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Comparison of immune responses between chicken lines after vaccination with different infectious bursal disease (IBD) vaccines

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To my family and friends

Home is where the heart is

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List of abbreviations

°C	degree celcius
µg	microgram
µl	microliter
AGP	agar gel precipitation
ALV	avian leukosis virus
APP	acute phase protein
BA	Brucella abortus
BALT	bronchio-associated lymphoid tissue
BF	Bursa of Fabricius
BrL	Brown Leghorn
BSA	bovine serum albumin
BT	broiler-type
C.	*Campylobacter*
CALT	conjunctiva-associated tissue
CCLi	chemokine (C-C) motif ligand
CD	cluster of differentiation
CMI	cell-mediated immunity
ConA	Concanavalin A
CT	caecal tonsil
CXCLi	chemokine (C-X-C) motif ligand
d	distinct
dIBDV	distinct IBDV
dph	days post hatch
dpi	days post infection
ds	double-stranded
DT	dual-purpose type
E. coli	*Escherichia coli*
EID	embryonic incubation day
ELISA	enzyme-linked immunosorbent assay
GALT	gut-associated lymphoid tissue
GC	germinal center
Gzm-A	granzyme A
HA	high antibody
hvr	hyper-variable region
HVT	herpesvirus of turkeys
HVT-IBD	herpesvirus of turkeys – infectious bursal disease
HW	high weight
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
Icx	immune complex
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILTV	infectious laryngo tracheitis virus
iNOS	inducible nitric oxide synthase

KLH	keyhole limpet hemocyanin
LA	low antibody
LPS	lipopolysaccharide
LT	layer-type
LTA	lipoteichoic acid
LW	low weight
MALT	mucosa-associated lymphoid tissue
MBL	mannose-binding-lectin
MD	Marek’s disease
MDA	maternally-derived antibodies
MDV	Marek’s disease virus
MHC	major-histocompatibility-complex
NDV	Newcastle disease virus
NK	natural killer
NO	nitric oxide
ORF	open reading frame
p	projection
PALS	peri-arteriolar lymphoid sheath
PCR	polymerase chain reaction
PELS	peri-ellipsoid lymphoid sheath
PFN	perforin
PFN-Gzm-A	perforin-granzyme A
PHA	phytohemagglutinin
PGC	primordial germ cells
qRT-PCR	quantitative real-time reverse transcriptase PCR
RSV	Rous sarcoma virus
RT-PCR	reverse transcription polymerase chain reaction
SE	*Salmonella* Enteritidis
sIgM	surface IgM
SPF	specific-pathogen-free
SRBC	sheep red blood cells
ST	*Salmonella* Typhimurium
TCR	T-cell receptor
Th	T-helper
TGF	transforming growth factor
TNP	trinitrophenyl
TNP-KLH	trinitrophenyl -conjugated keyhole limpet hemocyanin
Treg	regulatory T-cell
VNT	virus neutralization test
VP	viral protein
vv	very virulent
vvIBDV	very virulent IBDV

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1. Summary

Comparison of immune responses between chicken lines after vaccination with different infectious bursal disease (IBD) vaccines.

Marina Dobner

This thesis was part of a collaborative research project, which had the aim to compare the use of a newly developed dual-purpose chicken breed (Lohmann Dual, dual- purpose type, DT) with high-performing layer- and broiler type chickens (Lohmann Brown Plus, LT; Ross 308, BT). The project was funded by the German Government's Special Purpose Fund held at Landwirtschaftliche Rentenbank, Boehringer Ingelheim Vetmedica GmbH, Big Dutchman International GmbH, and Lohmann Tierzucht GmbH. Different working groups addressed various important aspects, such as the nutrition, housing conditions as well as the behavior right up to the slaughtering process and meat quality comparing DT, LT and BT chickens. Our part of the project investigated the development of the immunocompetence and disease resistance comparing DT with high-performing LT and BT chickens. The infectious bursal disease virus (IBDV) was used a model pathogen.

Infectious bursal disease (IBD) is one of the most important immunosuppressive viral diseases in poultry. Economic losses due to increased mortality rates and susceptibility to secondary pathogens threaten the commercial poultry production. Field observations and experimental studies indicate that the genetic background of chickens influences the susceptibility to IBDV. Therefore, the understanding of genotype-dependent variations in immune responses upon IBDV vaccination are needed to ensure the health and protection of chickens in the field.

The aims of the first study were to determine genotype-associated differences in the development of the immune system and in innate and adaptive immune responses during the first weeks post hatch. Furthermore, the effect of the *in ovo* vaccination with a recombinant vectored herpesvirus of turkeys (HVT) – IBD vaccine on the investigated immune parameters was included (1st manuscript). The relative lymphoid organ

weights, the structural development and numbers of different immune cell populations in the bursa of Fabricius (BF) (CD4+, CD8+ T-cells) and in the spleen (CD4+, CD8+ T-cells, B-cells, macrophages) as well as the number of germinal centers (GC) in the spleen and caecal tonsil (CT) were determined. Besides the anti-IBDV specific humoral immune response, the secondary antibody response after vaccination with an infectious bronchitis virus (IBV) - Newcastle disease (NDV) vaccine was investigated. Additionally, isolated spleen leukocytes were stimulated *in vitro* with either Concanavalin A (ConA) or lipopolysaccharides (LPS) to evaluate the immune responsiveness of T-cells and macrophages. It was demonstrated that the genotype significantly influenced the immune organ development, the anti-IBDV specific antibody response as well as interferon (IFN) γ release after ConA stimulation and nitric oxide (NO) release after LPS stimulation. For all the investigated parameters, BT birds showed the most vigorous immune responses compared to LT birds, while DT birds took an intermediate position. Early HVT-IBD vaccination at embryonic incubation day (EID) 18 affected various investigated immune parameters including the numbers of GC in the spleens, CD4+ T-cell numbers in the BF and macrophage populations in the spleens. Furthermore, HVT-IBD affected the anti-IBV and -NDV antibody response, which varied between the genotypes.

The aim of the second study was to understand more about the role of T-cell mediated immune responses in the bursal recovery after vaccination with an intermediate plus IBDV strain. Birds were investigated up to 28 days post IBDV inoculation. Two experiments were conducted where the different chicken lines were inoculated at 28 or 35 days post hatch (dph) (Exp. 1, Exp. 2). Besides the commercial BT, LT and DT chicken lines also the highly susceptible specific-pathogen-free (SPF) LT chickens were included in the comparison (2nd manuscript). In order to determine the time point when anti-IBDV specific maternally derived antibodies (MDA) were below the breakthrough titer of the vaccine, the Deventer formula was applied. The anti-IBDV specific antibody response was determined by ELISA. The BF was investigated for macroscopic and microscopic lesions, B-cell depletion and repopulation. Furthermore, the development of intrabursal CD4+ and CD8+ T-cell accumulation, the number of IBDV antigen positive cells as well as regulatory T-cell (Treg)-related cytokine mRNA

expression (interleukin (IL)-10, transforming growth factor (TGF) β4) in the BF was investigated. SPF chickens confirmed the IBDV inoculation in both experiments. The immune parameters for BT, LT and SPF chickens were comparable between the two experiments. DT chickens showed a high deviation in the decrease of maternally derived antibody (MDA) levels from the other chicken lines, which led to a delayed induction of immune responses after vaccination specifically in Exp. 1. The study revealed a significant genotype-influence on the duration of the bursal recovery process. Inoculated BT groups showed a faster recovery indicated by a continuous decline in intrabursal CD4+ and CD8+ T-cells, bursal lesion scores and IBDV-antigen loads. Significantly elevated levels of IL-10 mRNA expression in the BF were associated with a prolonged intrabursal T-cell accumulation and bursal lesions. We may speculate that Tregs and IL-10 might be involved in the recovery process after IBDV infection. This might be a result of a different T-cell regulation between the chicken lines.

Taken together, our results provide new insights into genotype-associated variations in the immune responses after vaccination with different IBDV vaccines. Based on our studies, we suggest that genotype-dependent vaccination schedules should be implemented in the field. Furthermore, this study stresses the importance of the T-cell immunity in chickens, which should be investigated in the future with respect to defining different T-cell subpopulations and their functions.

2. Zusammenfassung

Vergleichsstudie über die Immunantwort zwischen Hühnerlinien nach Vakzination mit unterschiedlichen Impfstoffen gegen das Infektiöse Bursitis Virus (IBDV).

Marina Dobner

Diese These war Teil eines Forschungsverbundprojektes, welches das Ziel hatte den Einsatz einer neuen Zweinutzungshuhngenetik (Dualhuhn, Lohmann Dual, DT) mit hochleistenden Lege- und Masttyplinien (Lohmann Brown plus, LT; Ross 308, BT) zu vergleichen. Die Förderung erfolgte aus Mitteln des Zweckvermögens der Landwirtschaftlichen Rentenbank, Boehringer Ingelheim Vetmedica GmbH, Big Dutchman International GmbH und Lohmann Tierzucht GmbH. Mehrere Arbeitsgruppen befassten sich mit unterschiedlichsten Aspekten zur Tiergesundheit, zu den Haltungsbedingungen, zur Tierernährung, zum Verhalten bis hin zum Schlachtprozess und der Fleischqualität. Unser Projektteil untersuchte die Entwicklung der Immunkompetenz und Krankheitsresistenz des Dualhuhns im Vergleich zu hochleistenden BT- und LT- Hühnerlinien. Das Infektiöse Bursitis Virus (IBDV) wurde als Modellerreger verwendet.

IBDV verursacht eine der wichtigsten immunsuppressiven Erkrankungen des Geflügels. Wirtschaftliche Verluste können aufgrund von erhöhter Mortalität und Empfänglichkeit gegenüber Sekundärerregern entstehen und stellen eine Bedrohung für die kommerzielle Geflügelhaltung dar. In Feld- als auch in experimentellen Studien wurde gezeigt, dass der genetische Hintergrund der Hühner die Empfänglichkeit gegenüber IBDV beeinflusst. Daher ist es notwendig, Genotyp-bedingte Unterschiede in der Immunantwort nach IBDV-Impfung aufzudecken, um die Gesundheit und den Schutz der Hühner im Feld sicherstellen zu können.

Die Ziele der ersten Studie waren es, sowohl Genotyp-assoziierte Unterschiede in der Entwicklung des Immunsystems als auch in der angeborenen und erworbenen Immunantwort zu bestimmen. Außerdem wurde der Effekt eines rekombinanten

Putenherpesvirus (HVT) – Infektiöse Bursitis (IBD) (HVT-IBD) Vektorimpfstoffes auf die untersuchten Immunparameter in die Untersuchungen mit einbezogen (1. Manuskript). Die relativen Immunorgangewichte, der strukturelle Aufbau und die Anzahl unterschiedlicher Immunzellpopulationen in der Bursa Fabricii (BF) (CD4+, CD8+ T Zellen) und in der Milz (CD4+, CD8+ T-Zellen, B-Zellen, Makrophagen) sowie die Anzahl der Germinalzentren (GC) in Milz und Zäkaltonsille wurden bestimmt. Neben der anti-IBDV spezifischen humoralen Immunantwort, wurde die sekundäre Antikörperantwort nach Impfung mit einem Infektiöse Bronchitis Virus (IBV) - Newcastle Disease Virus (NDV) Impfstoff untersucht. Schließlich wurden aus der Milz isolierte Leukozyten *in vitro* mit Concanavalin A (ConA) oder Lipopolysacchariden (LPS) stimuliert, um die Immunreaktivität von T Zellen und Makrophagen zu untersuchen. Der Genotyp hatte einen signifikanten Einfluss auf die Immunorganentwicklung, die anti-IBDV spezifische Antikörperantwort und die Interferon (IFN) γ und Stickstoffmonoxid (NO) Freisetzung nach Stimulation mit ConA und LPS. BT-Hühner zeigten die stärkste Immunantwort für alle untersuchten Immunparameter im Vergleich zu den LT-Hühnern, während die DT Tiere eine Zwischenstellung einnahmen. Die *in ovo* HVT-IBD Impfung beeinflusste verschiedene Immunparameter, welche zwischen den Genotypen variierten. Es waren sowohl die Anzahl der GC und Makrophagen in den Milzen, die Anzahl der CD4+ T Zellen in der BF als auch die anti-IBV und –NDV Antikörperantwort betroffen.

Das Ziel der zweiten Studie war es, mehr über die Rolle der T-Zell vermittelten Immunantwort in der Regenerationsphase nach Impfung mit einem intermediär plus IBDV Lebendimpfstoff zu erfahren. Die Hühner wurden bis 28 Tage nach IBDV Inokulation untersucht. In zwei Experimenten wurden die unterschiedlichen Hühnerlinien an 28 oder 35 Tagen inokuliert. Neben den kommerziellen BT, LT und DT Hühnern wurden zusätzlich die hochempfänglichen, spezifisch-pathogen freien (SPF) LT-Hühner in den Vergleich miteinbezogen (2. Manuskript). Um zu bestimmen, wann die maternalen anti-IBDV-Antikörpertiter (MDA) unterhalb des Durchbruchtiters des Impfstoffes lagen, wurde die Deventer Formel angewendet. Die anti-IBDV-spezifische Antikörperantwort wurde bis 28 Tage nach Infektion untersucht. Die BF wurde auf makroskopische und mikroskopische Läsionen, auf B-Zell-Depletion und

Repopulation untersucht. Außerdem wurde sowohl der Verlauf der intrabursalen CD4+ und CD8+ T-Zell Akkumulation, die Anzahl IBDV-Antigen positiver Zellen als auch die regulatorischen T-Zell (Treg)–assoziierten Zytokinexpression (Interleukin (IL)-10, Transforming growth factor (TGF)β4) untersucht. Der Verlauf der IBDV Infektion in den SPF Hühnern war zwischen den Versuchen vergleichbar. Auch die Ergebnisse der BT- und LT-Hühner waren für beide Experimente vergleichbar. Der Abfall der MDA der DT-Hühner wich stark von den anderen Genotypen ab, was zu einer verzögerten Induktion der Immunantworten, insbesondere in Exp. 1 führte. Die Studie zeigte einen signifikanten Einfluss des Genotyps auf die Dauer der Regenerationsphase der BF. Inokulierte BT Gruppen zeigten eine schnellere Regeneration, welche durch einen kontinuierlichen Abfall der intrabursalen CD4+ und CD8+ T-Zellen, der Bursa Läsions-Scores und der IBDV-Antigen Menge gekennzeichnet war. Signifikant erhöhte IL-10-mRNA-Level in der BF waren mit einer verlängerten intrabursalen T-Zell Akkumulation und Bursaläsionen assoziiert. Wir vermuten, dass Tregs und IL-10 an der Regeneration nach einer IBDV-Infektion beteiligt sind. Dies könnte durch eine unterschiedliche T-Zell Regulation zwischen den Hühnerlinien bedingt sein.

Insgesamt zeigen unsere Ergebnisse Genotyp-assoziierten Unterschiede in den Immunantworten nach Applikation unterschiedlicher IBDV Impfstoffe. Basierend auf unseren Untersuchungen, sollte ein Genotyp-abhängiges Impfregime im Feld umgesetzt werden. Außerdem zeigt unsere Studie die Bedeutung der T-Zell Immunität beim Huhn auf, die zukünftig insbesondere in Bezug auf unterschiedliche T-Zell Subpopulationen und deren Funktionen näher untersucht werden sollte.

3. Introduction

The production of poultry meat and eggs plays a major role in the supply of animal protein for the global population. Over many decades chickens were bred to achieve high-performing livestock animals. They were either selected for fast growth, improved body weight gain within a short period of time by low feed conversion rates for meat-type birds (broiler-type chickens, BT) or for a preferably high egg-production and longevity within one production cycle for layer-type chickens (LT). Future predictions suggest that the global production of poultry will outreach the production of other livestock species, such as pigs and cattle. Therefore, securing the poultry health constitutes the foundation to further continue the supply of reliable and affordable protein sources.

This thesis was part of a collaborative research project “Integhof”, which addressed the use of a newly developed dual-purpose chicken line as a step towards better animal welfare and health. It was speculated that due to the reduction in performance intensity for both egg- and meat traits the general health might be improved. The goal of this part of the project was to compare the dual-purpose chicken with a high-performing BT and LT chicken line with respect to its immunocompetence and disease resistance. In this context the infectious bursal disease virus (IBDV) was used as a model pathogen.

Immunosuppressive, viral diseases, such as infectious bursal disease (IBD), pose a continuous threat to the health of chicken flocks worldwide. Thus, suitable immunoprophylactic measures play a particularly important role to maintain poultry health and productivity.

The Infectious bursal disease virus (IBDV) belongs to the family of *Birnaviridae* (bi-segmented, double stranded RNA viruses) and represents a highly contagious agent for young chickens. The virus is transmitted by direct contact with infected animals but also by contaminated litter, faeces, equipment, feed or staff clothing. After oral or oculonasal inoculation, the virus replicates in B-lymphocytes and also macrophages of gut-associated tissues and subsequently reaches its target organ, the Bursa of Fabricius (BF). IBDV mainly targets actively dividing immunoglobulin (Ig)M-bearing

B- cells in the BF, which are destroyed by the virus. Furthermore it also affects the function of other immune cell populations, such as macrophages. Consequently, infected birds suffer from severe immunosuppression, which cannot only lead to a higher susceptibility to secondary infections but also to a depression in vaccine responses against other pathogens. Substantial economic losses can therefore be directly caused by increased mortality rates but also indirectly by the reduced performance with a decreased feed conversion efficiency and body weight development.

Currently, IBD associated losses can only be prevented by vaccination programs. In the field, BT as well as LT chickens are vaccinated against IBDV.

In developed countries, where animal welfare standards are highly rated in the society as well as in politics, the high performance pressure on livestock animals is frequently criticized. It is suggested that animals with a reduced performance might be able to use more of their resources for other important traits, such as the immune responses. The use of so-called dual-purpose breeds (dual-purpose type, DT), which are used for both meat- and egg production, addresses several animal welfare aspects including less performance pressure for both production traits.

Field observations and also experimental studies suggest that the genetic background of chickens influences IBDV pathogenesis. Efforts have been made to understand more about the mechanisms of genetic resistance to different diseases in the chicken. The major histocompatibility complex (MHC) is associated with the susceptibility to several infectious diseases. Genetically based differences in innate immune responses in association with disease susceptibility or resistance may exist but their role in IBDV-infection is still not clear.

More studies are needed to determine immune-related differences between chicken lines to ensure the advancement of prophylactic strategies for a better control of immunosuppressive, viral diseases.

Two main objectives were addressed in this thesis:

1) In the first study we wanted to compare the development of innate and acquired immune parameters during the first five weeks post hatch between BT, LT and DT chickens. In addition we determined possible immunostimulatory effects of a recombinant vector herpesvirus of turkeys (HVT) IBD (HVT-IBD) vaccine on these immune parameters to identify possible variations between the chicken lines (Manuscript 1, Chapter 6).

2) Subsequently the impact of genotype-associated differences on IBDV pathogenesis and immune responses were addressed after inoculation with an intermediate plus IBDV strain. For that reason, particular attention was given to the recovery phase and T-cell mediated immune responses as well as the cytokine expression pattern in the BF (Manuscript 2, Chapter 7).

Therefore, the thesis provides an important contribution to the understanding of IBD and the basis for the development of new prophylactic approaches.

4. Literature review

4.1 Genetic selection of high-performing chicken genotypes

4.1.1 History

The domestication of chickens has already started thousands of years ago during the Neolithic period in China, Europe and Western Asia (WEST and ZHOU 1988). Culture, religion and entertainment might have been the first reasons for the domestication (CRAWFORD 1990; TIXIER-BOICHARD et al. 2011). After the domestication, various chicken breeds with special morphological features developed in different parts of the world. In the beginning of the 20th century, the selection for descriptive traits, such as comb types as well as plumage and skin colors, was intensively studied and it was demonstrated that these traits followed the inheritance based on Mendelian rules (HUNTON 2006). Soon, researchers also moved quantitative traits, such as egg production, body weight, growth and conformation, into their focus. It was shown, that the inheritance of quantitative traits could not be explained by Mendelian genetics (HUNTON 2006). Technological and mathematical advancements facilitated the breeding for specific traits by improved data recording and statistical methods. The understanding of quantitative genetics initiated long-term selection experiments in various types of chickens and the establishment of several breeding companies (HUNTON 2006; TIXIER-BOICHARD et al. 2012). Nowadays, only a few major breeding companies dominate the global market, specialized in the breeding of either layer-type (egg production) or broiler-type chickens (meat production). The negative correlation between reproductive and meat traits might have contributed to this development.

4.1.2 Problems associated with genetic selection

The unilateral genetic selection of chickens for a high performance in egg- or meat production led to health associated problems, which include metabolical, physiological, behavioral and also immunological disorders (RAUW et al. 1998).

