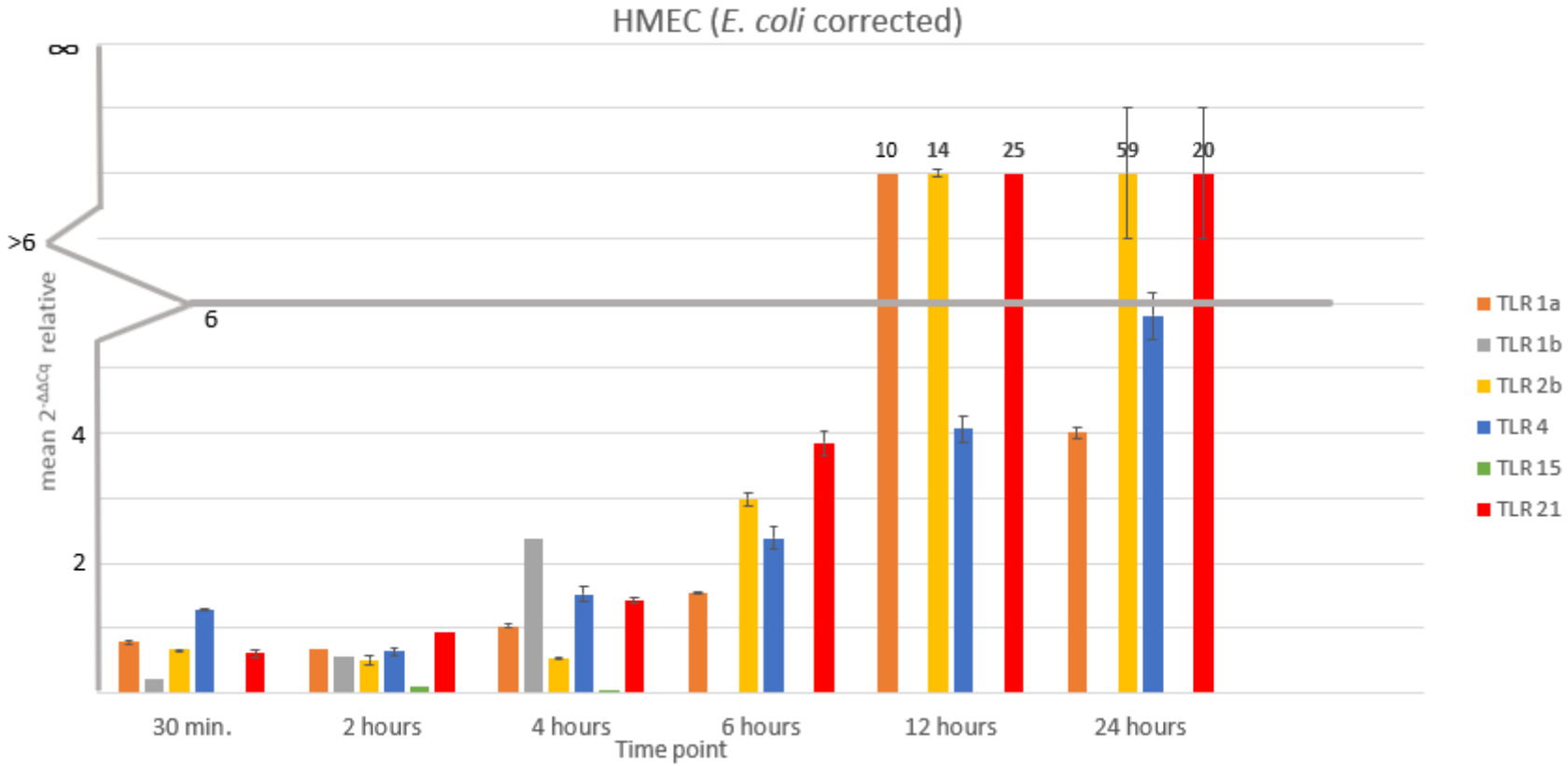

Fig. 12: Expression differences of TLRs as average values of $2^{-\Delta\Delta Cq}$ in HMEC (*E. coli* corrected) infected HD11 cells at 30min, 2h, 4h, 6h, 12h and 24 h after infection (n=1, in duplicate). The expression of uninfected cells was adjusted to 1 to provide a proportional view. Absent values are due to an undetectable expression in case of TLR 2a and 3 and a lack of signal from several

samples necessary for the calculation of HMEC (*E. coli* corrected) in case of TLR 1b, 5, 7 and 15. Only values outside the range of the respective uninfected cells' standard deviation are graphically represented.