The selection of BT chickens mainly concentrated on fast growth, improved feed conversion rates and high breast meat proportion (HAVENSTEIN et al. 1994a, b). The growth rate of broiler chickens increased over 400% comparing broiler strains from 1950 and 2005 (ZUIDHOF et al. 2014).

The fast body weight gain over a very short period of time may lead to skeletal problems in BT chickens due insufficient bone structure maturation during the production period. Also metabolical disorders, such as pulmonary hypertension which leads to the ascites syndrome is a consequence of the selection for rapid growth and body weight (JULIAN 1998; KALMAR et al. 2013).

The selection for specific production traits in LT chickens mainly focused on livability, egg weight, age at sexual maturity and still most importantly the number of produced eggs per hen housed (ARTHUR and ALBERS 2003). Also in LT chickens skeletal problems are frequently recorded due to nutritional imbalances of calcium, phosphorus and vitamin D supply. Nevertheless, many different factors contribute to the manifestation of osteoporosis (WHITEHEAD et al. 2003). It was also reported that selection towards a lower body weight and high production performance resulted in an increasing incidence of feather pecking and cannibalism in specifically brown feathered LT birds (PREISINGER 1997; KJÆR and MENCH 2003).

In the last years, the social concern and criticism about the negative effects of the breeding towards high-performing chickens continuously increased. Therefore, new breeding efforts, focusing on the health of the animals, were established. In some countries including Germany, additional animal welfare concerns, such as the killing of one-day old male layer-type chickens in the egg-production industry, increased the pressure on the breeding companies.

4.1.3 Dual-purpose breeds

In the conventional poultry production of the 1950s-1960s the use of dual-purpose breeds was very common (WINDHORST et al. 2014). The negative correlation between body weight gain and egg-production led to the genetic selection of breeds specialized in only one specific production trait (egg- or meat production).

Consequently, dual-purpose breeds were then mostly used in the hobby area. In the recent years, dual-purpose breeds came back into the focus of breeding companies due to increasing societal and political discussed animal welfare concerns. Several “old breeds” were tested in the field but were shown to possess a low performance in both egg- and meat production (LANGE et al. 1995; HAHN et al. 1995). Some worldwide acting and also smaller breeding companies produced dual-purpose chicken lines, such as the Lohmann Dual (Lohmann Tierzucht GmbH), the Novogen-Dual (Novogen S.A.S), the Dominant Red Barred D 495 (Dominant CZ), Walesby Special (Hölzl Hatchery) using crosses (hybrids) of BT and LT chicken lines. Male and female chickens show a more moderate production performance, which is still economically acceptable. Organic breeding companies developed their own dual-purpose breeds (Coffee&Cream, Bresse x Gauloise, Ökologische Tierzucht GmbH). The problem of the killing of one-day old male LT chickens can thereby be circumvented and furthermore the production and performance intensity, which is also criticized, will be reduced (DAMME et al. 2015). Additionally, the reduction in production performance might lead to a greater health in the flocks and therefore lead to a reduction in the use of antibiotics and medications in general.

4.2 The immune system of chickens

The immune system of avian species, including the chicken, shows several differences to the mammalian immune system and owns its unique features. Most studies on the avian immune system have used chickens as a model. Therefore, this literature review will mainly focus on studies conducted in chickens.

The discovery of the BF as the main organ for the generation of the B-cell- and therefore the antibody-repertoire in avians, was the first step for comparative immunology and for subsequent investigations on the B-cell development in mammalian species. Several differences exist between the avian and the mammalian immune system. Some of these differences will be addressed in the following chapters with respect to their importance for the topics of this thesis.

4.2.1 The structure of the immune system

The immune system is mainly divided into the innate (unspecific) and the adaptive (specific) immunity. The two arms of the immune system can be separated by their reaction pattern and their specificity but only the close interaction and cooperation between them leads to efficient immune responses (JUUL-MADSEN et al. 2014).

Innate immunity

The major feature of the innate immune system is the fast reaction of associated cells to limit the spread of invading pathogens until the cells of the adaptive immune system are ready to respond (MEDZHITOV and JANEWAY 1997, 1998). Importantly, different soluble factors of the innate immune system are released to subsequently attract and recruit specialized immune cells. This fast reaction is possible due to the high frequency of the expression of innate recognition receptors but comes along with lower specificity of these receptors. Besides the cellular components of the innate immune system, such as macrophages, natural killer cells (NK-cells), granulocytes and thrombocytes, the epithelial surfaces as physical barriers and chemical responses define important parts. Acute phase proteins (APP), antimicrobial peptides and the complement system are parts of the active chemical responses upon pathogen invasion (JUUL-MADSEN et al. 2014). Furthermore, natural antibodies, which possess antigen-binding abilities in non-immunized animals, play an important role as the first line of defense in cooperation with the complement system. These antibodies were connected with disease resistance in chickens, but still not much is known about the regulation of immune responses by these cells (HÄRTLE et al. 2014; VAN DER KLEIN et al. 2015).

Adaptive immunity

The adaptive immune responses on the other hand take a longer time to develop but the responses are highly specific. The specific immune responses can be separated into the cell-mediated immunity (CMI), which is mainly based on T-cells, and the humoral immunity, which includes the B-cells and the antibody development.

B-cells and antibodies

Like mammals, chickens express the Ig heavy chain isotypes IgM, IgA and IgY, which is the counterpart of mammalian IgG (DAHAN et al. 1983; PARVARI et al. 1988; MANSIKKA 1992; HÄRTLE et al. 2014). Different from mammals, chickens lack the expression of IgE and IgD isotypes (HÄRTLE et al. 2014). Chicken IgM, IgA and IgY were shown to have the same functions as the mammalian counterparts, but chicken IgY shows slight structural and functional differences (RATCLIFFE 2006). The generation of antibody diversity in chickens was shown to differ from mammals, as it is mainly generated by a process called gene conversion (RATCLIFFE 2006).

T-cells

The major T-cell lineages in chickens can be divided into three subgroups, which can be distinguished by different T-cell receptors (TCR). γδ T-cells are TCR1+ cells whereas two subsets of αβ T-cells exist, which are either TCR2+ or TCR3+ cells (CHEN et al. 1986; CIHAK et al. 1988; SOWDER et al. 1988). Additional CD4 and CD8 surface molecules define CD4+ helper T-cell populations, which mainly interact with MHC II presenting cells, whereas CD8+ cytotoxic T-cells interact with MHC I presenting cells (CHAN et al. 1988).

In contrast to rodents and primates, chickens are one of the species which possesses a larger proportion of γδ T-cells in the peripheral lymphocyte population (20-60%) (KUBOTA et al. 1999). In the last years it has become clear that several different γδ T-cell subsets exist in various tissues of chickens. γδ T-cells have been described as innate-like cells, since several functional and phenotypic characteristics are similar to innate immune cells, such as NK cells (HUHLE et al. 2017). Several investigators succeeded in determining suitable surface markers to differentiate between different γδ T-cell subsets in the chicken (TREGASKES et al. 1995; BERNDT et al. 2006; HUHLE et al. 2017). Spontaneous, cytotoxic function in the spleen was recently shown to be associated with CD8+ γδ T-cells (FENZL et al. 2017).

Further T-cell subsets, such as the regulatory T-cells (Tregs) have been well described in mammalian species, especially mouse and human. $CD4^+CD25^+$ forkhead box

protein 3 (*foxp3*)$^+$ cells are described as the “natural” Tregs with *foxp3* being the unique marker. Due to its nuclear expression, $CD4^+CD25^+$ are commonly used for the detection of Tregs. CD25 is a general marker for T-cell activation in avians (HALA et al. 1986). In avian species the existence of *foxp3* has not been determined yet but $CD4^+CD25^+$ and $CD4^+TGF\beta^+$ cells were described as avian Treg subsets (SHANMUGASUNDARAM et al. 2011; GURUNG et al. 2017). Avian Tregs were shown to possess similar cytokine mRNA expression patterns compared to mammalian Tregs, which include IL-10 and TGFβ (WORKMAN et al. 2009; SHANMUGASUNDARAM et al. 2011; SELVARAJ 2013). The avian equivalent to mammalian TGFβ1 is TGFβ4 (JAKOWLEW et al. 1997). The role of Tregs as suppressor T-cells in avian infectious diseases has not been fully elucidated. Further studies are needed to learn more about the role, functions and working mechanisms of T-cells in avian infectious diseases.

4.2.1.1 Important lymphoid organ and structures

The chicken’s primary lymphoid organs are the Bursa of Fabricius (BF) and the thymus. All other immune organs and tissues are secondary lymphoid organs. The most important secondary lymphoid organ is the spleen. Since chickens don’t possess any encapsulated lymph nodes like mammals, different lymphoid tissues and nodules are widely dispersed throughout the body. Most of the lymphoid tissues can be described as mucosa-associated lymphoid tissues, consisting of the gut-associated lymphoid tissues (GALT), the respiratory-associated lymphoid tissues, such as the bronchio-associated tissue (BALT), the conjunctiva-associated tissue (CALT), the Harderian gland and the reproductive-associated lymphoid tissues. The most important immune structures in the GALT are the Peyer’s Patches, the caecal tonsils (CT), the Meckel’s Diverticulum and tonsils located in the oesophagus, pylorus and as mentioned above, the BF (OLÁH et al. 2014).

Bursa of Fabricius

The BF is a unique organ in birds, which is responsible for primary B-cell lymphopoiesis and maturation as well as for antibody development (GLICK 1991; RATCLIFFE 2002). In 1956 it was shown that after surgical removal of the BF in neonatal chickens,

subsequent antibody production against *Salmonella* Typhimurium O antigen was suppressed (GLICK et al. 1956). As a diverticulum of the cloaca, the BF is connected with the proctodeum. The bursal lumen consists of different folds or plicae, which contain the bursal follicles (OLÁH and GLICK 1978). Each bursal follicle is divided into a cortical and a medullary zone, which are separated by the epithelial cells of the cortico-medullary boundary. The cellular composition of the bursal follicles consists of mainly lymphocyte populations but also epithelial cells and bursal secretory cells (CIRIACO et al. 2003), which are only present in the bursal medulla (GALLEGO et al. 1996). 98% of the BF-lymphocyte populations consist of B-cells. Very few T-cells can be found in the bursal cortex and sometimes in the medulla, whereas macrophages are only present in the interfollicular, connective tissue (KINCADE and COOPER et al. 1971; POTWOROWSKI 1972; MAST and GODDEERIS et al. 1998; OLÁH et al. 2014).

Spleen

As in mammals, the avian spleen is divided into red and white pulp. Whereas the red pulp consists of both non-lymphoid and lymphoid cells, the white pulp is mainly populated by lymphocytes (OLÁH et al. 2014). The white pulp consists of two morphologically distinct compartments, the peri-arteriolar lymphoid sheath (PALS), which surrounds the central arteries and the peri-ellipsoid lymphoid sheath (PELS), which surrounds the penicillary capillaries (JEURISSEN 1993; JEURISSEN et al. 1994). The PELS consists of ellipsoid reticular cells, B-cells and an outer ring of macrophages, which was suggested to form the equivalent of the marginal zone in mammals (JEURISSEN et al. 1992). The different compartments in the spleen contribute to the induction of adaptive (PALS) as well as both innate and adaptive immune responses (PELS).

Germinal centers (GC)

The GC develop in all peripheral lymphoid organs after antigen contact and thereby increase with age (JEURISSEN and JANSE 1994; YASUDA et al. 1998). After antigenic contact, the initial response by IgM+ molecules is taken over by a high production of IgY molecules (Ig isotype switch) (FELLAH et al. 2014). The GCs are the

site where Ig isotype switching takes place and B-cell memory develops (YASUDA et al. 2003). The affinity maturation for the antibodies involved in the secondary responses also takes place in the GC (ARAKAWA et al. 1996).

4.2.2 The development of the immune system

Many parts of the immune system in the chicken already develop during embryogenesis. Neonate chickens get exposed to pathogens of the outside environment as soon as they hatch. Studies on germ-free birds showed that prior to hatch the immune tissues start developing independent of antigen contact, but the development is driven further after hatching by antigen contact (HEGDE et al. 1982). At hatch, some parts of the immune system are not fully developed but will mature in the following weeks after hatch (MAST and GODDEERIS 1998, 1999). Most cellular components are already present prior to hatch but the tissue structure and numbers of immune cell populations change rapidly after hatch (JEURISSEN et al. 1989; BUCY et al. 1990; PARAMITHIOTIS and RATCLIFFE 1994). Therefore, the maternally derived antibodies (MDA), which are transferred through the hen's yolk, play an important role in the protection of the chickens during the first weeks of life until the immune system is fully developed.

BF and B-cell development

The chicken B-cell development takes place in three different stages: a pre-bursal, bursal and post-bursal stage (HOUSSAINT et al. 1983). The colonization of the BF with progenitor B-cells occurs at a specific time period between EID 8 and 12 (LE DOUARIN et al. 1975; HOUSSAINT et al. 1976). Ig-expressing B-cells subsequently proliferate (MCCORMACK et al. 1989). The emigration of B-cells to secondary lymphoid organs occurs at the time around hatch (COOPER et al. 1969; PARAMITHIOTIS and RATCLIFFE 1993). First IgM+ cells in the BF can be found between EID 12-14 and outside the BF around EID 17. IgY+ cells start to appear at around hatch and cannot be found outside the BF until four dph (KINCADE and COOPER et al. 1971). The separation between the cortical and medullary zone of the bursal follicles becomes clearer during the first weeks post hatch with the bursal cortex

being fully developed at 14 dph. It is suggested that the B-cell emigration to the periphery originates from the bursal cortex (PARAMITHIOTIS and RATCLIFFE 1994). The BF reaches its maximum size at around eight to ten weeks of age and subsequently involutes with sexual maturity. This time period represents the post-bursal stage, where post-bursal stem cells are important for the B-cell maintenance (TOIVANEN and TOIVANEN 1973).

Thymus and T-cell development

The thymus gets colonized in three different waves starting at embryonic incubation day (EID) six, the second at EID 12 and the third at EID 18, which lasts until after hatching (COLTEY et al. 1989). In contrast to mammals, chickens are one of the species which possesses a larger proportion of γδ T-cells in the peripheral lymphocyte population (20-60%) (KUBOTA et al. 1999). γδ T-cells migrate to the periphery at different time points during embryogenesis and are most frequently found in epithelial-rich tissues, such as the intestine. During embryogenesis, the spleen is also populated with thymic T-cells but some cell types (TCR3+) can only be found in the spleen as early as two days post hatch (FELLAH et al. 2014).

Spleen

Some immune organs, such as the spleen play an important role in the first days and weeks post hatch to compensate other not fully developed immune tissues. The structural development of the spleen has also been associated with its functionality (JEURISSEN et al. 1994). During embryogenesis functionally important structures, such as the peri-arteriolar lymphoid sheaths (PALS) and the peri-ellipsoid lymphoid sheaths (PELS), representing the T- and B-cell compartments of the spleen, are fully developed around the time of hatch, but mature during the first week of life (MAST and GODEERIS et al. 1998, 1999).

4.2.3 Influence of the genetic background on immune responses and disease susceptibility

Several studies indicate an association between performance traits and immune responses. It was suggested that the genetic selection process towards opposing production traits in BT and LT chickens might have affected the immune responses in different ways (HAVENSTEIN et al. 1994a; LESHCHINSKY et al. 2001; KOENEN et al. 2002). Not many studies addressed the correlation between egg-performance traits and associated differences in immune responses (SIEGEL et al. 1982). Comparative studies on immune responses between BT and LT chicken lines and on the association between the selection towards special immune-related traits and immune responses are summarized in Table 1 and 2.

BT chickens were suggested to be more specialized in short-term, innate immune responses whereas LT might mount more vigorous long-term cellular immune responses after administration of TNP-KLH (KOENEN et al. 2002). Nevertheless, other studies on cellular immune response comparisons between BT and LT lines demonstrated opposite results (LESHCHINSKY and KLASING 2001). The relative growth of important immune organs, such as the BF and the spleen has decreased with the selection for increased body weight (CHEEMA et al. 2003). Furthermore the selection for increased body weight gain was shown to have a negative effect on the ability to mount humoral immune responses (MILLER et al. 1992; CHEEMA et al. 2003). Also in LT chicken lines, higher body weights were correlated with a lower humoral immune response to different viral and bacterial antigens (GROSS et al. 1980; SIEGEL et al. 1982; PARMENTIER et al. 1996). Nevertheless, differences in immune responses against different viral and bacterial infections were also shown to vary within different BT and LT chicken lines differing in their production performance (VAN HEMERT et al. 2006; BLOHM et al. 2016; KJAERUP et al. 2017; GILES et al. 2019).

Table 1. Antigen-dependent differences in immune responses between LT and BT chicken lines

Type of investigation	Antigen	LT	BT	Reference
Humoral immune responses	NDV	↑	↓	VAN EK et al. 1987
	ST	↓	↑	GROVES et al. 2015
	C. jejuni	↓	↑	HAN et al. 2016
	TNP-KLH	IgG ↑	IgM ↑	KOENEN et al. 2002
Cellular immune responses	TNP-KLH + ConA	↑	↓	KOENEN et al. 2002
	ConA	↓	↑	LESHCHINSKY and KLASING, 2001
	PHA	↓	↑	LESHCHINSKY and KLASING, 2001
	PHA	↓	↑	CORRIER et al. 1990
	LPS	↑	↓	LESHCHINSKY and KLASING, 2001
Cytokine expression	LPS	↑	↓	LESHCHINSKY and KLASING, 2001
	SE	Spleen: CXCLi2, IL-10, IL-12α, CCL2i ↑	Caecum: IL-12α, IL12β, CCLi2 ↑	CHEESEMAN et al. 2007
Mortality after challenge	SE	↑	↓	GUILLOT et al. 1995

*C. Campylobacte*r, ConA concanavalin A, CCLi2 C-C motif ligand 2, CXCLi2 C-X-C motif ligand 2, Ig immunoglobulin, LPS lipopolysaccharide, NDV Newcastle disease virus, PHA phytohemagglutinin, TNP-KLH trinitrophenyl-conjugated keyhole limpet hemocyanin, SE *Salmonella* Enteritidis, ST *Salmonella* Typhimurium;
↑ = higher, ↓ = lower

Table 2. Differences between chickens selected for specific traits.

Selected for	Antigen/ investigation	Genotype	HA-line	LA-line	Reference
High or low antibody response to SRBC	SRBC	LT	↑	↓	KREUKNIET et al. 1990 PARMENTIER et al. 1996
	ConA	LT	↑	↓	SCOTT et al. 1991
		LT	-	-	KREUKNIET et al. 1994
		LT	↓	↓	PARMENTIER et al. 1994
		LT	↓	↑	GEHAD et al 1999
	PHA	LT	↑	↓	SCOTT et al. 1991
		LT	-	-	KREUKNIET et al. 1994
	BSA	LT	BSA: ↑	BSA:↓	PARMENTIER et al. 1994
	Phagocytic activity	LT	-	-	KREUKNIET et al. 1995
	Spleen (PELS structure)	LT	↑	↓	KREUKNIET et al. 1996
	E.coli, NDV, IBV, IBDV	LT	↑	↓	PARMENTIER et al. 1996
	BA	LT	↑	↓	DUNNINGTON et al. 1992 SCOTT et al. 1994
	LPS LTA KLH	LT	↑	↓	PARMENTIER et al. 2006

Table 2 continuing

			HW-line	LW-line	
High body weight	SRBC	LT	↓	↑	GROSS et al. 1980
	SRBC	LT	↓	↑	SIEGEL et al. 1982
	SRBC	BT	↓	↑	MILLER et al. 1992
	SRBC, *E.coli*, NDV, IBV, IBDV,	LT	↓	↑	PARMENTIER et al. 1996
	Immune organ weight	BT	↓	↑	CHEEMA et al. 2003
			High-line	Low-line	
High or low serum IgM or IgG	BA	LT	IgM ↑ IgG ↑	IgM ↓ IgG ↓	SARKER et al. 2000
			High-MBL	Low-MBL	
High or low MBL serum concentration	IBV - viral load	LT	↓	↑	KJAERUP et al. 2014
	- humoral immune response		-	-	
	- γδ CD8α T-cells		↑	↓	
	- monocytes		↑	↓	
	IBV - innate gene expression	LT	↑	↓	HAMZIC et al. 2016
	- adaptive gene expression		↑	↓	
	E. coli - body weight gain	LT	↑	↓	NORUP et al. 2009
	- T-cell proliferation		↑	↓	

Table 2 continuing

			High-line	Low-line	
High or low cutaneous hypersensivity reaction	NDV -antibody development	?	↑	↓	AHMED et al. 2007
High or low antibody response to NDV, IBDV, IBV	Macrophage activity / phagocytosis	LT	↑	↓	GUIMARAES et al. 2011

BA Brucella abortus, BSA bovine serum albumin, BT broiler-type, CD cluster of differentiation, ConA concanavalin A, *E. Escherichia*, Ig Immunoglobulin, HA high antibody, HW high weight, IBV infectious bronchitis virus, IBDV infectious bursal disease virus, KLH keyhole limpet hemocyanin, LPS lipopolysaccharide, LA low antibody, LW low weight, LT layer-type, LTH lipoteichoic acid, MBA mannose-binding lectin, NDV Newcastle disease virus, PELS peripheral lymphoid sheath, PHA phytohemagglutinin, SRBC sheep red blood cells; ↑ = higher response, ↓ = lower response, - = no difference

The chicken MHC is strongly associated with disease resistance and susceptibility. Viral infections, such as Marek's Disease, Avian leucosis and Rous sarcoma virus are associated with the MHC (summarized in Table 3). Bacterial and parasitic infections have been linked to the MHC, which are also summarized in Table 3.

Table 3. MHC-associated differences in the disease resistance against different pathogens.

Pathogen	Reference
MDV	HANSEN 1967 BACON1977 BACON and WITTER 1994 KAUFMAN and VENUGOPAL 1998 LAMONT et al. 1998 JAROSINSKI et al. 2002 DAALGARD et al. 2009 PARVIZI et al. 2009 CHAKRABORTY et al. 2019
RSV	SCHIERMAN and COLLINS 1987 AEED et al. 1993 WHITE et al. 1994 KAUFMAN and VENUGOPAL 1998 TAYLOR et al. 2004
ALV	YOO and SHELDON 1994 MAYS et al. 2005
NDV	RUSSELL 1994 NORUP et al. 2011
ILTV	POULSEN et al 1998
IBV	JOINER et al 2007 BANAT et al 2013 SMITH et al 2015 DA SILVA et al 2019
Pasteurella multocida (Fowl cholera)	LAMONT et al. 1987

Table 3 continuing

Salmonella spp.	COTTER et al. 1998
Staphylococcus aureus	COTTER et al. 1992
Clostridium perfringens (Necrotic enteritis)	DINH et al. 2014
Coccidiosis	LILLEHOJ et al. 1989 CARON et al. 1997 PINARD-VAN DER LAAN et al. 1998
Helminths	SCHOU et al. 2003 NORUP et al. 2013 PLEIDRUP et al. 2014
Northern fowl mite	OWEN et al. 2008 MURILLO et al. 2016
Skeletal disease (BT birds)	JOINER et al. 2005
SRBC	BACON et al. 1987 DIX and TAYLOR 1996 KARACA et al. 1999
BA	KARACA et al. 1999

ALV avian leukosis virus, BA Brucella abortus, BT broiler-type, IBV infectious bronchitis virus, ILTV infectious laryngotracheitis virus, MDV Marek's disease virus, NDV Newcastle disease virus, RSV rous sarcoma virus, SRBC sheep red blood cells

Different immune cell populations, such as macrophages, T-cells and B-cells were also shown to differ in their proliferation after stimulation with different mitogens (Table 4). The ratio of different T-cell subsets was also suggested to be influenced by the MHC (Table 4).

Table 4. MHC-associated differences in immune cell numbers, -activity and - proliferation.

Immune cells	Reference
Macrophages	PUZZI et al. 1990 HUSSAIN and QURESHI 1997 HUSSAIN and QURESHI 1998 DIL and QURESHI 2002a DIL and QURESHI 2002b DAWES et al. 2014 COLLISSON et al. 2017 CHAKRABORTY et al. 2019
CD4:CD8 lymphocyte ratio	HALA et al. 1991 EWALD et al. 1996
CMI (ConA and PWM)	GEHAD et al. 1999
PBLs (PHA)	BACON and PALMQUIST 2002

CD cluster of differentiation, CMI cell-mediated immunity, ConA concanavalin A, PBL peripheral blood leukocytes, PHA phytohemagglutinin, PWM pokeweed mitogen

Interestingly, the selection for specific performance parameters, such as egg production, hatchability, body weight, fertilization rate, adult and embryonic mortality was shown to promote special haplotypes to appear more frequently after some generations (GAVORA et al. 1986; LAMONT et al. 1987b; LAKSHMANAN et al. 1997) and that some haplotypes might be beneficial for disease resistance and production traits (B^2, B^{21}) (LAMONT 1998).

4.3 Infectious Bursal Disease (IBD)

4.3.1 Introduction

Infectious bursal disease (IBD), also known as “Gumboro disease” is an acute, highly contagious immunosuppressive disease of young chickens, caused by the infectious bursal disease virus (IBDV) (ETERRADOSSI and SAIF 2013). Its widespread distribution poses a great health and subsequent economic threat to the poultry industry worldwide. Figure 1 demonstrates the worldwide reported IBDV distribution from July-December 2018. Depending on the virus strain, it may lead to increased mortality rates and more importantly to severe persistent or transient immunosuppression causing subsequent secondary infections and depression in vaccine responses.

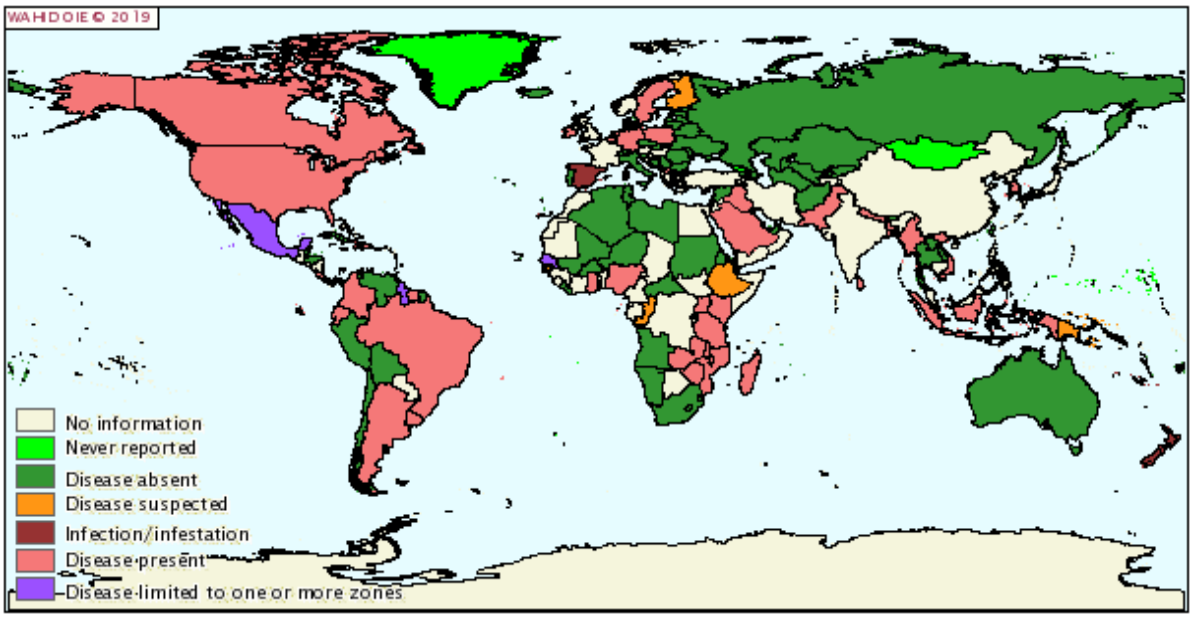

Figure 1. Worldwide reported cases of IBDV (July- December 2018) (World Animal Health Organization (OIE), 2019).

4.3.2 History

In 1962, a newly emerged disease, named „avian nephrosis“, was described in Gumboro in the US Federal state of Delaware. At the same time a nephropathogenic strain of Infectious Bronchitis, the so called “gray strain” (WINTERFIELD and HITCHNER 1962), was circulating in the field and led to similar kidney lesions in chickens and therefore complicated the pathogen identification. Later on it was shown that this similar pathology was based on two different infectious agents by isolating the “infectious bursal agent” in embryonated eggs (WINTERFIELD and HITCHNER 1962). Due to its pathognomonic bursal lesions the name “Infectious Bursal Disease” was proposed (HITCHNER 1970). When in 1972 Infectious Bursal Disease (IBD) Virus (IBDV) was shown to be an immunosuppressive pathogen (ALLAN et al. 1972; FARAGHER et al. 1972), research on the development of efficient IBD control strategies was increasing. A second serotype was reported in 1980 (MCFERRAN et al. 1980). The disease spread rapidly throughout the USA (LASHER and DAVIS 1997) and other parts of North America as well (LUCIO et al. 1972; IDE and STEVENSON 1973). Other continents, including Europe, Asia, Africa, the Middle East, India and Australia were also affected (EL-ZEIN et al. 1974; FIRTH 1974; ONUNKWO 1975; GYENING and CORKISH 1976; MANDA and PARANJAPE 1979). In Germany the “infectious bursa disease” emerged in 1965/66 (LANDGRAF et al. 1967).

4.3.3 Infectious Bursal Disease Virus (IBDV)

4.3.3.1 Virus taxonomy

Initially it was suggested that IBDV should be assigned to picornaviruses (CHO and EDGAR 1969; LUNGER and MADDUX 1972) or to reoviruses (KÖSTERS et al. 1972; HARKNESS et al. 1975). It was shown that IBDV consists of a two segmented double-stranded RNA (dsRNA) (MÜLLER et al. 1979a) and with other viruses with similar properties they were placed in the family of *Birnaviridae* (DOBOS et al. 1979). The family of *Birnaviridae* includes four genera, from which the genera *Aquabirnavirus, Blosnavirus* and *Avibirnavirus* infect non-mammalian vertebrates and the genus *Entomobirnavirus* infects insects (DELMAS et al. 2018). IBDV is assigned to the genus *Avibirnavirus*.

4.3.3.2 Morphology and genomic structure

IBDV has a single-shelled, non-enveloped virion with icosahedral symmetry and a diameter of 55-65nm (HIRAI and SHIMAKURA 1974; HARKNESS et al. 1975; PATTISON et al. 1975; NICK et al. 1976; OZEL and GELDERBLOM 1985). The genome of the dsRNA virus consists of 2 segments (*bisegmented* RNA virus) designated A and B (DOBOS et al. 1979; MÜLLER et al. 1979a; JACKWOOD et al. 1984). The smaller segment B codes for the virus polymerase protein (VP) 1 (MORGAN et al. 1988). The larger segment A consists of two open reading frames (ORF). The larger one codes for a precursor protein, which is processed into the structural proteins VP2 and VP3 by the viral protease VP4 (AZAD et al. 1985; HUDSON et al. 1986; JAGADISH et al. 1988). The smaller ORF overlaps the N-terminal region of VP2 and encodes the non-structural VP5 (MUNDT et al. 1995).

The structural protein VP2 is responsible for the induction of virus-neutralizing antibodies and plays the major role in the host-protective immune responses (BECHT et al. 1988; FAHEY et al. 1989; HEINE et al. 1991). Hence, the VP2 protein has been used for the development of viral vector and subunit vaccines against IBDV. Hydrophilic parts within the projection (P) domain of VP2 form the variable domain (BAYLISS et al. 1990). This hypervariable region (hvr) has been shown to be involved in antigenic variation processes (VAKHARIA et al. 1994; ETERRADOSSI et al. 1997; LETZEL et al. 2007).

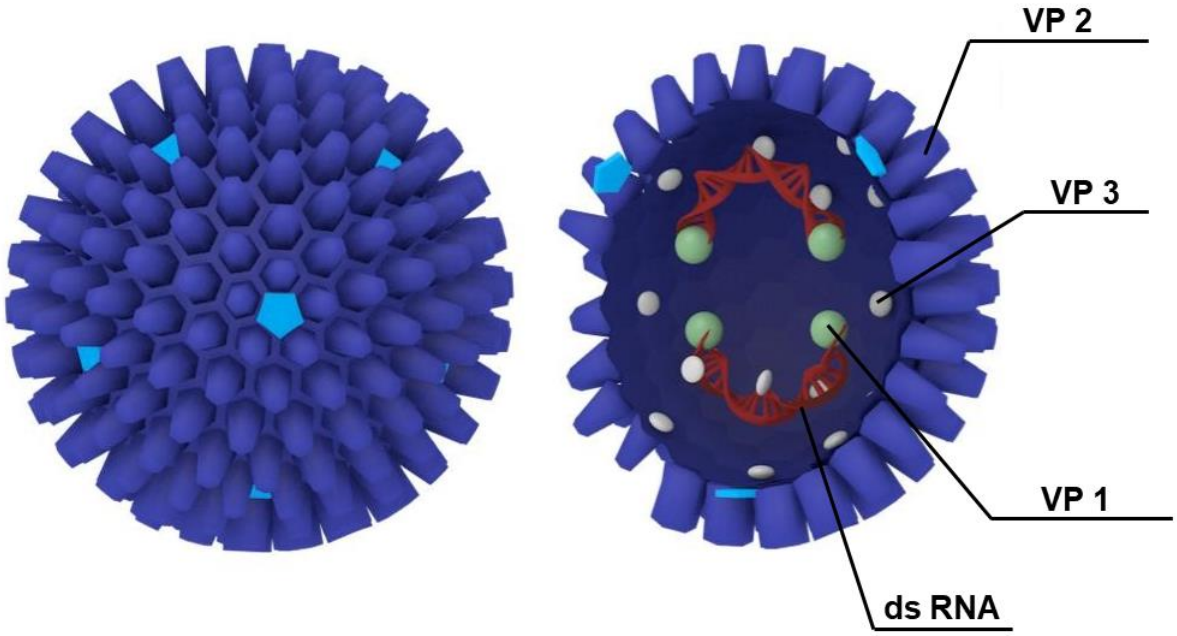

Figure 2. Schematic illustration of IBDV.

VP = viral protein; ds RNA = double-stranded RNA

4.3.3.3 Strain classification

Two serotypes of IBDV (serotype 1 and 2) can be distinguished by virus neutralization test (VNT) (MCFERRAN et al. 1980). First it was believed that the serotype 2 only exists in turkeys (JACKWOOD et al. 1982) but later on this serotype was also isolated from chickens (ISMAIL et al. 1988). As infection experiments have shown, only serotype 1 is pathogenic for chickens. Nevertheless, a serological response to serotype 2 can be observed (JACKWOOD et al. 1985; ISMAIL et al. 1988).

Based on their antigenicity, serotype 1 IBDV strains can be separated into classical “standard” strains, antigenic variant strains and “very virulent” (vv) IBDV strains. In 1985/1986 frequent IBDV vaccination failures were reported from the field (ROSENBERGER et al. 1985). The isolated strains were described as “variant” strains and showed differences to the classical/standard strains in the structure of epitopes (ROSENBERGER and CLOUD 1986; SNYDER et al. 1988). Variant strains changed over time by antigenic drift and therefore the vaccines, deriving from standard strains, weren’t protective anymore (JACKWOOD and SAIF et al. 1987; SNYDER et al. 1988).

Despite of classical IBDV strains, local variant strains of IBDV have also been reported in Australia (SAPATS and IGNJATOVIC 2000). In the late 1980s, so called very virulent strains developed in the Netherlands and Belgium (CHETTLE et al. 1989; VAN DEN BERG et al. 1991). Mortality rates after infection with vvIBDV now increased up to 60% in layers and 30 % in broilers and reached 100 % in experimentally infected specific pathogen free (SPF) chickens (VAN DEN BERG et al. 1991; NUNOYA et al. 1992). Not only Europe and Asia (NUNOYA et al. 1992; LIN et al. 1993) were affected by the occurrence of vvIBDV, but also in South America (DI FABIO et al. 1999; IKUTA et al. 2001) and in Africa (HORNER et al. 1994) cases due to vvIBDV were described. The United States reported first outbreaks of vvIBDV infection in 2008 in California (JACKWOOD et al. 2009; STOUTE et al. 2009). Australia and New Zealand stay free of vvIBDV infection until now.

Based on the antigenicity of vvIBDV strains, it was shown that they are similar to classic serotype 1 IBDV strains (VAN DEN BERG et al. 1991; ETERRADOSSI et al. 1999; ABDEL-ALIM and SAIF 2001). Further studies demonstrated that vvIBDV strains also differ antigenically to classical IBDV strains showing unique amino acid residues within the hvr VP2 (BROWN et al. 1994; ETERRADOSSI et al. 1997; ETERRADOSSI et al. 1998; ZIERENBERG et al. 2000; VAN DEN BERG et al. 2004; JACKWOOD and SOMMER-WAGNER 2011). Nevertheless, the term vvIBDV is rather based on its pathogenicity than on antigenic differences and should not be mixed up with the antigenic designations of classic and variant IBDV strains (VAN DEN BERG et al. 2004; JACKWOOD 2017). Strains classified as vvIBDV can also show differences in their pathogenicity (VAN DEN BERG et al. 2004). The VP2 protein was shown not to be the only genome part involved in the pathogenicity and virulence of vvIBDV (BOOT et al. 2000). The segment B of IBDV also contributes to the pathogenicity and virulence of vvIBDV strains (BOOT et al. 2005; GAO et al. 2007; ESCAFFRE et al. 2013). First in the U.S. and then also in Europe interserotypic reassortant vvIBDV strains were reported, which consisted of the serotype 1 vvIBDV segment A and serotype 2 IBDV segment B (JACKWOOD and SOMMER-WAGNER 2011; SOUBIES et al. 2017). In South America IBDV strains were reported, which are genetically distinct from the other traditional IBDV lineages (dIBDV) (HERNANDEZ et al. 2015; TOMAS et al. 2019). It

was shown that dIBDV strains are also present in Europe and Asia (DOMANSKA et al. 2004; JEON et al. 2009; YAMAZAKI et al. 2017).

More recent studies suggested to classify worldwide distributed IBDV strains into genogroups, based on the nucleotide sequencing of the hvr of VP2 (MICHEL and JACKWOOD 2017; JACKWOOD et al. 2018). Based on these studies, IBDV isolates can be classified into seven major worldwide distributed genogroups (Genogroup 1-7). The three major genogroups (1-3) contain the as previously classified classical strains (Genogroup 1), the antigenic variant strains (Genogroup 2) and the vvIBDV strains (Genogroup 3). Genogroup 4 contains the recently determined dIBDV strains.

4.3.4 Epidemiology

Infections with serotype 1 IBDV are distributed worldwide. Serotype 1 and 2 were isolated from many different species, but chickens and turkeys are the only natural host species. Nevertheless, clinical signs can only be observed in chickens. Different free-living bird species were also found to be positive for anti-IBDV antibodies (WILCOX et al. 1983; OGAWA et al. 1998b). Mosquitoes and the meal worm were suggested to function as vectors (SNEDEKER et al. 1967; HOWIE and THORSEN 1981; MCALLISTER et al. 1995). IBDV has a high tenacity in the environment. If the cleaning and disinfection of the stables is not carried out properly, the virus can survive and the following flocks can be infected again (BENTON et al. 1967).

4.3.5 Clinical signs

Clinical signs of IBDV can be observed within two to four days of incubation. Chickens with clinical signs show whitish, watery diarrhea, anorexia, severe prostration, ruffled feathers and death (COSGROVE 1962). A reduced feed consumption and increased water consumption can be observed in the infected flocks (LASHER and SHANE 1994). The mortality rate increases the first two days after infection and clinical symptoms might be observed during five to seven days after infection (PARKHURST 1964). The clinical outcome of the disease and the mortality rates vary depending on the age and chicken breed, the virulence of the infecting strain, as well as the level of MDA. During necropsy, infected birds might show severe dehydration and

hemorrhages in the thigh and pectoral muscles (COSGROVE 1962; WANG et al. 2009; ETERRADOSSI and SAIF 2013).

At the age of three to six weeks, chickens are the most susceptible to infection (ETERRADDOSSI and SAIF 2013). Birds infected at a younger age might develop a subclinical infection leading to a severe immunosuppression (ALLAN et al. 1972; FARAGHER et al. 1974).

4.3.6 Pathology and histopathology

The BF is the target organ of the virus. Following infection, macroscopic lesions can be observed. As early as three days post infection (dpi) the BF increases in size and weight due to edema and hyperemia (SAIF 1998). The BF is surrounded by a gelatinous, yellowish transudate. Sometimes hemorrhagic foci can be observed on the surface of the bursal mucosa. After four dpi, the size and weight of the BF decreases again, first reaching its normal weight and subsequently turning atrophic (CHEVILLE 1967). Other immune tissues, such as the spleen, thymus, CT and the bone marrow might also be affected and show more severe lesions after vvIBDV infection (TANIMURA and SHARMA et al 1997). The spleen might be slightly enlarged and might show diffuse grey spots on the surface (RINALDI et al. 1974). Renal lesions might also occur due to a strong dehydration of the birds (COSGROVE 1962).