4.1.2. LMH cell line

Results of the expression of the respective TLRs of LMH cells are given in detail below. Raw data on the TLR-expression of all groups is attached at the end of this work (**Add. Tab. 2**).
Because of a lack of measurable expressions of naive LMH cells or LMH cells infected with HMEC or EC at several time points, only a few comparisons were feasible for including in figures. Values resulting from only one biological sample are given in the text.
The microscopic examination of the viability of naive LMH cells at the end of the experiment revealed no substantial loss of the initial cell amount. In contrast, for infected LMH cells a visible loss of s cells compared to the naive cells was observed, indicating a poorer viability of these cells until the end of the experiment.

4.1.2.1. TLR 1a

Except at 24 hpi, the naive cell lines showed a continuous expression of TLR 1a-RNA at the measured time points (**Add. Tab. 2**).
In the LMH+EC group, TLR 1a-RNA was exclusively higher expressed compared to uninfected cells after 6 hours of incubation.
In HMEC infected cells the expression of TLR 1a was once below those of the naive cells at 4 hpi.

4.1.2.2. TLR 1b

No expression of TLR 1b-RNA could be detected at any time point in LMH cells.

4.1.2.3. TLR 2a

No expression of TLR 2a-RNA could be detected at any time point in LMH cells.

4.1.2.4. TLR 2b

Except at 24 hpi the uninfected cells showed an expression of this TLR with comparable lower levels after 30 minutes and 12 hours of incubation (**Add. Tab. 2**).
In only *E. coli* infected cells TLR 2b-RNA expression was lower than in the uninfected cells at 12 hpi. The same result was detectable for the HD11+HMEC group. These values resulted both from only one biological sample.

4.1.2.5. TLR 3

Naive cells showed an expression of this TLR after 2, 4 and 6 hours of incubation (**Add. Tab. 2**).
In the HD11+EC group TLR 3-RNA was less expressed than those of the naive cells after 6 hours of incubation. The same result was detected in the HMEC group.

4.1.2.6. TLR 4

Naive cells showed a constant expression of this TLR after 2, 4 and 6 hours of incubation (**Add. Tab. 2**).
In EC group TLR 4 was exclusively higher expressed compared to uninfected cells after an incubation period of 6 hours. The same result was detected in the HD11+HMEC group, showing at 4 hpi a lower expression of this TLR.

4.1.2.7. TLR 5

Naive cells showed an expression only after 2, 4 and 6 hours of incubation. At the last mentioned time point a slight increase of the expression was noticed. (**Add. Tab. 2**).
No TLR 5 expression values outside the naive cells' standard deviation could be detected at any time point in infected LMH cells.

4.1.2.8. TLR 7

No expression of TLR 7-RNA could be detected at any time point in LMH cells.

4.1.2.9. TLR 15

No expression of TLR 15-RNA could be detected at any time point in LMH cells.

4.1.2.10. TLR 21

Except at 30 min and 24 hours of incubation the uninfected cells showed an expression of this TLR with varying signal levels (**Add. Tab. 2**).
No TLR 21 expression values outside the naive cells' standard deviation could be detected at any time point in infected LMH cells.

4.1.2.11. HMEC (*E. coli* corrected)

After the *E. coli* correction of the HMEC values exclusively TLR 2b, 3 and 4 showed variations in their expression levels compared to the uninfected cells at 6 hpi. The RNA level was higher for TLR 2b (11.84) and 4 (20.67) and lower for TLR 3 (0.04).

5. Discussion

H. meleagridis is an extracellular protozoan that causes histomonosis in poultry (Tyzzer et al. 1920). The impact of this disease on the poultry industry has worsened over the past few years at a global scale and there is evidence to suggest that the increase in infections is in response to the ban of various prophylactic and therapeutic drugs (Liebhart et al. 2017). In the search for alternative treatments, the role of the immune system in defending *H. meleagridis* became the centre point of several studies (Mitra et al. 2018). Zhou et al. (2013) determined chTLR expression following infection by intracellular parasite *E. tenella*, but there is no information about the role of TLRs during extracellular parasitic infection in chickens. This study attempts to address the lack of information on this topic. *H. meleagridis* infects the caecum and the liver of host birds. By the use of HD11 cells, direct contact between macrophages and the parasite could be investigated and LMH cells revealed the response of hepatocytes *in vitro*. So far, there are only a few publications focusing on TLR expression in HD11 and LMH cell cultures (Iqbal et al. 2005a, Brownlie et al. 2009, Ciraci and Lamont 2011, Han et al. 2010, Weder 2012, Qi et al. 2017). However, there is no published work that covers the TLR response against an extracellular parasite using these cell lines.