Microscopically, lesions can be detected in several immune organs and tissues, such as the BF, spleen, thymus, Harderian gland and CT. Within 24 hours after infection necrosis and degeneration of mainly B-cells in the medullary area of the bursal follicles can be seen followed by an infiltration of heterophils and reticuloendothelial cells (NAQI and MILLAR 1979; ETERRADOSSI and SAIF 2013). After three to four days edema and hyperemia can also be observed microscopically, contributing to the increase in size and weight of the organ. After the first inflammatory reaction, cystic cavities develop in the medullary areas of the bursal follicles (HELMBOLDT and GARNER 1964; CHEVILLE 1967). The interfollicular tissue might show fibroplasia. At this time, the normal structure of the BF cannot be recognized anymore.

Early after infection, the spleen shows a perivascular hyperplasia of the reticuloendothelial cells. At around three dpi lymphoid necrosis in the GC of the spleen can be observed (HELMBOLDT and GARNER 1964). Some lymphocyte necrosis can also be observed in the thymus within three days. A variant virus strain caused less microscopic lesions in the thymus compared to a classic strain (SHARMA et al. 1989). Infection with vvIBDV strains can lead to a greater loss in thymic weight (TANIMURA and SHARMA 1997).

CTs might also show an infiltration of heterophils and lymphocyte necrosis. After IBDV infection, the Harderian gland can either show a great decrease in the number of plasma cells or might show plasma cell necrosis depending on the age of the chickens at infection (DOHMS et al. 1981; DOHMS et al. 1988).

Depending on the virus strain, lesions might develop differently. vvIBDV strains lead to more severe lesions and a longer persistence, but bursal lesions are comparable to the lesions induced by classic strains. Variant strains were shown to induce a higher bursal damage but inflammatory reactions were absent (SHARMA et al. 1989).

4.3.7 Pathogenesis and immunosuppression

After oral infection IBDV antigen can initially be found in macrophages and other lymphoid cells in the caecum at 4 hours pi and subsequently in the duodenum and jejunum (MÜLLER et al. 1979b). Through the portal vein the virus reaches the liver and from there it spreads to all the other organs, including the BF. Immature IgM+ B-cells, as the main target cells of the virus (KAUFER et al. 1980) are destroyed due to necrosis and apoptosis depending on the infecting virus strain (VASCONCELOS et al. 1994; RODRIGUEZ-LECOMPTE et al. 2005). Different proteins, such as the λ light chain of surface IgM+ (sIgM+), the heat shock protein 90 (HSP90) and the α4β1 integrin were suggested to be receptors for IBDV (OGAWA et al. 1998a; LIN et al. 2007; DELGUI et al. 2009). Figure 3 demonstrates the development of IBDV immunopathogenesis during the acute and recovery phase after infection.

Infiltration of macrophages, mast cells and heterophils during the acute phase of IBDV infection indicates the activation of the innate immune system (KHATRI et al. 2005;

RAUTENSCHLEIN et al. 2007b; WANG et al. 2008). IBDV also replicates in macrophages, which play a major role in the distribution of the virus (HIRAI and CALNEK 1979; BURKHARDT et al. 1987; MAHGOUB et al. 2012; INGRAO et al. 2013).

During the acute phase of infection, T-cells and macrophages infiltrate into the BF (TANIMURA and SHARMA 1997; WITHERS et al. 2005), which is accompanied by the release of various cytokines. Macrophages can directly be activated by IBDV and produce different pro-inflammatory cytokines and chemokines, such as interleukin (IL) -1β, IL-6, IL-8 and nitric oxide (NO) (KIM et al. 1998; KHATRI et al. 2005). Bursal T-cells also show up-regulation of different pro-inflammatory cytokines, such as IL-1β, IL-6, chemokine (C-X-C) motif ligand (CXCLi2) and interferon (IFN)-γ (ELDAGHAYES et al. 2006). The high release of IFN-γ by bursal T-cells also leads to further stimulation of macrophages to release more pro-inflammatory factors, such as IL-1β, IL-6 and inducible nitric oxide synthase (iNOS) (PALMQUIST et al. 2006). IL-6 and IFN-γ can also increase systemically (vvIBDV), emphasizing the role of the innate immune response during the acute phase of infection (RAUW et al. 2007; INGRAO et al. 2013). Anti-inflammatory cytokines, such as IL-10 are either up- or such as transforming growth factor (TGF)- β4 down-regulated during the acute phase of IBDV infection (ELDAGHAYES et al. 2006; LIU et al. 2010; RASOLI et al. 2015).

After IBDV infection, chickens show severe immunosuppression in both the humoral and the cell-mediated immunity. Primarily, IBDV leads to a destruction of mainly B-cells but several studies also showed the suppression of cell-mediated immune responses (CONFER et al. 1981; SHARMA et al. 1989; KIM et al. 2000; RAUTENSCHLEIN et al. 2003). It is believed that due to an overproduction of IFN-γ and subsequent stimulation of macrophages, convalescent chickens are compromised in their T-cell immune responsiveness (RAUW et al. 2007). The activation of T cells may last up to 12 weeks post infection depending on the infecting virus strain (KIM et al. 1999). The severity of immunosuppression will depend on the virus strain and infection dose as well as on the age and the breed of the chickens.

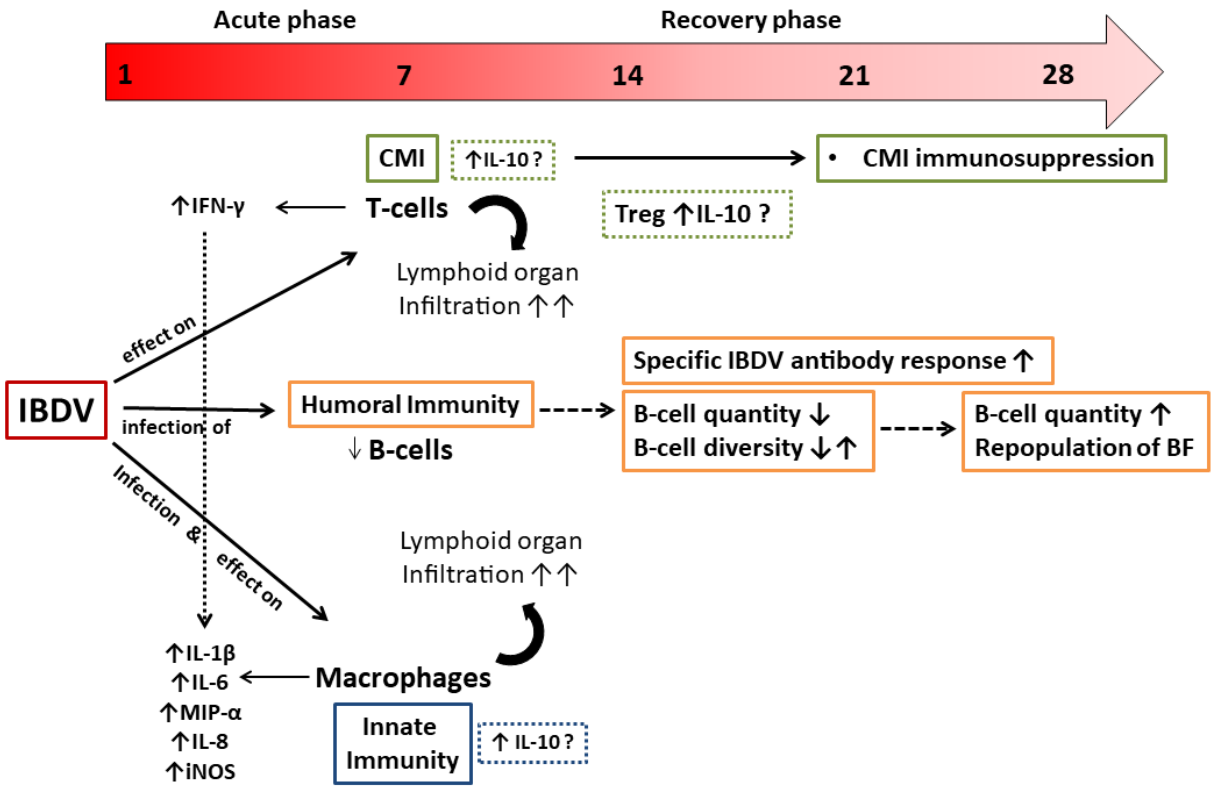

Figure 3. Immunopathogenesis of IBDV infection.

CMI cell-mediated immunity, BF bursa of Fabricius, IL interleukin, IFN interferon

4.3.8 Recovery after IBDV infection

The BF is the most affected and damaged organ by IBDV. As mentioned already, other immune organs and tissues can also be affected, but show a faster recovery after infection compared to the BF (ETERRADOSSI and SAIF 2013). Immature IgM+ B-cells are the main target cells of the virus. After infection a massive depletion in IgM+ and IgG+ B-cell populations takes place, but after some time both IgM+ and IgG+ B-cells repopulate the bursal follicles and the BF regains its normal structure (KIM et al. 1999; SHARMA et al. 2000). It was shown that after infection with a classical IBDV strain the bursal follicles can get repopulated with IgM+ B-cells after 12 weeks of infection, while depending on the virulence of the strain the BF returns to its normal weight after seven weeks pi (KIM et al. 1999). In the case of vvIBDV infection, bursal follicles may not be repopulated with B-cells (WILLIAMS and DAVISON 2005). After infection of two day

old chickens with classical IBDV, it was shown that two kinds of follicles develop during the recovery process. There are large, well-structured follicles, which are populated by bursal stem cells, which survived the IBDV infection. On the other hand small follicles without any structure and functional B-cells might develop (WITHERS et al. 2006). Birds with undifferentiated small follicles were not able to mount a humoral immune responses against IBDV and *Salmonella* Enteritidis.

T-cells were suggested to not only contribute to the immunopathological changes and immunosuppression but to also influence the recovery process after infection and contribute to virus clearance (KIM and SHARMA 2000; KIM et al. 2000; RAUTENSCHLEIN et al. 2002; WILLIAMS and DAVISON 2005; RAUF et al. 2011). It was suggested that clearance of the virus might be mediated by cytotoxic T-cells through Fas/FasL and perforin-granzyme A (PFN-Gzm-A) cytolytic pathways (RAUF et al. 2011, 2012).

4.3.9 Diagnosis

The first indication for IBDV infected chicken flocks might be the clinical picture and specific characteristics of the development of the disease (ETERRADOSSI and SAIF 2013). Characteristic pathological findings such as the bursal edema of infected birds during the acute phase will allow the confirmation of the disease. It is necessary to not only pick birds for necropsy, which show clinical symptoms but also deceased and supposedly “healthy” birds, to detect the distinctive changes in the BF during the course of infection. Histopathological investigations should also be applied to detect subclinical infections in the chicken flocks. A further confirmation of IBDV infection would be the immunohistochemical detection of the viral antigen in the respective immune organs (MÜLLER et al. 2003). For the detection of viral antigen, several serological tests, such as the agar-gel precipitation (AGP) test, enzyme-linked-immunosorbent assay (ELISA) and the virus neutralization test (VNT) are available. Furthermore, real-time reverse transcription polymerase chain reaction (RT-PCR), as a molecular tool for the detection of IBDV can be used (MOODY et al. 2000; KUSK et al. 2005; MICKAEL and JACKWOOD 2005). Real-time RT-PCR methods were developed, which include both IBDV segments and can distinguish between vv,

variant, classical and vaccine strains (PETERS et al. 2005; KONG et al. 2009; GHORASHI et al. 2011; HERNANDEZ et al. 2011; JACKWOOD et al. 2011; TOMAS et al. 2012).

4.3.9.1 Serological tests

IBDV antibodies can be detected by different serological tests. To detect the different serotypes of IBDV and antigenic variations, the VNT would be the method of choice (SKEELES et al. 1979). The most commonly used method in the field is the ELISA, which is also the most rapid test available. It has to be kept in mind that the ELISA cannot differentiate between the different serotypes and different commercially available ELISA systems might show varying sensitivity and specificity (ETERRADOSSI and SAIF 2013). Nevertheless, it represents the most feasible way to determine the flock specific immunity for the development of appropriate immunization measures.

4.3.10 Control and prevention of IBDV

Besides biosecurity measures, the most important way to protect chickens from IBDV infection is a successful vaccination regime. Especially the breeding flocks have to be vaccinated against IBDV to transmit anti-IBDV MDA to their progeny during the early phase post hatch. Parental flocks can be vaccinated with a live vaccine followed by an oil-emulsified, inactivated booster vaccination to ensure a prolonged protection of the progeny (LUCIO and HITCHNER 1979). Monitoring the antibody titers of the breeding flock and their progeny is therefore essential for a succeeding protection of the chickens in the field.

90% of the U.S hatcheries use automatic *in ovo* application systems for vaccination, especially for the broiler industry (Peebles et al. 2018). The fast vaccine application to a large number of eggs, the reduction of labor costs and the early induction of immune responses are advantages of *in ovo* application systems.

4.3.10.1 Conventional live and inactivated vaccines

Conventional live vaccines can be administered by different immunization routes. The most common applicable routes are via eye-drop or drinking water during the first three

weeks after hatch (VAN DEN BERG 2000). Mostly, attenuated live vaccines based on classical and variant strains are used and can be classified into different groups depending on their virulence. The categories are mild, mild intermediate and intermediate plus “hot” vaccines (MÜLLER et al. 2012; ETERRADOSSI and SAIF 2013). Depending on the presence of maternally derived anti-IBDV antibodies the protection by mild vaccines might not be as effective as by intermediate plus vaccines. On the other hand, the more virulent vaccines might lead to more severe bursal lesions and immunosuppression in the chickens (TSUKAMOTO et al. 1995; RAUTENSCHLEIN et al. 2005). Further safety concerns are directed at the generation of new reassortant viruses by the application of live virus vaccines (HE et al. 2014; ALKIE and RAUTENSCHLEIN 2016).

Since MDA might interfere with the replication of the vaccine strains, serological profiling of the flocks is necessary to determine the optimal time point for vaccination (KOUWENHOVEN and VAN DEN BOS 1992, 1994; DE WIT 1998, 2001). The Deventer formula was developed to estimate the right time point of vaccination based on the half-life time of the MDA, which is depending on the genetic background of the chickens, furthermore the age of chickens at sampling, the breakthrough level of the vaccine and the percentage of the birds having antibody levels below the breakthrough level of the vaccine (DE WIT 1998, 2001).

Inactivated vaccines are mostly administered parenterally to breeding flocks as a booster vaccination to transmit uniform and high antibody levels to the progeny chickens (MAAS et al. 2001). Prior to vaccination with inactivated vaccines, the birds have to be exposed to a suitable replicating antigen (priming) (MÜLLER et al. 2012). Therefore, inactivated IBDV vaccines are mostly used in combined vaccination programs (prime and boost regimes) in breeding flocks.

4.3.10.2 Immune-complex (Icx) vaccines

IBDV Icx vaccines obtain a mixture of IBDV-specific antibodies and the infectious virus (WHITFILL et al. 1995; MÜLLER et al. 2012). The advantage of these kinds of vaccines is that they can be administered *in ovo* to a high amount of birds and they were shown to be effective in the presence of MDA (JOHNSTON et al. 1997;

GIAMBRONE et al. 2001). On the other hand, Icx vaccines might lead to a severe immunosuppression especially when administered to young chickens.

4.3.10.3 IBD vector vaccines

Similar to the Icx vaccines, the vector vaccines are also suitable for *in ovo* administration at EID 18 as well as at one day post hatch. Different live attenuated vaccine viruses were genetically modified to express the immunogenic VP2 of IBDV, such as the avian adenovirus CELO (FRANCOIS et al. 2004), Newcastle disease virus (NDV) (HUANG et al. 2004), Marek's disease virus (MDV) (TSUKAMOTO et al. 1999) and HVT (BUBLOT et al. 2007). The HVT was shown to be the most suitable vector virus since it has a large genome, it is effective in the presence of MDA and is able to induce a life-long immunity (BUBLOT et al. 2007; LE GROS et al. 2009; BARON et al. 2018). For the protection against Marek's disease (MD) and IBD, different recombinant HVT-IBD vector vaccines are commercially available (VAXXITEK®HVT-IBD, Boehringer Ingelheim; VECTORMUNE®HVT, Ceva Animal Health, LLC). The recombinant Vaxxitek HVT-IBD vaccine contains the VP2 gene from the classical Faragher 52/70 strain while the Vectormune HVT-IBD vaccine contains the VP2 gene from the Delaware variant E strain (INGRAO et al. 2017). Both vaccines consist of the same HVT strain FC126 (DARTEIL et al. 1995; BUBLOT et al. 2007; PEROZO et al. 2009). In contrast to recombinant HVT-IBD vector vaccines, live vaccines might lead to a transient immunosuppression, indicated by systemic B-cell depletion and induction of bursal lesions and thus might impair the humoral immune response against other vaccines (KIM et al. 1999; RAUTENSCHLEIN et al. 2005; RAUTENSCHLEIN et al. 2007a; PRANDINI et al. 2016; ROH et al. 2016).

4.3.11 Influence of the genetic background on IBDV susceptibility

Layer-type hybrids were suggested to be more susceptible to IBDV infection than broiler-type birds at the same age (WINTERFIELD and THACKER 1978; VAN DEN BERG et al. 1991). In field as well as experimental studies it was shown that commercial LT chickens show higher mortality rates compared to commercial BT chickens (VAN DEN BERG et al. 1991; BUMSTEAD et al. 1993). In addition to higher mortality rates, higher bursal lesion scores and a higher viral replication were also

associated with the susceptibility to IBDV infection (BUMSTEAD et al. 1993; MOODY et al. 2000). A comparison of IBD susceptibility between SPF BT and LT chickens also demonstrated that the SPF BT chickens showed less morbidity, mortality and less gross as well as histological lesions (SILVA et al. 2016). White Leghorns were suggested to develop the most severe disease and highest mortality rates but in other studies Brown Leghorn lines were recorded to show higher mortality rates (BUMSTEAD et al. 1993; ETERRADOSSI and SAIF 2013). Two LT chicken lines, which were designated as a resistant (6_1: White Leghorn) and a susceptible (BrL: Brown Leghorn) chicken line to IBDV (BUMSTEAD et al. 1993) switched their status of resistant and susceptible over time (SMITH et al. 2015). In the latter study it was suggested, that the BrL chicken line might be more capable of initiating a stronger innate immune response. The stronger innate immune response might lead to a faster viral clearance and milder outcome of the disease or important immune function genes might be highly upregulated. BT chickens, which were selected for high or low antibody responses against *E. coli*, showed a higher anti-IBDV specific antibody development (PITCOVSKI et al. 2001). Another study reported that the selection of BT chickens towards a high antibody response to sheep red blood cells (SRBC) might improve IBDV disease resistance (JAIN et al. 2013).

Investigations on the association between IBDV infection and the MHC delivered opposing results. A study conducted by FADLY and BACON (1992) demonstrated that the mortality rates after IBDV infection were influenced by the MHC-B-haplotype of the chickens. A different study didn’t see any correlation between mortality rates and the MHC-B-haplotype (NIELSEN et al. 1998). HUDSON et al. (2002) looked at differences between BT breeder chicken with different MHC-B-haplotypes and didn’t find a correlation between viral loads and CD4+ and CD8+ T-cell numbers in the BF. Antibody responses against live attenuated and inactivated IBDV vaccines were shown to differ between chickens with different MHC-B-haplotypes (JUUL-MADSEN et al. 2002, 2006).

Other studies also observed differences between and within commercial BT and LT chicken lines during the acute phase of IBDV infection (ARICIBASI et al. 2010;

TIPPENHAUER et al. 2013), which led to the assumption that especially differences in early innate immune responses influence the outcome of the disease. In these studies, differences in T-cell modulation between the lines were suggested to play an important role in IBDV pathogenesis.

5. Goals and Objectives

The overall goal of the study was to identify differences between chicken lines in innate and acquired immune responses and associated disease susceptibility to IBDV.

The objectives of the project were the following.

In the first study the objectives were to compare the development of the immune system as well as innate and adapted immune responses with and without vaccination with a recombinant vector HVT-IBD vaccine between BT, LT and DT chickens during the first five weeks post hatch.

The following parameters were determined:

a. Anti-IBDV specific humoral immune response
b. Secondary antibody responses after vaccination with IBV and NDV
c. Spleen and BF organ weight development
d. Immune cell populations in different immune organs
    - CD4+ and CD8+ T-cells in the BF
    - CD4+, CD8+ T-cells , B-cells and macrophages in the spleen
e. In vitro cell proliferation tests of spleen cell cultures
    - Mitogenic IFNγ assay after ConA stimulation (T-cells)
    - NO-test after LPS stimulation (macrophages)

In the second study the objective was to compare the IBDV pathogenesis and the recovery process after inoculation with an intermediate plus live IBDV vaccine strain between the chicken lines. Highly susceptible SPF LT chickens were included as a reference group.

The following parameters were determined:

a. Detection of anti-IBDV antibodies (MDA and seroconversion)
b. Bursal lesions by scoring of
    - BF / body weight ratio
    - Histology

- B-cell detection

c. IBDV antigen in the BF

d. T-cell system
   - Accumulation of CD4+ and CD8+ T-cells
   - IL-10 and TGFβ4 cytokine detection

6. Immune responses upon *in ovo* HVT-IBD vaccination vary between different chicken lines

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Authors' contributions:

MD, MA and SR conceived and designed the experiments; MD performed the experiments; MD analyzed the data; MD and SR wrote the paper. All authors read and approved the final manuscript.