In this work, naive HD11 cells showed an expression of all TLRs except TLR 2a and 3 with time dependent variations. The continuative expression of TLR 1a, 1b, 2b, 4 is in agreement with previous findings (Iqbal et al. 2005a). The absence of detectable RNA of TLR 2a and 3 in the uninfected HD11 cells is in contrast with findings from the previously mentioned author. This disagreement might be due to differences between the culture supplementations used in this and the above mentioned study. Conversely, the results characterizing the expression of TLR 5 at comparatively low levels in the uninfected cells conformed with results from Iqbal et al. (2005a). It is reasonable to conclude that the absence of TLR 5 expression at late timepoints might be due to a decrease of the already low expression levels into a non-detectable range. Regarding TLR 7, high expression levels were observed at the first two time points in our data. This result was in accordance with two previous studies in which the performed incubation time of the cells was not mentioned (Iqbal et al. 2005a, Philbin et al. 2005). The continuous expression of TLR 15 in naive HD11 cells was consistent with Higgs

et al. (2006), describing TLR 15 expression predominantly in lymphoid tissues. Regular TLR 21 expression in the uninfected HD11 cells was also in agreement with the detection of this TLR in, inter alia, macrophages (Brownlie et al. 2009).

Naive LMH cells showed an expression of TLR 1a, 2b, 3, 4, 5 and 21 with time dependent variations. Weder (2012) examined TLR 3, 7 and 21 expressions in uninfected LMH cells over 24 hours with positive results for all three TLRs. Except for TLR 7, these findings conformed with the results of our study. A possible reason for the discrepancy concerning TLR 7 might be the different culturing method of the cells used in the aforementioned study. These variations could be caused by the use of a different culture media and supplementations. The expression of TLR 21 in naive LMH cells was also observed by Brownlie et al. (2009).

Comparisons between the naive HD11 and LMH cells showed a more distinct expression of TLRs in the macrophage-like cell line. Heightened expression might be due to the immunological cell derivation of this cell culture system as TLRs, which are receptors of the innate immunity, show consequently higher expression levels in tissues with larger immunological compartments (Juul-Madsen et al. 2012).

HD11 cells infected with *E. coli* would be expected to express TLR 1a, 1b, 2a, 2b, 4, 5, 15 and 21 because cell parts and products of *E. coli* act as specific ligands (Iqbal et al. 2005a, Juul-Madsen et al. 2012, Brownlie et al. 2009, de Zoete et al. 2011). Except TLR 4 and 15 all remaining mentioned TLRs showed generally lower RNA levels compared to naive cells. In case of TLR 2a and 5 detectable signals were almost completely lacking. Technical and/or material shortcomings might be an explanation for these two TLRs because of the general absence of signals for almost all samples. The lower expression of TLR 1a, 1b, 2b and 21 could be due to the *in vitro* infection of the cells potentially being limited by the culturing system. Furthermore, it cannot be excluded that specific molecules regularly expressed *in vivo* were not produced (Law et al. 2013). Further research would be necessary to clarify the insufficiency of the *E. coli* PAMPs to active the respective receptors.
Because of the almost equal amount of *E. coli* cells in the HMEC the same TLRs as expected for the EC infected cells are assumed to be expressed in the HMEC infected cells with variations due to the additional parasites' influence. The RNA levels of TLR 1a, 1b, 2b, 4 and 21 were above those of the naive cells mainly at late time points. Earlier after infection

the expression varies showing predominantly lower RNA levels in the HMEC infected cells, indicating a possible time-dependent response to the contact with the parasite. The higher expression of TLR 5 has also been expected for HMEC infected HD11 cells because of flagellin as ligand for this TLR (Iqbal et al. 2005a). Possible reasons for the non-occurrence of this expression might be a structural difference between bacterial flagellin and the protozoan flagella (Brown 1945, Jones and Aizawa 1991). Another reason could be the absence of a flagellum of the tissue form of histomonads (Hess and McDougald 2020), indicating a possible irrelevance of the protozoan flagellum as virulence factor of the tissue form of the parasite. In general, a divergent TLR expression following a bacterial and a parasitic infection is obvious because of the different host cell interaction. However, variations caused by different numbers of *E. coli* cells in the EC and HMEC could mostly be excluded because of the determination of the bacterial growth during the experiment.