The extent of contribution from Marina Dobner to this article:

Scientific design: 40 %
Laboratory work: 90 %
Data analysis: 90 %
Scientific writing: 60 %

6.1 Abstract

The genotype of chickens is assumed to be associated with variable immune responses. In this study a modern, moderate performing dual-purpose chicken line (DT) was compared with a high-performing layer-type (LT) as well as a broiler-type (BT) chicken line. One group of each genotype was vaccinated *in ovo* with a recombinant herpesvirus of turkeys expressing the virus protein VP2 of the infectious bursal disease virus (HVT-IBD) while one group of each genotype was left HVT-IBD unvaccinated (control group). Genotype associated differences in innate and adapted immune responses between the groups were determined over five weeks post hatch. HVT-IBD vaccination significantly enhanced humoral immune responses against subsequently applied live vaccines compared to non-HVT-IBD vaccinated groups at some of the investigated time points ($P < 0.05$). In addition HVT-IBD vaccination had depending on the genotype a significant impact on splenic macrophage as well as bursal CD4+ T-cell numbers ($P < 0.05$). On the other hand, the detectable genotype influence on Interferon (IFN) γ and nitric oxide (NO) release of *ex vivo* stimulated spleen cells was independent of HVT-IBD vaccination. The results of our study suggest considering a genotype specific vaccination regime in the field.

6.2 Introduction

Since the 1930s-1940s, an intense genetic selection process in chickens was initiated in poultry production, for mainly two important, opposite production traits (Havenstein, 2006; Hunton, 2006). Chickens were either selected for high egg-production (layer-type chickens, LT) or for improved feed conversion rate, fast growth and high breast meat proportion (meat- or broiler-type chickens, BT). It is believed that the unilateral, selective breeding towards high production traits might have led to disadvantages with respect to other important traits, such as behavior, physiology (Julian, 2005; Kalmar et al., 2013; Kjaer and Sørensen, 1997; Rauw et al., 1998) and immunocompetence (Havenstein et al., 1994; Kreukniet et al., 1996; Qureshi and Havenstein, 1994). It was shown that immune responses not only vary between BT and LT genotypes (Koenen et al., 2002; Leshchinsky and Klasing, 2001; Qureshi and Miller, 1991) but also differ within different BT and also LT lines (Kjaerup et al., 2017; Miller et al., 1992; Parmentier et al., 2006). Different studies indicated that the LT genotype's immune system might not be as compromised as the immune system of BT genotypes (Havenstein et al., 1994; Koenen et al., 2002; Leshchinsky and Klasing, 2001). Therefore, it could be suggested that the selection towards a fast growth might have been more affected with regard to the immune system than the selection towards high egg-production (Siegel et al., 1982).

An alternative to high-performing chicken genotypes would be the use of dual-purpose breeds, which can be used for both egg- and meat production. The Lohmann Dual (dual-purpose type, DT) is one recently developed modern dual-purpose chicken genotype. Different to traditional dual-purpose breeds, which show low performance in both reproductive- and meat traits, the combination of layer and broiler lines in Lohmann Dual led to a moderate egg production on the female side and improved body weight gain when compared to conventional layer-type birds (Icken and Schmutz, 2013).

In general, innate as well as adaptive immune responses differ between chicken lines. *In vitro* mitogen assays in cell cultures have been used to demonstrate the influence of the genetic background on cellular-mediated immune responses (CMI). By stimulating T-cells with plant lectins, such as phytohaemagglutinin (PHA) or

concanavalin A (ConA) (Hovi et al., 1978; Toivanen and Toivanen, 1973), the CMI can be assessed (Li et al., 1999) and measured by the release of IFNγ (Lambrecht et al., 2004; Lowenthal et al., 1995). Differences between chicken lines in T-cell proliferation rates have already been recorded (Bacon and Palmquist, 2002). It was also shown, that chicken lines differ in their responsiveness to lipopolysaccharides (LPS), as strong inducers of the innate immunity and can be separated into hypo- and hyper-responders to LPS (Hussain and Qureshi, 1998). Because DT chickens have genetic characteristics of both layer- and broiler-type chickens, differences in the responsiveness of immune cells can be expected compared to conventional lines.

Immunocompetence and robustness in livestock animals, including poultry, gained importance in the recent years due to upcoming demands for the reduction of antibiotic treatments and medications in general. Vaccination is one of the most important tools to prepare the chickens for a challenging field environment. Especially during the first weeks post hatch, when parts of the immune system are still developing (Mast and Goddeeris, 1999), vaccination might advance the development of the immune system. In veterinary medicine live vector vaccines are widely used, particularly viral vectors such as adenoviruses, poxviruses and herpesviruses (Baron et al., 2018). The herpesvirus of turkeys (HVT) belongs to the genus *Mardivirus*, is closely related to the Marek's disease virus (MDV), but represents its own species *meleagrid herpesvirus type 1* (MeHV-1) (Osterrieder et al., 2006) and was shown to be apathogenic in chickens and turkeys (Fabricant et al., 1982). It is successfully used as a vector virus in recombinant vaccines against Newcastle Disease (ND), Infectious bursal disease (IBD), Infectious laryngotracheitis (ILT) and avian influenza (AI) (Darteil et al., 1995a; Palya et al., 2016; Reddy et al., 1996; Vagnozzi et al., 2012). HVT might modulate host immune responses by inducing the release of different immunostimulatory cytokines and interferons (Abdul-Careem et al., 2008; Karaca et al., 2004) depending on the time of inoculation and therefore might influence the outcome of immune responses in general. Interestingly, the induction of cytokines such as IFNγ and IL-10, which play a major role during the pathogenesis of MDV (Abdul-Careem et al., 2007; Jarosinski et al., 2005), have also been associated with HVT. Nevertheless, the effects of HVT alone or as a recombinant live vaccine have mostly been investigated in experimental setups

using one genetic chicken line or chicken lines with different MHC-haplotypes (Quere et al., 2005).

Differences in innate and adapted immune responses between chicken lines, selected for different performance traits might also lead to different immune responses to HVT, which hasn't been investigated before. The recombinant vectored vaccine HVT–infectious bursal disease virus (IBD) vaccine (HVT-IBD) is a vectored vaccine expressing the IBD virus protein VP2 (Darteil et al., 1995a). It can either be administered *in ovo* on embryonation day (ED) 18 or at one day post hatch (ph). Previous studies have demonstrated that this vaccine may also enhance the immune response after vaccination against other pathogens (Prandini et al., 2016).

The aim of the present study was to determine if the development of the immune system as well as innate and adapted immune responses differ between high performing LT and BT chickens compared to the DT birds with a special focus on the first 5 weeks ph. We also investigated if the early vaccination with HVT-IBD influences the investigated immune parameters differently depending on the respective genotype.

6.3 Material and Methods

Experimental chickens

Embryonated eggs of three different commercially available chicken genotypes (broiler-type (BT) = Ross 308, layer-type (LT) = Lohman Brown-Plus, dual-purpose type (DT) = Lohmann Dual) were provided by the BWE Brüterei Weser-Ems (BT), the Geflügelzuchtbetrieb Gudendorf-Ankum (LT) and Lohmann Tierzucht GmbH, Cuxhaven, Germany (DT), respectively. Birds were hatched together at the Clinic for Poultry in Hannover and birds from each genotype were randomly assigned to two subgroups (n=35 in Exp.1, n=30 in Exp.2). Each genotype group was either vaccinated with the HVT-IBD vaccine (+HVT-IBD) or with the diluent of the vaccine (control groups, –HVT-IBD). Additionally, +HVT-IBD as well as –HVT-IBD groups received a combined live Infectious bronchitis (IBV) and Newcastle disease (NDV) vaccine by eyedrop route. The chickens were housed in floor pens with wood shavings and had free access to food and water from the first day post hatch on. All animals were fed with the same commercial chicken diet for rearing layer-type birds (allmash-A, deuka, Deutsche Tierernährung Cremer GmbH, Germany). All experiments and procedures were authorized by the State Office for consumer protection and food quality of Lower Saxony (LAVES; approval number: 33.12-42502-04-15/1827) in Germany.

Experimental design

Overall two experiments (Exp. 1 and Exp. 2) were conducted for this study. Groups (n=30-35) were inoculated *in ovo* at ED 18 (+12 hours) with the recombinant vectored HVT-IBD vaccine (Vaxxitek®HVT-IBD, Boehringer Ingelheim, Germany) or with the diluent of the vaccine (control groups). On the first day ph all birds from both groups were vaccinated against IBV) and (NDV (Nobilis® Ma5+Clone30, MSD Tiergesundheit, Unterschleißheim, Germany) with one dose per eyedrop route. All vaccines were prepared and applied according to the manufacturer's instructions. Until 35 dph blood samples of 15 birds per group were collected weekly from the brachial vein (vena ulnaris) for antibody detection by using appropriate ELISA systems. The same 15 birds per group and genotype were used for continuous blood sampling throughout the

experiment (Fig. 1).. On days 14, 28 and 35 days ph seven (Exp.1) or five (Exp.2) birds per group were stunned and exsanguinated for necropsy. Samples of the bursa of Fabricius (BF), spleen and cecal tonsils (CT) were collected for histological and immunohistochemical investigations at days 14 and 28 ph. Body weights and organ weights of spleens (14, 28 and 35 dph) and BF (14 and 28 dph) of each bird were recorded to calculate organ/body weight ratios ($\frac{Organ\ weight}{Body\ weight} \times 1000$). In Exp. 2 at days 14, 28 and 35 ph, the spleens were also used for the isolation of leukocytes to investigate the immune responsiveness by mitogen-assay and nitric oxide (NO) test, respectively.

Histological examinations

Samples of BF, spleen and CT were fixed in 10% phosphate-buffered formalin and stored at 4 °C until use. The different tissue samples were sectioned at 2-3 µm and stained with haematoxylin and eosin (H&E). Tissues were investigated microscopically for differences in the structural development. Germinal centers (GC) in the spleen and CT were counted in three randomly selected microscopic fields of five (Exp.2) – seven (Exp. 1) birds per group and day at a magnification of 100x ± standard deviation (SD). Results are presented as a summary of both experiments with n=12 birds/group.

Immunohistochemical detection of immune cells

At necropsy, tissue samples were immediately snap frozen in liquid nitrogen and stored at -80 °C until use. The samples were sectioned at -28 °C (3-5 µm) and processed as previously described (Han et al., 2016; Schwarz et al., 2011). Mouse anti-chicken monoclonal, unlabeled antibodies for the detection of CD4+ T-cells (Clone CT4) and CD8+ T-cells (Clone EP42) (only Exp. 1) in the BF and macrophages (Clone KUL01), B-cells (Clone Bu-1) and CD4+ T-cells in spleen were purchased from Southern Biotech (Birmingham, AL, USA) and used at a dilution of 1:500. The Vectastain® *Elite* ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) provided the secondary, biotinylated goat-anti-mouse IgG Fc antibodies and biotinylated horseradish peroxidase (HRP) enzyme. In a last step the DAB (3,3'-diaminobenzidine) Peroxidase Substrate Kit (Vector Laboratories Inc.) was used for visualization. Positively stained

cells were counted in three randomly selected microscopic fields at a magnification of 400x per bird. Results are presented as a summary of both experiments with n=12 birds/group. Data are expressed as means of one group per day ± SD.

Antibody detection by ELISA

Blood samples were collected and serum prepared following standard procedures to detect anti-VP2 IBDV specific IgG-type antibodies (anti-VP2 antibodies), anti-IBV and anti-NDV antibodies with commercially available ELISA kits (Synbiotics IBD ProFLOK+®, ND Synbiotics ProFLOK+®, IB Synbiotics ProFLOK®, Synbiotics Corporation, San Diego, CA, USA). Following the manufacturer's instructions of the respective ELISA kits sera were tested for the antibodies mentioned above and mean antibody levels per group ± SD were calculated.

Isolation and preparation of leukocytes for *ex vivo* culture

In Exp. 2, spleens from five birds per group were collected during necropsy. The spleens were placed immediately in ice-cold phosphate buffered saline (PBS) (PBS Dulbecco; Biochrom GmbH, Berlin, Germany) with 2% Penicillin/Streptomycin (10.000 U/ml; 10.000 µg/ml) (Biochrom) and 2 % fetal bovine serum (FBS; Biochrom). The cells were isolated following a density centrifugation protocol (Archambault et al., 1976). Briefly, the spleens were smashed through a 70 µm nylon cell strainer (Falcon®, Corning, NY, USA) and washed in cold PBS. Afterwards the cells were pelleted at 425x *g* for 10 min at 4 °C. The supernatants were discarded and the cell pellet was resuspended in 7 ml of PBS at room temperature (RT). Biocoll Separating Solution (Biochrom) was adjusted to a specific density of 1.09 g/ml and used to underlay the obtained cell suspension. After centrifugation at 1180x *g* for 20 min mononuclear cells were collected from the interphase. The cells were kept in RPMI 1640 (Biochrom) with 10% fetal bovine serum (FBS), 1% L-Glutamin (Biochrom) and 2% Penicillin/Streptomycin (Biochrom). All samples were counted for vital cells by Trypan blue staining. 100 µl of medium containing 10^7 live cells per spleen and bird were seeded into sterile 96 round-bottom well cell culture plates (Sarstedt AG & Co, Nümbrecht, Germany).

Nitric oxide (NO) test

Lipopolysaccarides (LPS) from *Escherichia coli (E. coli)* 0127:B8 (Sigma-Aldrich GmbH, Munich, Germany) were used for the stimulation of spleen cells at a concentration of 10 µg/ml and incubated for 48 hours in a humidified atmosphere at 41°C and 5% CO_2. Cells incubated in medium without LPS were used as negative controls. All samples were tested in triplicates. After 48 hours of stimulation cell culture supernatants were transferred into 96 flat-bottom well plates (Greiner CELLSTAR®, Merck, Sigma-Aldrich GmbH, Munich, Germany) and immediately used for the detection of NO. Since NO is a highly diffusible, gaseous molecule its intermediate product nitrite (NO_2) can be measured by the Griess reaction, which has previously been described (Green et al., 1982). Therefore, equal volumes of the Griess reagent (Griess A: 1g sulphanilamid in 100 ml distilled water with 2.5% H_3PO_4; Griess B: 0.1 g N-(1-naphthyl) ethylendiamin dihydrochlorid 0.1% in 100 ml distilled water with 2.5% H_3PO_4) were added to each sample and the optical density (OD) values were determined at 570 nm with a microplate reader (Tecan Trading AG, Männedorf, Switzerland). A log_2 serial dilution of sodium nitrite ($NaNO_2$) was used to generate a standard curve to calculate NO_2 levels in µmol (µM). Per bird, mean NO levels of non-stimulated triplicates were subtracted from the mean NO levels of the stimulated triplicates. NO_2 levels are expressed as means of five birds per group and day in µM ± **SD.**

Mitogenic (IFNγ) assay and detection of IFNγ-release

ConA (Carl Roth GmbH + Co.KG, Karlsruhe, Germany) was used for stimulation of spleen cells at a concentration of 5 µg/ml and incubated for 48 hours in a humidified atmosphere at 41°C and 5% CO_2. All samples and controls were also tested in triplicates. Supernatants of ConA stimulated spleen cells were transferred to 96 flat-bottom well plates and stored at -20 °C until use. IFN*γ*-production was measured with a commercially available chicken IFN*γ* ELISA kit according to the manufacturer's instructions (IFN*γ* Chicken Matched Antibody Pair, Fisher Scientific GmbH, Germany). IFNγ concentrations were calculated based on the standard curve provided with the ELISA kit. In contrast to the manufacturer's instructions, culture supernatants were

diluted 1:128 to obtain OD values in the range of the standard curve. Per bird, mean IFNγ levels of non-stimulated negative triplicates were subtracted from the mean IFNγ levels of the stimulated triplicates. IFNγ concentrations are expressed as mean values of five birds per group and day in pg/ml ± SD.

Statistical analysis

All statistical analyses were carried out with the program Statistix 10.0 (Analytical software, Thallahassee, FL, USA). Hatch rates were analyzed using the Fisher's Exact Test. Differences between genotypes were analyzed using One-way analysis of variance (Completely Randomized Design) or Kruskal Wallis all-pairwise comparisons test. Differences between +HVT-IBD and corresponding –HVT-IBD control groups were analyzed by using Wilcoxon Rank Sum Test or Two Sample *t*-test. A factorial analysis of variance was used to statistically analyse the impact of the genotype, the HVT-IBD vaccine and the age of the birds on the numbers of GC, immune cell populations and NO and IFNγ release in *ex vivo* spleen cultures. A normal distribution of all data was considered before starting the analyses of the data. $P < 0.05$ was considered as statistically significant.

6.4 Results

Hatchability

In general, hatch rates were varying between the different genotypes. In both experiments BT and LT chickens had higher hatch rates compared to the DT chickens. Nevertheless, hatch rates were only differing significantly ($P < 0.05$, Fisher's Exact Test) between the genotypes in Exp. 2 (BT: 97%, LT: 96%, DT: 75%) but not in Exp. 1. No effect of HVT-IBD vaccination on the hatchability of the chickens was observed in BT and DT birds in all experiments. HVT-IBD vaccinated LT birds on the other hand had a significantly ($P < 0.05$, Fisher's Exact Test) higher hatch rate in Exp. 1 compared to HVT-IBD-non-vaccinated control birds (LT +HVT-IBD: 96% vs. LT –HVT-IBD: 78%). This result was not repeated in Exp. 2.

Body and relative organ weights

Body weights, BF and spleen weights of all birds were recorded during the necropsies. Body weights in control groups differed significantly ($P < 0.05$) as expected between BT and DT as well as LT chickens at all investigated time points (Fig. 2 a). LT chickens had the lowest body weights followed by the DT and BT chickens. The relative bursal and spleen weights differed between the genotypes, while LT chickens had higher relative bursal and spleen weights compared to DT and BT chickens (Fig. 2 b+c). DT chickens took an intermediate position. No significant differences in relative bursal or spleen weights were observed between female and male chickens of one genotype (S1 Fig). Nevertheless, male DT chickens had significantly higher relative spleen weights compared to female and male BT chickens, whereas the female DT chickens' relative spleen weights didn't differ significantly from female and male BT chickens at 14, 28 and 35 dph (S1 Fig). HVT-IBD vaccination didn't influence the body weight development after hatch. In most groups, HVT-IBD vaccination didn't have a significant effect on the organ weights. +HVT-IBD LT groups showed significant higher relative bursal weights at 14 and 28 dph as well as a higher relative spleen weights at 28 and 35 dph compared to the corresponding –HVT-IBD control groups ($P < 0.05$; Fig. 2 b+c).

–HVT-IBD DT birds showed a significantly higher relative bursal weight compared to +HVT-IBD DT birds at 14 dph.

Histological examinations

All sampled immune organs were investigated for differences between genotypes considering the structural development of the sampled organs. No differences in the structural development were observed between the genotypes at all investigated time points. Numbers of GC were counted in the CT and in the spleens (S2 Fig). As expected, the numbers of GC in the CT and in the spleens increased with the age of the birds in all groups (Fig. 3). Depending on the genotype, the numbers of GC in the spleens and CT varied at 28 dph but not at 14 dph (Fig. 3 b + d), demonstrating a significant influence of the genotype and the age of the birds (Table 1). BT birds showed the highest numbers of GC in the spleens and CT in both +HVT-IBD and –HVT-IBD groups on 28 dph (Fig. 3 b + d). Differences in the number of GC between +HVT-IBD or –HVT-IBD genotype groups were only significant in the spleens (Fig. 3 b). Independent of the genotype and age of the birds HVT-IBD vaccination significantly influenced the number of GC in the spleens and CT (Table 1). All HVT-IBD vaccinated groups had higher numbers of GC in the spleens on 28 dph compared to the control groups but these differences were only significant in BT chickens ($P < 0.05$).

Immunohistochemical staining

IBDV infection and vaccination can lead to an accumulation of T-cells in the BF, the primary target organ of the virus, depending on the virulence of the virus strain (Khan and Hashimoto, 1996; Rautenschlein et al., 2003). Therefore, we investigated possible changes in the number of different T-cell subsets in the BF, namely CD4+ and CD8+ T-cells at different time points after *in ovo* HVT-IBD vaccination. The staining of CD8+ T-cells was only conducted in Exp. 1 and not repeated in Exp. 2, since no changes in the number of CD8+ T-cells were observed between the groups (data not shown). The major functional compartments of the spleen, such as the peri-arteriolar lymphoid sheath (PALS) consisting of T-cells expressing CD4+, the peri-ellipsoid lymphoid sheath (PELS) consisting of B-cells and another outer ring of macrophages were

investigated for the respective immune cell populations between the groups (Fig. 4, S3 Fig). As expected, the development of PALS and PELS in the spleen was completed at 14 dph in all the investigated groups (Mast and Goddeeris, 1998, 1999). But interestingly, differences in the numbers of splenic macrophages between the groups were detected at 14 dph (Fig. 4 c, g, h), whereas no differences in the numbers of CD4+ T-cells and B-cells in the spleen were observed between the groups.