Infected HD11 cells (*H. meleagridis* (*E. coli* corrected)) showed higher expression levels of TLR 1a, 1b, 2b, 4 and 21 at varying time points compared to non-infected cells. These results are in concordance with those detected for the HMEC samples, proving the practicability of the used *E. coli* correction method. The first occurrence of a higher level of TLR 1a and 1b between 4 and 6 hpi might be due to a time-dependent response to the contact with the parasite. The following complete disappearance of TLR 1b in HMEC infected cells could be explained by a recurring decrease of the expression level in an undetectable range. Similar to TLR 1a TLR 2b was expressed at higher levels after 6 hours of incubation The continuing higher expression levels at the following time points confirmed the results of St. Paul et al. (2013) and implicate *H. meleagridis* as a possible ligand for TLR 2b. Zhou et al. (2013) demonstrated higher expression levels of TLR 4 after an *E. tenella* infection of monocyte-derived macrophages. Interestingly, the same result was observed following infection with *H. meleagridis* in the present work. Nevertheless, a direct comparison of the two studies is limited because of the parasites' differences in host cell parasitism (intercellular versus extracellular appearance). This might explain differences in the response of TLR 15 in HD11 cells between both parasites. Anyhow, a similar significant expression pattern as observed for TLR 4 could be demonstrated for TLR 21 at late time points in the infected HD11 cells, leading us to the assumption to play an important role in the immune response to an *H. meleagridis* infection which needs to be further investigated in future studies.

The absence of higher TLR 7-RNA amounts were not expected to be increased since ssRNA from viruses were shown act as a PAMP for this TLR (Diebold et al. 2004). The high

expression levels of TLR 15 in HD11 cells after an *E. coli* infection reduce the interpretive scope concerning TLR 15 expression after parasitic infection, therefore further research would be necessary.
In general, a mostly divergent TLR expression pattern following a bacterial and a parasitic infection was observed underlining the different host cell interactions.

LMH cells infected with EC and HMEC would also be expected to express the typical TLRs stimulated by bacterial PAMPs (Iqbal et al. 2005a, Juul-Madsen et al. 2012, Brownlie et al. 2009, de Zoete et al. 2011). In case of the EC samples, only TLR 1a and 4 showed this higher expression level respectively at 6 hpi. The lower signal levels of TLR 2b after 12 hours of incubation despite the presence of bacterial PAMPs might be related to the microscopically observed poor viability of the LMH cells. The expression levels of TLR 3 below those of the naive cells at 6 hpi can be due to the absent contact with double-stranded RNA, the defined PAMP for TLR 3 (Iqbal et al. 2005a).
The HMEC infected cells showed a similar expression profile as the *E. coli* infected cells, with additionally occurring lower expression levels of TLR 1a and 4 at 4 hpi. Infected LMH cells (*H. meleagridis* (*E. coli* corrected)) showed a higher expression of TLR 2b and 4 at 6 hpi. TLR 3-RNA was at a lower level at the same time point. The absence of further results before this point in the experiment is due to missing HMEC and/or EC values, which, if provided, enable the calculation of the HMEC (*E. coli* corrected). The omission of TLR signal from all infected LMH groups at earlier time points might be explained by a delay in the immune response of the infected LMH cells. Khvalevsky et al. (2007) demonstrated an induction of cell apoptosis following an expression of TLR 3 in hepatoma cell lines. This finding is not completely in concordance with our results but might be an explanation for the observed poor viability of the LMH cells at the end of the experiment leading to the absence of further detectable TLR signals at late time points. A further potential reason for this poor viability might be suboptimal culture conditions and a future optimisation would be necessary to exclude this deficiency about the cells' viability.
Weder (2012) exclusively investigated the expression of 3 TLRs (TLR 3, 7 and 21) in LMH cells. TLR 3 and 7 showed a decrease 24 hpi whereas TLR 21 underwent no obvious changes following a Gallid Herpesvirus-1 infection. These results are in agreement with Iqbal et al. (2005a) defining viral RNA as ligands for TLR 3 and 7. Consequently, the differences of the used pathogens and their presence intra-, respectively extracellular might explain deviations to our results.

Similarities between the expression patterns of TLRs from infected HD11 and LMH cells were identified for 2b and 4, however, it should be noted that HD11 cells showed a high viability compared to LMH cells at the end of the experiment by microscopic examination which could explain a more distinct expression of these respective TLRs. Conclusively, at 6 hpi both TLRs were higher expressed in infected HD11 and LMH cells.
Additionally, the reference gene expression must be considered to influence the calculated TLR values to a varying extend for both cell lines. For example, TFRC is known reacting with an up-regulation to an infection process (Tacchini et al. 2008). Consequently, a higher expression of TFRC would result in an apparently reduction of the others genes' expression through the calculation of the values used in this study. This TFRC up-regulation was partially observable in our investigations and must be kept in mind in the result interpretation. For the HMEC (*E. coli* corrected) values this mentioned influence of the reference genes is probably negligible, what might explain the visible detectability of the higher expression levels of the sensitive TLRs in this group.

The cause of the different TLR expression patterns of HD11 and LMH cells after infection with *H. meleagridis* was most probably based in their different cell functions. HD11 derived from macrophages, antigen presenting cells with an essential role in innate and acquired immunity. It could therefore be expected that macrophages interacted directly with *H. meleagridis* and specifically responded against the pathogen during infection. The origin of the LMH cell culture system was chicken hepatocytes with main functions in the liver metabolism and detoxification. In the liver, Kupffer cells which are specialized macrophages and part of the mononuclear phagocyte system are responsible for immunological features. Additionally, this would explain a lower TLR reactivity of the LMH cells towards the *H. meleagridis* infection. Conversely, cultured mouse hepatocytes showed an expression of TLR 1-9 after a stimulation with LPS, proving that hepatocytes by themselves are able to perform an immunological response (Liu et al. 2002). Consequently, we expected a similar result for this study concerning the expression of avian TLRs. The prevalence of histomonads in liver tissue was another reason for this assumption. However, it should again be underlined, that a direct comparison of *in vitro* and *in vivo* cells is limited because of the different environment and the possible absence of specific molecules regularly expressed *in vivo*. This statement is verified by comparing the before mentioned results from Liu et al. (2002) with the *in vivo* findings from Ojaniemi et al. (2005), detecting only TLR 2 in high

levels in mouse liver after LPS injection. However, the comparison of our results with those from mammals is limited because of species specific differences (Davison 2009). Anyhow, infected LMH- and HD11 cells showed higher RNA levels of the same TLRs compared to the control group with the exception of TLR 1a and 21. Microbial DNA as trigger for the expression of TLR 21 might explain the high RNA levels of this TLR in HD11 cells according to results from Brownlie et al. (2009). Overall, our findings demonstrate that TLR 1a, 2b, 4 and 21 are involved in the recognition of *H. meleagridis*. This recognition is expected to induce a signalling cascade according to the activated TLRs resulting in the expression of pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (CXCLi1, CXCLi2, CCLi4, IL-8) as immune mechanism against the invading histomonads (Kogut et al. 2006, Takeuchi and Akira 2010). From other parasites a variety of PAMPs are known triggering the immune response of mammals (Aguirre-García et al., 2019). In the present work it could be shown that specific TLRs of chicken cells can be expressed following contact to *H. meleagridis* which argues for the presence of associated PAMPs of the flagellate. Further research applying a higher number of samples for all groups at all time points would be necessary to proof the results for statistical relevance. Additionally, several study conditions have to be optimized for future studies, including an exact quantification of the cell number during and at the end of the experiment, the use of further intern RT-qPCR references and the use of other cell lines, perhaps even primary cell lines to exclude *in vitro* variations of the immune response, especially of the hepatocytes.

6. Summary

Histomonas meleagridis is an extracellular parasite with a raising economic impact in the poultry industry, leading to mortalities in turkeys and economic losses in chicken flocks. Ulceration and inflammation of the liver and caeca as typical pathological findings result from the infiltration of histomonads in the mentioned organs.

The ban of preventive and curative drugs against histomonosis in recent years led to intensified research focused on finding new strategies against the disease, including studies on the immune response against *H. meleagridis*.