The numbers of macrophages in the spleens and the numbers of CD4+ T-cells in the BF of each genotype per day were summarized for the two experiments. The investigated immune cell populations decreased over time per microscopic field in all genotype groups (Fig. 4 a - d). Therefore, independent of HVT-IBD vaccination and genotype, the age of the birds had a significant influence on the number of CD4+ T-cells in the BF and on the number of macrophages in the spleen (Table 1).

CD4+ T-cells in the BF

No significant differences were observed in the numbers of CD4+ T-cells per microscopic field between genotypes independent of the vaccination status. After HVT-IBD vaccination significant differences in CD4+ T-cell numbers were detected (Table 1; effect of Genotype x HVT-IBD, *P*-value = 0.02). Between +HVT-IBD DT birds and the corresponding –HVT-IBD controls the numbers of CD4+ T-cells differed significantly at 14 and 28 dph (Fig. 4 a+b and e+f; $P < 0.05$). A similar trend was observed for the other genotypes but the differences were statistically not significant ($P > 0.05$).

Macrophages in the spleen

The number of macrophages was influenced by the HVT-IBD vaccination depending on the genotype (Table 1; effect of Genotype x HVT-IBD *P*-value = 0.01). +HVT-IBD BT chickens had significant higher numbers of macrophages compared to +HVT-IBD LT chickens at 14 dph (Fig. 4 c and g+h; $P < 0.05$). +HVT-IBD DT chickens took an intermediate position. +HVT-IBD BT and DT chickens also showed a higher number of macrophages at 14 dph compared to the corresponding –HVT-IBD control groups. Likewise, at 28 dph +HVT-IBD BT chickens showed a higher number of macrophages

compared to –HVT-IBD controls but the differences weren't significant (Fig. 4 d; $P > 0.05$).

Humoral immune responses

Anti-VP2 IBDV specific IgG-type antibody titers

Anti-VP2 antibodies were determined weekly after administration of the HVT-IBD vaccine on ED 18 (+12 hours) *in ovo*. Due to the different age and vaccination schedules of the respective parental flock the maternal antibody status differed between the genotypes in Exp. 1 and 2. LT and DT chickens had very similar initial anti-IBDV maternal antibody titers at 7 dph whereas the BT chickens started with a lower level of anti-IBDV maternally derived antibodies (MDA) in both experiments (Fig. 5 a+b). After 21 dph all HVT-IBD vaccinated groups showed an increase in circulating anti-VP2-antibodies. In Exp. 1 +HVT-IBD BT and LT chickens had significantly ($P < 0.05$, Wilcoxon Rank Sum Test) higher Anti-VP2 antibody titers compared to their corresponding –HVT-IBD control groups (statistical evaluation is not specifically highlighted in Fig. 5 a+b, $P < 0.05$) starting at 21 dph. In Exp. 2 +HVT-IBD BT and DT chickens had significantly ($P < 0.05$, Wilcoxon Rank Sum Test) higher anti-VP2 antibody titers compared to their corresponding –HVT-IBD control groups (statistical evaluation is not specifically highlighted in Fig. 5 a+b, $P < 0.05$) starting at 21 dph. To further emphasize the stronger and faster increase of anti-VP2 antibodies in BT chickens, all antibody titers were additionally expressed as a percentage of the initially measured mean antibody level of each genotype group (Fig. 5 c+d). BT chickens in Exp. 1 showed a lower mean anti-VP2 antibody titer at 7 dph than in Exp. 2. Despite the lower initial mean anti-VP2 antibody titer at 7 dph in Exp.1, the anti-VP2 antibody response earlier than in Exp. 2 (Fig. 5).

Anti-IBV and –NDV antibody titers

All groups received the IB/ND live vaccine on the day one ph. At 7 dph anti-IBV MDA were comparable between LT and DT chickens. BT chickens showed lower anti-IBV antibody levels at 7 dph (Fig. 6 a, b, c) compared to LT and DT chickens. The decrease of anti-IBV MDA was comparable between +HVT-IBD and –HVT-IBD groups of all

chicken genotypes. BT chickens showed a faster decrease of anti-IBV antibody titers, which reached the cut-off value of the ELISA at 28 dph and increased subsequently (Fig. 6 a). At 35 dph anti-IBV antibody titers between HVT-IBD vaccinated genotype groups differed significantly ($P < 0.05$; DT=3.4 ± 0.6 (A), LT=2.9 ± 0.7 (B), BT=2.4 ± 0.7 (C) (antibody titers in log 10)). In general anti-IBV antibody titers of +HVT-IBD and –HVT-IBD groups were comparable between the groups but at 7 dph +HVT-IBD LT chickens showed significantly higher anti-IBV antibody titers compared to the corresponding –HVT-IBD control group (Fig. 6 c).

No differences in anti-NDV antibody titers between +HVT-IBD genotype groups were observed at all investigated time points. –HVT-IBD BT chickens had lower anti-NDV antibody titers compared to –HVT-IBD DT and LT chickens (Fig. 6 d, e, f). At 35 dph –HVT-IBD DT chickens had the lowest anti-NDV antibody titers compared to –HVT-IBD BT and LT chickens. At different time points +HVT-IBD genotype groups showed significantly higher anti-NDV antibody levels compared to the corresponding –HVT-IBD control groups (+HVT-IBD BT chickens at 7 and 21dph, +HVT-IBD DT chickens at 35 dph and +HVT-IBD LT chickens at 7 and 14 dph (Fig. 6 b, e, f, $P < 0.05$).

NO test

NO release was detected in supernatants of *ex vivo* LPS stimulated spleen cells of all genotypes as an indicator for macrophage activity. High variations after stimulation between individuals of one group were observed. Genotype differences in NO release appeared between –HVT-IBD genotypes as well as between +HVT-IBD genotypes. At 28 dph spleen cells of –HVT-IBD BT birds produced higher NO amounts compared to spleen cells of –HVT-IBD LT birds. At 35 dph +HVT-IBD and –HVT-IBD BT spleen cells showed a significantly higher NO release compared to LT and DT spleen cells (Fig. 7, $P < 0.05$). In general, the genotype and the age of the birds had the highest impact on the release of NO in spleen cell cultures (Fig. 7, Table 2). No differences between +HVT-IBD genotype groups and their corresponding –HVT-IBD control groups in NO release were observed.

Mitogenic (IFNγ) assay

IFNγ release into the supernatants of *ex vivo* ConA stimulated spleen cells of all genotypes was determined. *Ex vivo* stimulated spleen cells of BT birds produced the highest amounts of IFNγ at all investigated time points. Independent of vaccination status splenocytes of LT and DT birds produced lower amounts of IFNγ compared to splenocytes of BT birds at all investigated time points. Values were significantly different between the groups at 35 dph (Fig. 8; $P < 0.05$). LT spleen cells tended to release the lowest amounts of IFNγ. The HVT-IBD vaccination didn't have any impact on the release of IFNγ into the supernatants (Fig. 8; *P*-value=0.45). The genotype had the highest impact on IFNγ-release of *ex vivo* ConA stimulated chicken spleen cells (Table 2; *P*-value < 0.001).

6.5 Discussion

Several studies demonstrated the association between differences in performance traits and immune responses (Gross et al., 1980; Siegel et al., 1982; Sumners et al., 2012). In this study various immune parameters were selected for comparison between a high performing layers and broilers and DT chickens with moderate meat and egg production traits. Therefore, humoral immune responses after vaccination with different live vaccines, specifically the magnitude of antibody levels, and the IFN-γ as well as NO release of *ex vivo* spleen cell cultures were included. The focus was on the first 35 days ph. During this early stage of life, the immune system is still developing and the most significant differences are expected. In two experiments we either vaccinated the different genotypes with a recombinant vectored HVT-IBD vaccine or with placebo at EID 18 *in ovo* to additionally obtain information about the immunomodulating effect of the HVT-IBD vector vaccine on innate and adaptive immune responses between the genotypes in a comparable approach.

Body weight development between the different chicken lines differed significantly ($P < 0.05$). As expected, BT chickens showed the highest body weights at all investigated time points, followed by the DT and the LT chickens but showed significantly lower relative spleen and bursal weights compared to LT and DT chickens. Higher relative immune organ weights might be associated with the ability to mount higher humoral immune responses (Parmentier et al., 1995) but the selection for fast growth in broiler chickens might have resulted in a decrease in the relative growth of important immune organs (Cheema et al., 2003), which could also be confirmed in this study. Nevertheless, higher relative immune organ weights might not always be associated with a higher humoral immune response, since BT chickens of our study showed the strongest anti-VP2 antibody response. The DT chickens take an intermediate position in body weight development and relative growth of the spleen and the BF.

Most of the immune organs in chickens start to develop during embryogenesis and mature in the following weeks post hatch. The development of the bursal follicles with their typical differentiation into the cortical and medullary zone is apparent at the time around hatch (Paramithiotis and Ratcliffe, 1994; Ratcliffe, 2006) with the cortex being

fully developed at two weeks after hatch (Oláh et al., 2014). The spleen represents the most important secondary lymphoid organ in chickens. Its structural development has been associated with its functionality (Jeurissen et al., 1994) and is therefore of importance. In broilers as well as in layers, characteristic structures of the spleen can be found around hatch. However, the structure and especially the B-cell compartment (PELS) mature during the first week ph (Mast and Goddeeris, 1998, 1999). By histological and immunohistochemical investigations, no differences in the structural development were detected between the genotypes at the investigated time points 14 and 28 dph of neither the lymphoid organs. In the future, earlier sampling time points might be interesting to include. Different studies indicated that the numbers of immune cells are influenced by the origin of the sample itself, the genetic background, the age of the birds and the environment (Erf et al., 1998; Mast and Goddeeris, 1998; Montgomery et al., 1991; Seliger et al., 2012). Depending on the investigated samples and immune cell populations, the numbers of immune cells might either increase (Seliger et al., 2012) or decrease with the age of the chickens (Erf et al., 1998). In the present study the numbers of CD4+ T-cells in the BF and macrophages in the spleen didn't differ between the genotypes (-HVT-IBD) at 14 and 28 dph but in all groups the numbers of CD4+ T-cells in the BF as well as numbers of macrophages in the spleen decreased with the age of the birds.

GCs, which are important for antibody development, Ig isotype switch, affinity maturation and memory development of B-cells (Arakawa et al., 1996; Yasuda et al., 2003), are found in various peripheral lymphoid tissues including the spleen and the CT. The number of GCs increases with age after increased antigen contact (Jeurissen and Janse, 1994). Nevertheless, histological investigation of the number of GCs in the spleen and CT revealed a significant influence of the genotype on the number of GC in the spleens and CT independent of HVT-IBD and age of the birds ($P < 0.05$). In all genotype groups the numbers of GC in the spleen and CT increased over time. BT chickens developed significantly higher numbers of GC in the spleens compared to LT chickens at 28 dph ($P < 0.05$). Since GCs play an important role in antibody development, the higher numbers of GCs in the spleens of BT chickens may coincide

with the results concerning the higher anti-VP2 antibody development in BT birds compared to the other chicken genotypes.

The ability of macrophages to release NO and express inducible nitric oxide synthase (iNOS) has been shown to be genetically influenced by using chickens with different MHC-haplotypes (Dil and Qureshi, 2002; Hussain and Qureshi, 1998). It was demonstrated that macrophages, which derived from a broiler background (MQ-NSCU) and the layer MHC haplotype ($B^{15}B^{15}$), were classified as hyper-responders to LPS compared to macrophages from two other layer-type strains (GB1 $B^{13}B^{13}$ and GB2 $B^{6}B^{6}$). In the present study, spleen cell cultures of BT chickens released the highest amounts of NO after LPS stimulation. These results allow the speculation that differences in the release of NO might be related to different MHC-haplotypes of the chicken lines used in this study. The MHC-haplotypes of the chicken lines of this study are not known, but would be interesting to be determined in the future.

In different studies, BT chickens either showed a higher or lower proliferative response after stimulation of spleen cell cultures with PHA or ConA compared to LT chickens (Koenen et al., 2002; Leshchinsky and Klasing, 2001). In our study the release of IFNγ in spleen cell cultures, as an indicator of the CMI responsiveness was measured by ELISA. Spleen cells of LT chickens tended to release the lowest IFNγ amounts at all investigated time points while spleen cells from BT chickens showed the highest IFNγ release. The age of the birds at the time of the investigation played a significant role for the magnitude of the CMI response in all genotypes ($P < 0.05$). DT chickens showed a similar CMI responsiveness compared to the LT chicken genotype.

In addition to the investigations on the genotype effects, we also wanted to determine the effect of the HVT-IBD vaccine on the investigated immune parameters between the three genotypes. Several studies demonstrated that HVT alone or as a recombinant vaccine might play a role as a modulator of the host immune response by stimulating the release of T-helper (Th)–1 related cytokines, such as IFNγ, but also anti-inflammatory cytokines, such as IL-10 were detected (Abdul-Careem et al., 2008; Karaca et al., 2004). HVT was also associated with modifying the proliferation of different immune cell subsets (Ingrao et al., 2018; Kurukulasuriya et al., 2017).

Not only the safe and effective application of HVT-IBD *in ovo*, but also the potential to be cross-protective against MDV and to not interfere with maternally derived antibodies (Darteil et al., 1995; Goutebroze et al., 2003) makes it preferable to other vaccines against IBD. Maternally derived anti-IBDV antibodies differed between the lines because of different vaccination schedules of the parental flocks. In the field in Germany, parents of BT chickens usually receive a one time vaccination with live vaccines against IBDV, whereas the parental flocks of LT birds receive an IBDV live and IBDV inactivated boost vaccination. The study by Koenen et al. (2002) suggested that BT chickens might be more specialized in mounting short-term, humoral immune responses. BT birds showed a higher and faster IgM response after antigen contact but lower IgG response compared to LT birds. In the present study, BT chickens also showed a stronger and faster anti-VP2 antibody immune response compared to LT and DT chickens after vaccination with the recombinant live vaccine HVT-IBD. Even if it supposed that MDA levels do not interfere with HVT-IBD vaccination (Darteil et al., 1995), lower anti-VP2 antibody levels at the time of vaccination with HVT-IBD might have been advantageous for a faster anti-VP2 antibody development in BT chickens, besides the genotype difference.

As previous studies suggested an immunomodulating effect of this vaccine, we therefore included the effect of HVT-IBD vaccination on the humoral immune response after IB and ND vaccination (Prandini et al., 2016). With our study we could confirm these observations in all the investigated genotypes. + HVT-IBD vaccinated groups developed higher anti-IBV and anti-NDV antibody titers at some of the investigated time points compared to – HVT-IBD control groups. In murine infection models it was shown that some herpesviruses are able to induce heterologous immunity (cross-protection) against other pathogens due to latency and their continuing activation of immune parameters (Barton et al., 2007; White et al., 2012).

An enlargement of the spleen after HVT vaccination has been observed by Islam et al. (2002) and therefore suggested a reactive response in secondary lymphoid organs after HVT inoculation. We could confirm this observation in our study, where the relative immune organ weights of LT chickens were significantly higher in +HVT-IBD

groups at 28 and 35 dph. BT chickens of the present study didn't show an enlargement of the immune organs, but +HVT-IBD BT birds developed significantly higher numbers of GC in the spleens of the birds compared to –HVT-IBD controls ($P < 0.05$), which might indicate a higher immunoreactive response to the vaccination as well.

After IBDV infection and also vaccination CD4+, CD8+ T-cells and macrophages infiltrate into various lymphoid organs, especially the BF, as the target organ of the virus (Withers et al., 2006). Administration of recombinant HVT-IBD vaccines was shown to have a minor effect on the number of circulating and intrabursal T-cells (Rautenschlein et al., 2011) or might affect the numbers of CD8+ T-cell populations in the spleen (Ingrao et al., 2018). In the present study, CD4+ T-cell numbers reached higher levels in the BF in all the HVT-IBD vaccinated groups compared to non-vaccinated controls, which were only significant in DT chickens. In future studies earlier sampling time points during the first week post vaccination would be interesting to investigate. Significant differences between –HVT-IBD and +HVT-IBD groups in the number of CD4+ T-cells might have disappeared over time. In the spleen significantly higher numbers of macrophages were determined in +HVT-IBD BT and DT chickens at 14 dph. Therefore, our study suggests that depending on the chicken genotype and age of the chickens, the HVT-IBD vaccine might have a varying degree of impact on the number of immune cell populations.

The influence of HVT-IBD on the CMI and macrophage activity in spleen cell cultures was investigated in the present study. *In vitro* lymphocyte proliferation assays demonstrated that an infection or vaccination with IBDV might lead to a depression in the responsiveness of T-cells (Confer and MacWilliams, 1982; Corley and Giambrone, 2002; Rauw et al., 2007). In our study, no differences between +HVT-IBD and –HVT-IBD control groups in the CMI were observed, as measured by IFNγ release of spleen lymphocytes. We might suggest that HVT-IBD not only leads to a lower B-cell depression compared to other live vaccine against IBDV (Prandini et al., 2016), but might also not compromise the CMI. Except a higher number of macrophages in the spleen in +HVT-IBD BT and DT chickens, *in vitro* LPS stimulated splenic leukocytes of these groups didn't show an increased release of NO. This might be due to a loss in

viable cells during the splenic leukocyte isolation protocol. Therefore, it can be concluded that the HVT-IBD live vaccine might not prime or stimulate spleen leukocytes to release higher levels of NO, as it was demonstrated for other live virus vaccines (Liman and Rautenschlein, 2007; Rautenschlein and Sharma, 1999) or stimulatory effects might have disappeared over time. Therefore, earlier sampling time points might be taken into account.

Finally, our study clearly demonstrated a genotype effect on the developing immune system. These effects were observed for the parameters immune organ development, antibody response as well as IFNγ release after ConA stimulation and macrophage activation after LPS stimulation. For all the investigated parameters, BT birds showed the most vigorous responses compared to LT birds, while DT birds take an intermediate position. Based on our study we suggest considering the selection for immune related traits in performance tests of pure line chickens to further investigate if these traits could be used as potential selection traits. Early HVT-IBD vaccination at ED 18 affected various investigated immune parameters including the number of GC in the spleens, CD4+ T-cell numbers in the BF and macrophage populations in the spleens and the anti-IBV and anti-NDV antibody responses. Furthermore, it was demonstrated for the first time that the immune responses after HVT-IBD vaccination vary between genotypes. Subsequently we suggest that vaccination programs should be modified depending on the respective chicken genotype.

6.6 Acknowledgements

This project was part of the collaboration project “Integhof” and was supported by funds of the German Government’s Special Purpose Fund held at Landwirtschaftliche Rentenbank, Boehringer Ingelheim Vetmedica GmbH, Big Dutchman International GmbH, and Lohmann Tierzucht GmbH. Furthermore, we would like to thank Sonja Bernhardt, Melanie Bode and Martina Weding for their great help in animal care. For the excellent technical support we would like to thank Christine Haase and all other doctoral students at the Clinic for Poultry.

6.7 Figures and Tables

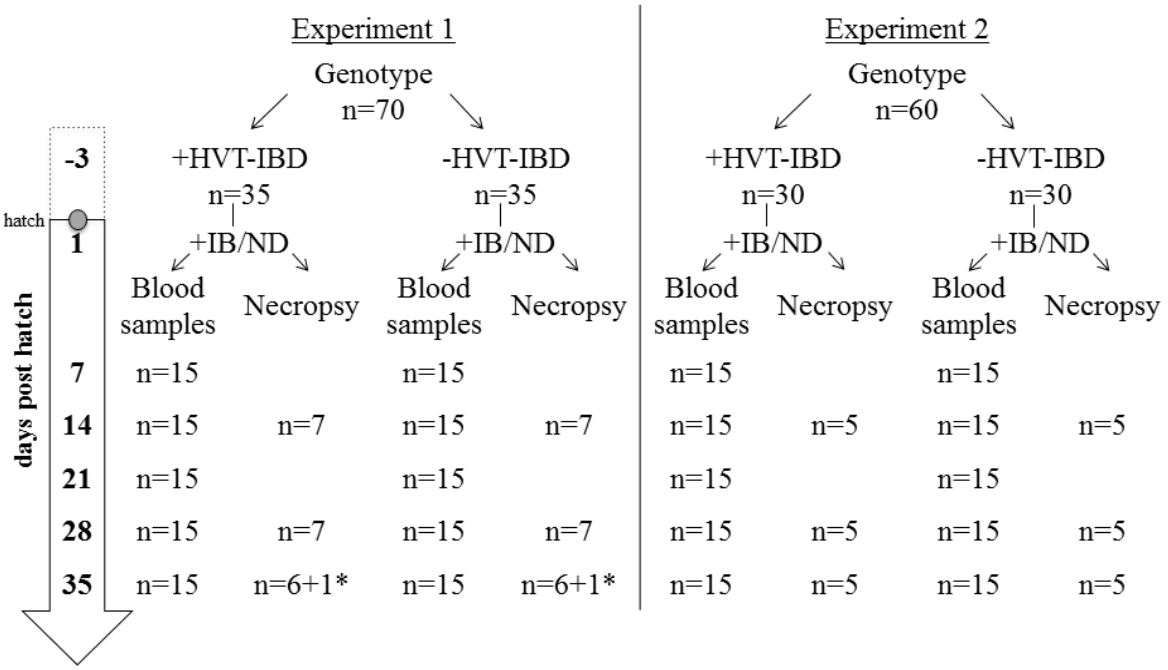


Fig. 1. Demonstration of the experimental setup and assignment of the different groups per genotype in Exp. 1 and Exp. 2. *1 animal from the blood sampling group was used for the collection of spleen samples.