For this study, cell lines of macrophages (HD11 cells) and hepatocytes (LMH cells) infected with cultivated *H. meleagridis* were investigated by RT-qPCR for the expression of toll-like receptors. This expression was determined after 30 minutes, 2 hours, 4 hours, 6 hours, 12 hours and 24 hours of incubation. Cells were additionally infected with E. coli only since histomonads could not be cultured axenically for excluding bacterial effects. TLR 2b and 4 showed higher expression levels compared to naive cells in both infected cell types. RNA levels above those of the control group for TLR 1a and 21 was only detectable in the infected HD11 cells. Compared to LMH cells, with exclusively higher expression levels at 6 hpi, the challenged HD11 cells showed continuously high expression levels noticeably at late time points. However, no obvious expression changes occurred for TLR 2a, 3, 5, 7 and 15 in HD11 cells. Infected LMH cells showed no expression for TLR 1a, 1b, 2a, 5, 7, 15 and 21 which might be related to a poor viability of the LMH cells at the end of the experiment compared to HD11 cells and the different cellular functions of the two cell populations. HD11 as a cell culture system of immune cells is expected to respond specifically against invading pathogens. LMH as a cell culture system derived from chicken hepatocytes is less responsible for immunological interactions.

In summary, TLR 1a, 2b, 4 and 21 demonstrated to play a role in the host defence against *H. meleagridis*. This defence is expected to be characterized by an induced signalling cascade resulting in the expression of pro-inflammatory cytokines and chemokines as immune mechanism against the invading histomonads.

7. Zusammenfassung

Histomonas meleagridis ist ein extrazellulärer Parasit, der bei Puten zu hoher Moralität führen kann und in Hühnerbetrieben ökonomische Einbußen verursacht. Ulzera und Entzündungen von Leber und Blinddärmen sind typische pathologische Befunde, welche aufgrund der Infiltration des Parasiten in das Gewebe der genannten Organe resultieren.

Aufgrund des Verbotes von Medikamenten zur Vorbeugung und Therapie der Histomonose in den letzten Jahren, wurde die Forschung für neue Bekämpfungsmöglichkeiten vorangetrieben. Dabei spielt die Rolle des Immunsystems der infizierten Tiere eine entscheidende Rolle.

Für die vorliegende Arbeit wurden Zellkulturen aus Makrophagen (HD11 Zellen) und Hepatozyten (LMH Zellen) mit kultivierten Histomonaden infiziert und daraufhin die Expression von Toll-like-Rezeptoren mittels RT-qPCR quantifiziert. Die unterschiedlichen Inkubationszeiten waren 30 Minuten, 2, 4, 6, 12 und 24 Stunden. Zusätzlich wurden die Zellen mit *E. coli* infiziert, um Einflüsse der Bakterien zu berücksichtigen, da Histomonaden nicht axenisch kultiviert werden konnten. TLR 2b und 4 zeigten erhöhte Expressionslevel im Vergleich zu den naiven Zellen in beiden infizierten Zelltypen. Eine erhöhte RNA Menge verglichen zur Kontrollgruppe konnte von TLR 1a und 21 nur in den infizierten HD11 Zellen festgestellt werden. Verglichen mit LMH Zellen, welche jeweils immer nur sechs Stunden nach der erfolgten Infektion eine höhere Expression gegenüber nicht infizierten Zellen aufgewiesen haben, zeigten HD11 Zellen eine länger andauernde Erhöhung. Allerdings kam es zu bestimmten Zeitpunkten bei TLR 2a, 3, 5, 7 und 15 in HD11-Zellen und häufiger in LMH-Zellen bei TLR 1a, 1b, 2a, 5, 7, 15 und 21 zu keiner oder einer nur gering von der Kontrollgruppe abweichenden Expression. Dies könnte mit der schlechteren Lebensfähigkeit der LMH Zellen am Ende des Experiments verglichen mit HD11 Zellen und den unterschiedlichen zellulären Funktionen der beiden Zelllinien zusammenhängen. HD11 Zellen sind Makrophagen die in der Immunantwort gegen eindringende Erreger involviert sind. Im Gegensatz dazu sind LMH Zellen Hepatozyten, deren Aufgabe im Leberstoffwechsel zu finden ist.

Zusammenfassend konnte gezeigt werden, dass TLR 1a, 2b, 4 und 21 im Immunsystem des Huhnes als Abwehr gegen *H. meleagridis* eine signifikante Rolle spielen. Diese Abwehr ist erwartungsgemäß durch eine ausgelöste Signalübermittlungskaskade gekennzeichnet, welche zur Expression von pro-inflammatorischen Zytokinen und Chemokinen als immunologischer Mechanismus gegen eindringende Histomonaden führt.

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duplicate). The expression of uninfected cells was adjusted to 1 to provide a proportional view. Absent values are due to an undetectable expression in case of TLR 2a and 3 and a lack of signal from several samples necessary for the calculation of HMEC (*E. coli* corrected) in case of TLR 1b, 5, 7 and 15. Only values outside the range of the respective uninfected cells' standard deviation are graphically represented.

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Tab. 1: Overview on investigations on avian TLRs and their functionality.
Tab. 2: Primers and probes used for RT-qPCR.

11. List of abbreviations

CFU	colony forming unit
Cq	cycle of quantification
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
EC	*E. coli* culture
E. tenella	*Eimeria tenella*
HD11	chicken macrophage-like cell line
H. gallinarum	*Heterakis gallinarum*
H. meleagridis	*Histomonas meleagridis*
HMEC	*H. meleagridis*+*E. coli* culture
HMEC (*E. coli* corrected)	*H. meleagridis*+*E. coli* culture (*E. coli* corrected)
hpi	hours post infection
IFN-γ	interferon Gamma
IgA	immunglobulin A
LMH	Leghorn Male Hepatoma cell line
LPS	lipopolysaccharide
PAMP	pathogen-associated molecular patterns
RPL13	ribosomal protein L13
rpm	repeats per minute
ssRNA	single-stranded RNA
TFRC	transferring receptor protein 1
TLR	toll-like receptor

Add. Tab. 1: RT-qPCR Cycle of quantification (Cq) from HD11 cells (technical duplicates) (no detectable RNA is described as n.d.).

		Cq	
	Naive HD11	HD11+HMEC	HD11+EC
30 min			
TLR 1a	19.775	25.565	19.995
	18.615	23.23	20.025
TLR 1b	22.2	n.d.	22.205
	21.1	28.01	22.105
TLR 2b	19.905	24.95	19.65
	18.26	22.84	19.215
TLR 4	20.585	25.14	20.14
	18.8	23.46	19.925
TLR 5	n.d.	n.d.	n.d.
	27.425	n.d.	n.d.
TLR 7	21.4	n.d.	20.54
	19.875	n.d.	n.d.
TLR 15	19.535	n.d.	19.215
	17.635	n.d.	18.85
TLR 21	21.235	27.625	20.755
	18.99	24.28	21.315
2 hours			
TLR 1a	19.48	21.68	19.9
	20.32	n.d.	18.83
TLR 1b	22.03	23.885	21.66
	21.725	n.d.	28.905
TLR 2b	19.66	20.59	19.625
	19.695	33.845	19.655
TLR 4	20.125	21.19	19.685
	20.235	29.59	19.435
TLR 5	26.91	n.d.	n.d.
	30.05	n.d.	n.d.
TLR 7	21.175	n.d.	20.39
	21.305	n.d.	18.73
TLR 15	19.145	23.11	16.98
	19.775	n.d.	16.015
TLR 21	20.78	21.54	20.595
	20.585	n.d.	20.3
4 hours			
TLR 1a	20.235	21.545	20.31
	20.42	22.335	19.385
TLR 1b	23.265	23.26	22.415

	23.315	n.d.	21.175
TLR 2b	19.375	21.11	19.9
	19.735	21.82	18.695
TLR 4	19.92	21.63	22.02
	20.325	22.35	18.78
TLR 5	26.475	n.d.	27.06
	n.d.	n.d.	26.13
TLR 7	n.d.	23.26	20.475
	n.d.	26.345	n.d.
TLR 15	19.975	24.73	17.395
	20.3		16.225
TLR 21	22.63	22.095	21.23
	21.31	22.565	19.96
6 hours			
TLR 1a	20.48	23.375	20.815
	19.93	22.72	20.515
TLR 1b	22.695	n.d.	n.d.
	22.255	n.d.	n.d.
TLR 2b	18.985	22.285	21.15
	19.49	20.15	19.835
TLR 4	19.545	20.965	19.95
	19.79	22.335	19.855
TLR 5	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 7	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 15	20.99	n.d.	n.d.
	19.83	n.d.	17.325
TLR 21	22.205	24.01	23.42
	20.39	23.375	21.93
12 hours			
TLR 1a	20.645	n.d.	21.125
	21.14	23.94	21.845
TLR 1b	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 2b	19.545	26.385	21.695
	20.21	23.185	22.685
TLR 4	20.33	26.64	19.735
	20.9	24.31	20.595
TLR 5	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 7	n.d.	n.d.	n.d.