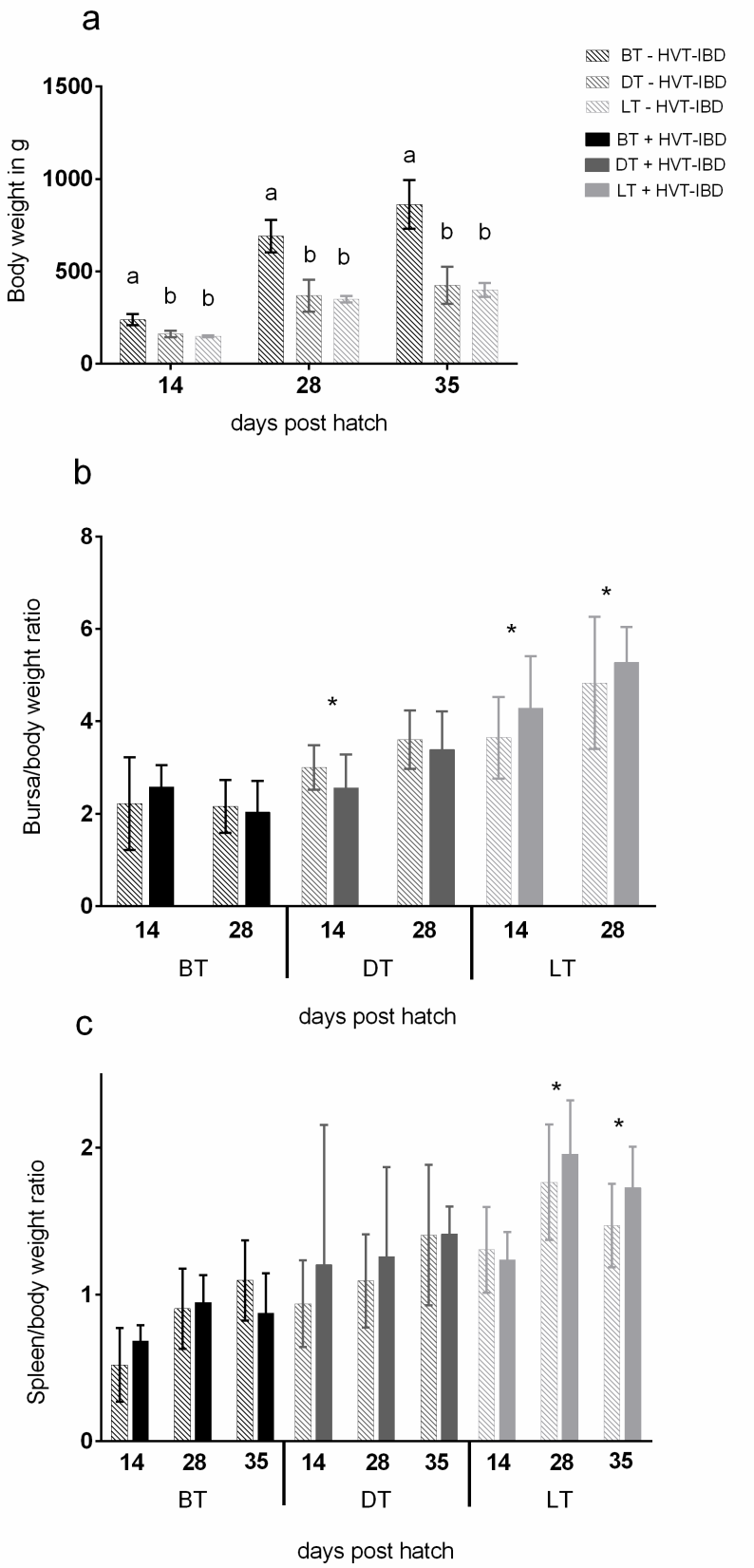

a
BT - HVT-IBD
DT - HVT-IBD
LT - HVT-IBD
BT + HVT-IBD
DT + HVT-IBD
LT + HVT-IBD
Body weight in g
1500
1000
500
0
a
b
b
a
b
b
a
b
b
14
28
35
days post hatch
b
Bursa/body weight ratio
8
6
4
2
0
*
*
*
14
28
14
28
14
28
BT
DT
LT
days post hatch
c
Spleen/body weight ratio
2
1
0
*
*
14
28
35
14
28
35
14
28
35
BT
DT
LT
days post hatch

Fig. 2. Body weight and relative organ weight development in BT, DT and LT chickens up to 35 dph. (a) Body weight at days ph in Exp. 2 as a representative experiment of non-vaccinated control groups; (b) Bursa/body weight ratios at days ph of +HVT-IBD groups (filled bars) and –HVT-IBD controls (open bars); (c) Spleen/body weight ratios at days ph of +HVT-IBD groups (filled bars) and –HVT-IBD controls (open bars). Data of Exp. 1 and Exp. 2 are presented as a summary of both experiments in in b and c. Data are expressed as means of n=5 (a) or n=12 (b) and (c) birds per group and day ± SD. * indicate significant differences between the different genotype groups in (a) and indicates significant differences between +HVT-IBD and –HVT-IBD control groups in (b) and (c) (One Way ANOVA Test, Two Sample T-test, both with $P < 0.05$).

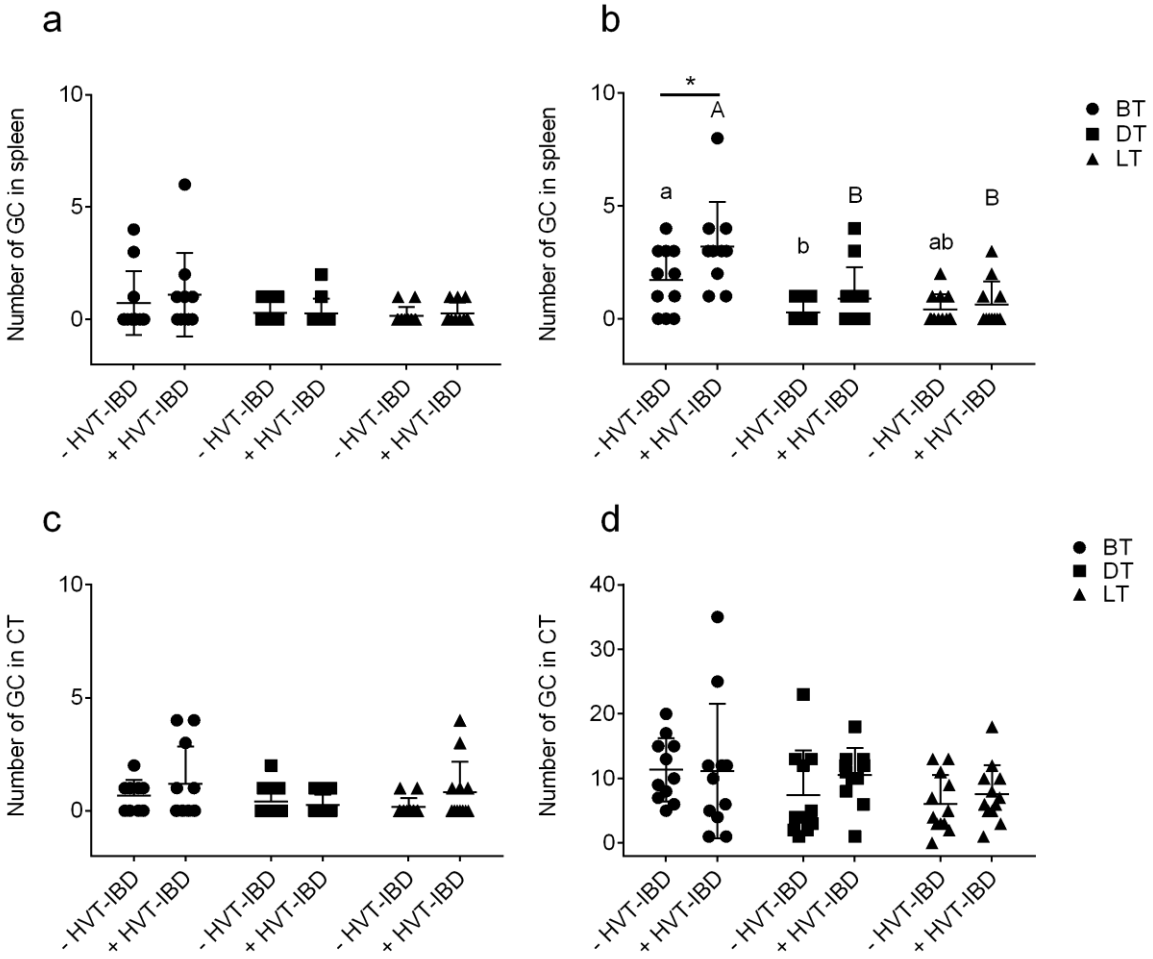
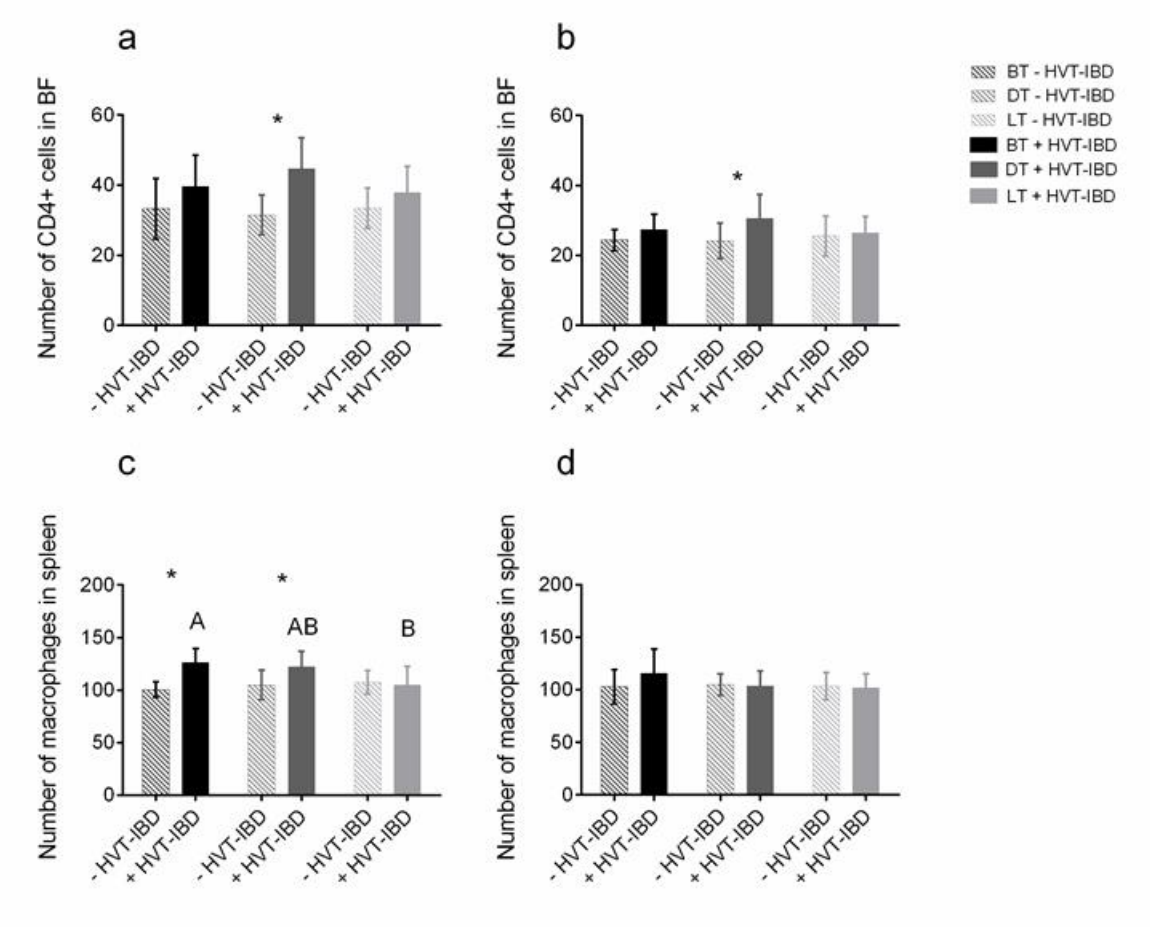


Fig. 3. Histological determination of the numbers of GC in the spleens (a + b) and CT (c + d) of BT, DT and LT chickens (n=12 birds/group) after HVT-IBD vaccination (+ HVT-IBD) or placebo inoculation (-HVT-IBD) at 14 dph (a + c) and 28 dph (b + d). Data of Exp. 1 and Exp. 2 are presented as a summary of both experiments. GCs were counted for each bird of one group in 3 different randomly chosen microscopic fields at a magnification of 100x. [a, b] indicate significant differences between –HVT-IBD control groups and [A, B] indicate significant differences between +HVT-IBD groups (Kruskal Wallis One Way ANOVA, $P < 0.05$). * indicate significant differences between +HVT-IBD and –HVT-IBD groups within one genotype (Wilcoxon Rank Sum Test, $P < 0.05$).


a
b
c
d
Number of CD4+ cells in BF
Number of macrophages in spleen
BT - HVT-IBD
DT - HVT-IBD
LT - HVT-IBD
BT + HVT-IBD
DT + HVT-IBD
LT + HVT-IBD
- HVT-IBD
+ HVT-IBD
A
AB
B

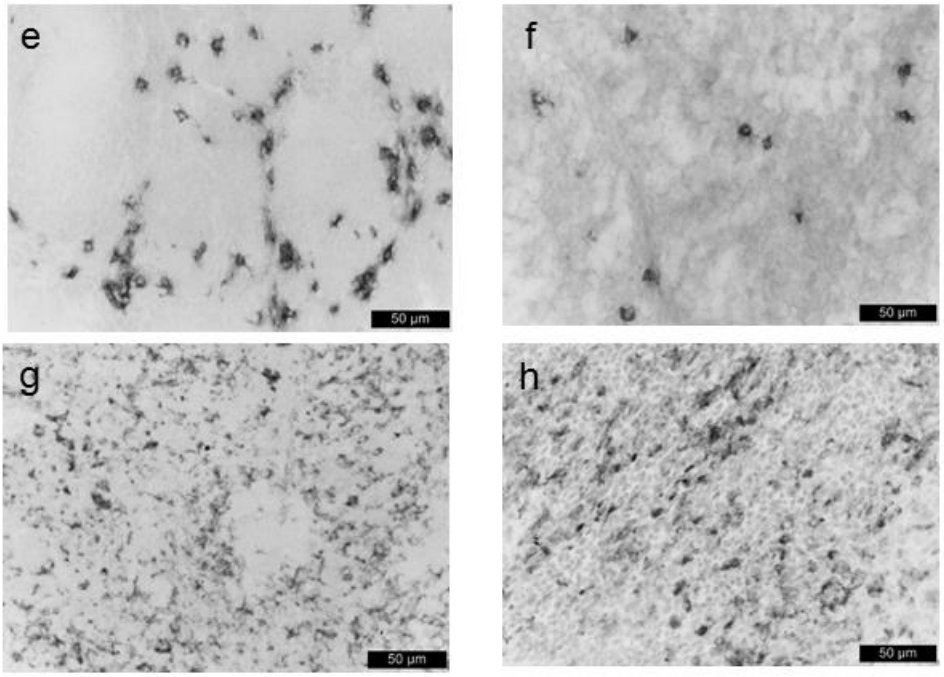

e
f
g
h
50 µm

Fig. 4. Immunohistochemical determination of CD4+ T-cells in the BF at 14 (a) and 28 dph (b) and macrophages in the spleen at 14 (c) and 28 dph (d) after HVT-IBD vaccination (+HVT-IBD) or placebo inoculation (-HVT-IBD). Data of Exp. 1 and Exp. 2 are presented as a summary of both experiments. e - h show representative staining of either CD4+ T-cells in the BF of DT +HVT-IBD (e) and –HVT-IBD (f) groups or macrophages of BT +HVT-IBD (g) and –HVT-IBD (h) groups at 14 dph. Immune cells were counted for each bird per group in 3 different randomly chosen microscopic fields at a magnification of 400x. ^{A,B} indicate significant differences between the genotypes of +HVT-IBD groups (Kruskal-Wallis One Way ANOVA, $P < 0.05$). * indicate significant differences between +HVT-IBD and the –HVT-IBD control groups (Wilcoxon Rank Sum Test, $P < 0.05$).

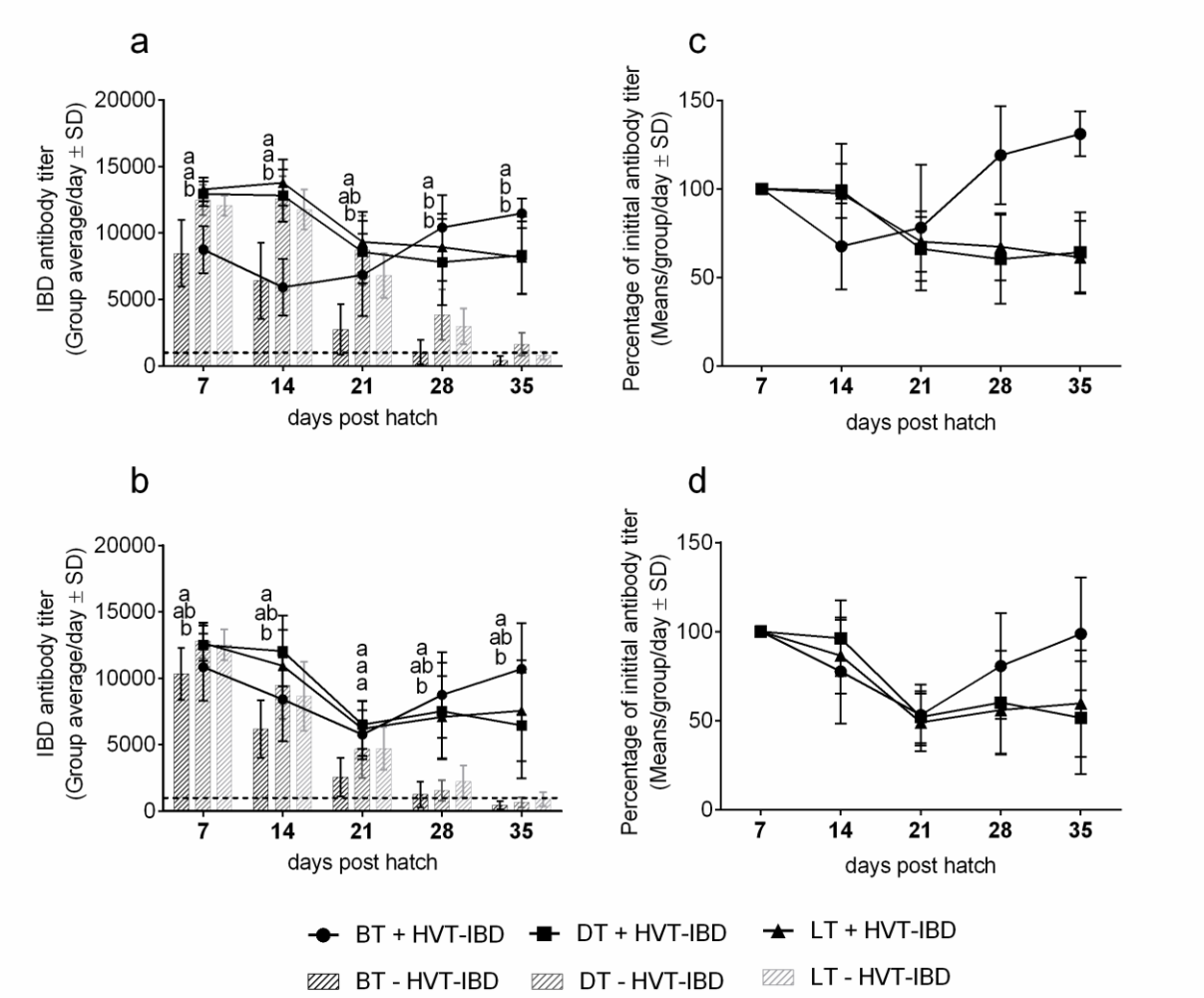


Figure 5. Anti-IBDV antibody titers after *in ovo* HVT-IBD vaccination in Exp. 1 (a+c) and Exp. 2 (b+d). Lines represent the HVT-IBD vaccinated groups (+ HVT-IBD), bar graphs show the corresponding non-vaccinated control groups (-HVT-IBD). c+d show the anti-IBD antibody development of +HVT-IBD groups as a percentage of the initially measured antibody titer at 7 dph per genotype. The initially measured mean antibody titer per genotype group was defined as 100%. Data are expressed as means of 15 birds per group and day ± SD. ^{a,b} indicate significant differences between the genotypes of +HVT-IBD groups at days ph (Kruskal-Wallis One-Way ANOVA or One-Way ANOVA test ($P < 0.05$)). The dotted line shows the Cut-off value of the ELISA.