	n.d.	n.d.	n.d.
TLR 15	22.17	n.d.	17.345
	23.275	n.d.	18.525
TLR 21	23.795	n.d.	23.29
	22.895	26.9	25.1
24 hours			
TLR 1a	21.155	30.785	21.44
	20.75	28.825	22.75
TLR 1b	23.93	n.d.	25.12
	22.9	n.d.	27.79
TLR 2b	20.245	28.385	23.56
	19.715	27.12	26.2
TLR 4	20.785	28.865	19.895
	20.1	26.45	21.71
TLR 5	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 7	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 15	22.35	n.d.	17.865
	21.975	n.d.	19.22
TLR 21	21.27	28.335	21.2
	20.445	30.1	28.2

Add. Tab. 2: RT-qPCR Cq values from LMH cells (technical duplicates) (no detectable RNA is described as n.d.).

		Cq	
	Naive LMH	LMH+HMEC	LMH+EC
30 min			
TLR 1a	25.77	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 2b	26.345	n.d.	33.61
	n.d.	n.d.	34.58
TLR 3	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 4	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 5	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 21	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
2 hours			
TLR 1a	23.285	n.d.	n.d.

	23.82	n.d.	n.d.
TLR 2b	23.1	n.d.	n.d.
	24.465	n.d.	n.d.
TLR 3	25.14	n.d.	n.d.
	25.545	n.d.	25.14
TLR 4	24.37	n.d.	n.d.
	25.3	n.d.	n.d.
TLR 5	25.08	n.d.	n.d.
	25.095	n.d.	n.d.
TLR 21	23.24	n.d.	n.d.
	24.25	n.d.	29.875
4 hours			
TLR 1a	23.74	27.83	n.d.
	22.855	24.77	n.d.
TLR 2b	23.84	28.725	n.d.
	23.205	27.535	n.d.
TLR 3	30.3	32.235	n.d.
	23.6	n.d.	n.d.
TLR 4	24.635	29.84	n.d.
	23.93	29.13	n.d.
TLR 5	26.6	n.d.	n.d.
	24.835	n.d.	n.d.
TLR 21		n.d.	n.d.
	31.095	n.d.	n.d.
6 hours			
TLR 1a	23.115	n.d.	23.1
	24.085	27.19	22.41
TLR 2b	24.155	26.615	23.825
	26.17	26.59	22.845
TLR 3	21.36	30.55	21.955
	22.595	31	21.39
TLR 4	24.985	27.685	24.44
	25.97	27.525	23.84
TLR 5	27.53	n.d.	26.12
	27	n.d.	25.48
TLR 21	23.345	n.d.	22.785
	31.38	n.d.	24.46
12 hours			
TLR 1a	n.d.	n.d.	n.d.
	24.55	n.d.	n.d.
TLR 2b	n.d.	n.d.	n.d.
	29.905	n.d.	n.d.

TLR 3	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 4	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 5	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 21	n.d.	n.d.	n.d.
	24.87	n.d.	n.d.
24 hours			
TLR 1a	n.d.	n.d.	24.785
	n.d.	n.d.	n.d.
TLR 2b	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 3	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 4	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 5	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.
TLR 21	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.

Add. Tab. 3: Amount of DH5α in all types of cultures used in the experiment.

	Dilution at the agar-plate			
	10^-6	10^-7	10^-8	10^-9
Histomonas culture-HD11	n.c. (not countable)	17	0	0
Histomonas culture-LMH	same	29	0	0
HD11*E.coli* DH5α **1:10 diluted** (day 2; over night in the fridge)	n.c.	23	2	0
LMH*E.coli* DH5α **1:10 diluted** (day 2; over night in the fridge)	n.c.	13	2	0

Add. Tab. 4: Efficiency and slope of the applied RT-qPCR genes.

Gene	Efficiency	Slope
chTLR 1a	96	-3.23
chTLR 1b	100.4	-3.46
chTLR 2a	96.3	-3.19
chTLR 2b	96.4	-3.52

chTLR 3	96.3	-3.44
chTLR 4	95.1	-3.20
chTLR 5	100.3	-3.51
chTLR 6	96.4	-3.35
chTLR 15	100.9	-3.69
chTLR 21	99.2	-3.40