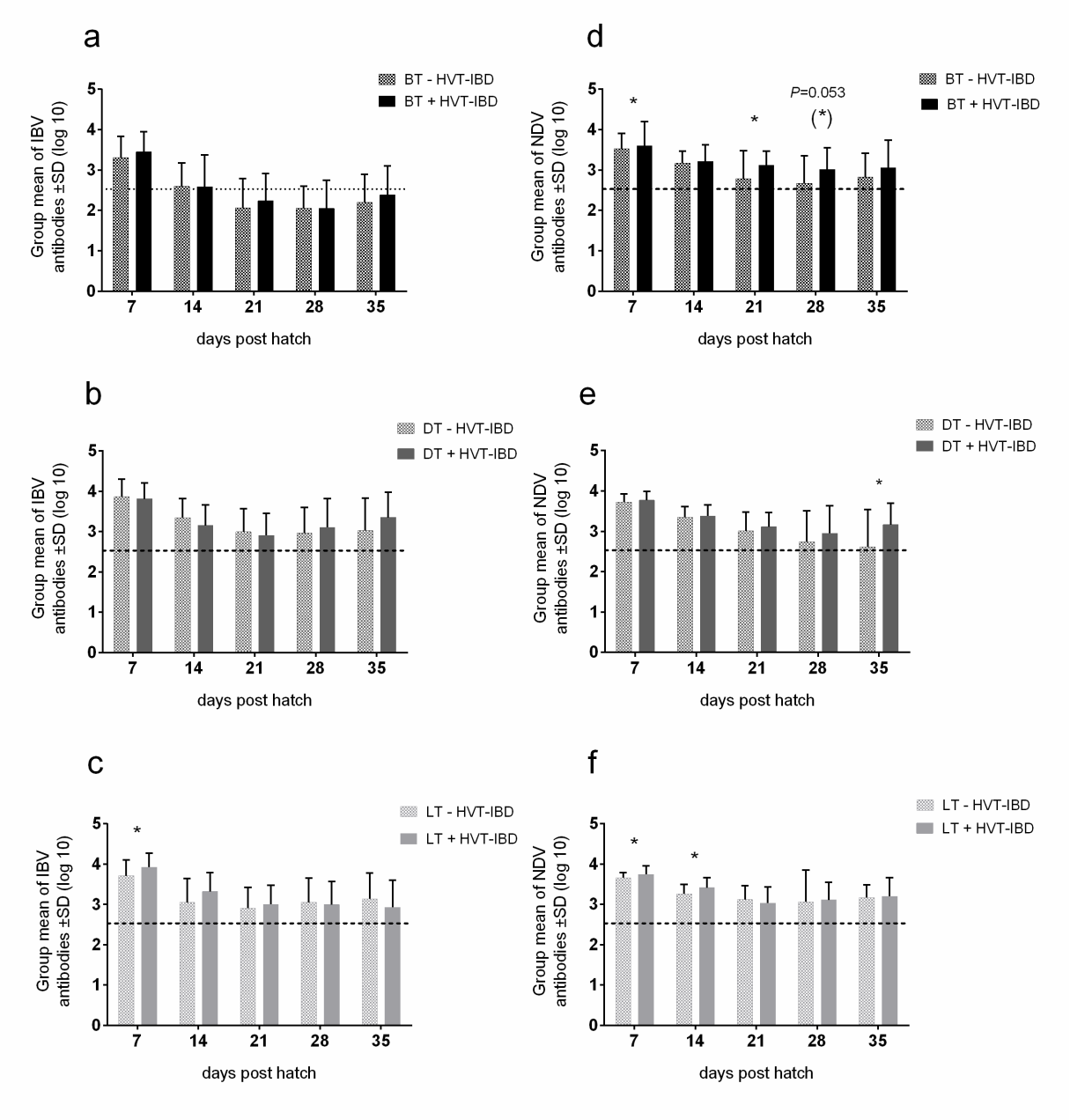


Figure 6. Anti-IBV antibody titers (a, b, c) and anti-NDV antibody titers (d, e, f) of HVT-IBD vaccinated (+HVT-IBD) and non-vaccinated control groups (- HVT-IBD). (a+d) BT chickens, (b+e) DT chickens, (c+f) LT chickens. Data are expressed as means of 30 birds per group and day ± SD and presented as a summary of Exp. 1 and Exp.2 (n=15 birds per experiment). * indicate significant differences between +HVT-IBD and –HVT-IBD control groups (Wilcoxon Rank Sum Test ($P < 0.05$).The dotted line shows the Cut-off value of the ELISA.

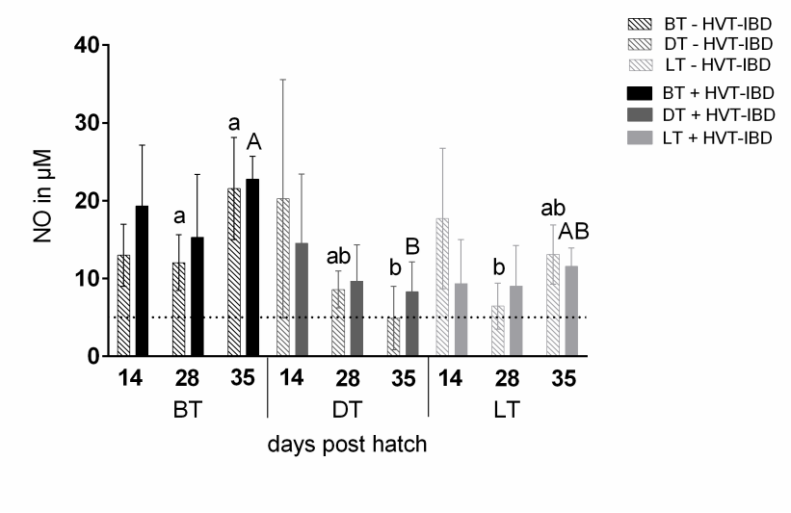


Figure 7. NO release in µM after *ex vivo* stimulation of spleen cultures of HVT-IBD vaccinated birds (+HVT-IBD) and control groups (- HVT-IBD) of Exp.2. Data are presented as means of 5 birds per group and day ±SD. ^{A,B} indicate significant differences between genotypes of +HVT-IBD groups per day (Kruskal-Wallis One Way ANOVA, $P < 0.05$). ^{a,b} indicate significant differences between –HVT-IBD control groups per day (Kruskal-Wallis One Way ANOVA, $P < 0.05$).

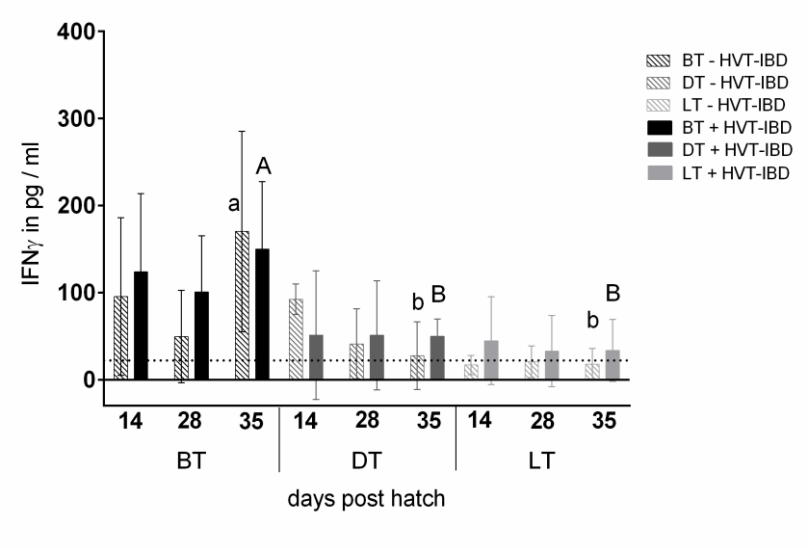


Figure 8. IFNγ release in pg/ml after *ex vivo* stimulation of spleen cultures of HVT-IBD vaccinated (+ HVT-IBD) and non-vaccinated control groups (-HVT-IBD) of Exp.2. Data are presented as means of 5 birds per group and day ± SD. ^{A,B} indicate significant differences between genotypes of +HVT-IBD groups per day (Kruskal-Wallis One Way ANOVA, $P < 0.05$). ^{a,b} indicate significant differences between –HVT-IBD control groups (One-Way ANOVA test, $P < 0.05$).

Table 1. *P*-values for different factors influencing the number of lymphocyte populations in the BF and spleen as well as numbers of GC in the spleen.

Tissue	Effect	*P*-values for		
		GC	Lymphocyte populations	
			CD4+	macrophages
BF	Genotype*		0.18	
	HVT-IBD**		< 0.001	
	Age***		< 0.001	
	Genotype x HVT-IBD		0.02	
Spleen	Genotype*	< 0.001		0.09
	HVT-IBD**	0.03		0.001
	Age***	< 0.001		0.02
	Genotype x HVT-IBD	0.23		0.003
CT	Genotype*	0.04		
	HVT-IBD**	0.34		
	Age***	< 0.001		
	Genotype x HVT-IBD	0.71		

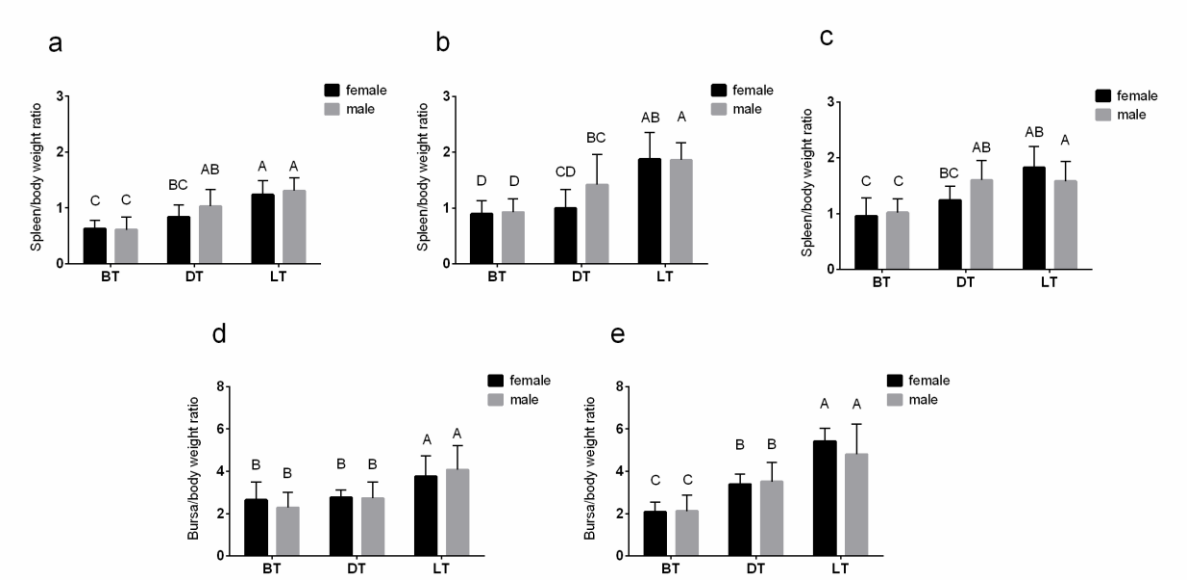
(*) independent of vaccination and age
(**) independent of genotype and age
(***) independent of genotype and vaccination

Table 2. *P*-values for different factors influencing the release of NO and IFNγ of *ex vivo* spleen cell cultures.

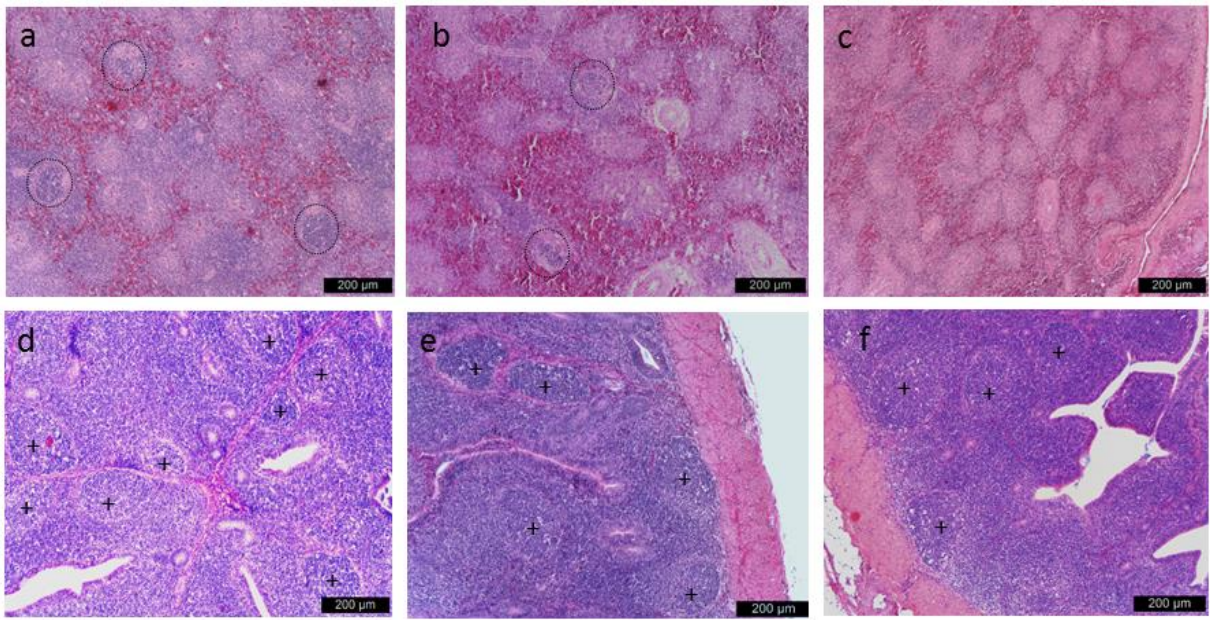
Effect	*P*-values for	
	NO	IFNγ
Genotype*	0.001	< 0.001
HVT-IBD**	0.86	0.45
Age***	0.01	0.2
Genotype x HVT-IBD	0.22	0.76
Genotype x Age	0.01	0.08

(*) independent of vaccination and age
(**) independent of genotype and age
(***) independent of vaccination and genotype

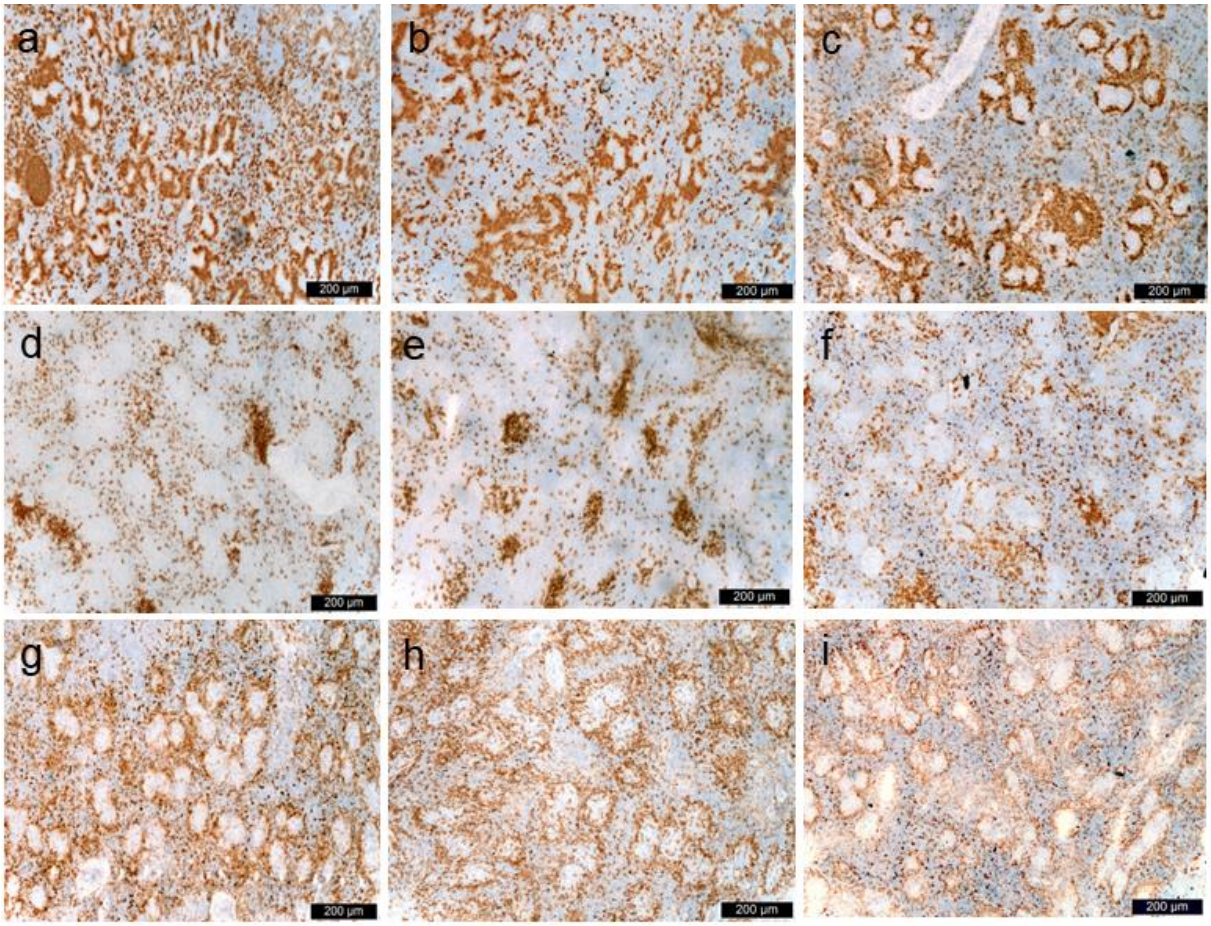
6.8 Supplementary Material



S1 Figure. Influence of the genotype and sex of birds (Genotype x Sex) on the relative spleen (a –c) and bursal weights (d +e) in BT, DT and LT chickens at 14 (a + d), 28 (b + e) and 35 dph (c). The data are presented as a summary of both experiments. +HVT-IBD and –HVT-IBD groups are considered as one group for the demonstration of sex-related differences in relative spleen weights between the chicken lines. ^{A,B,C,D} indicate significant differences between groups (One-Way ANOVA test, $P < 0.05$).



S2 Figure. Representative pictures of the histological detection of GC in the spleens (a – c) and CT (d – f) of +HVT-IBD BT (a + d), DT (b + e) and LT birds (c + f) at 28 days post hatch at a magnification of 100x. For the spleens the GC are encircled by a dotted line (a – c). (+) indicate the GC in the CT (d – f).



S3 Figure. Immunohistochemical detection of different immune cell populations in the spleens of the chickens at a magnification of 100x. Positively stained cells are shown in a brown colour (DAB-substrate). a – c demonstrate the immunohistochemical staining of B-cells (Bu-1+) in BT (a), DT (b) and LT (c) +HVT-IBD chickens at 14 dph. d – f demonstrate the immunohistochemical staining CD4+ T-cells in BT (d), DT (e) and LT (f) +HVT-IBD chickens at 14 dph, forming the PALS in the spleens. g – i demonstrate the immunohistochemical staining of macrophages (KUL01+) in BT (g), DT (h) and LT (i) +HVT-IBD chickens at 14 dph, forming the PELS in the spleens.

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7. Genotype-associated differences in bursal recovery after infectious bursal disease virus (IBDV) inoculation

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MD, MA and SR conceived and designed the experiments; MD performed the experiments; MD analyzed the data; MD and SR wrote the paper. All authors read and approved the final manuscript.

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Data analysis: 90 %
Scientific writing: 80%

7.1 Abstract

T-cell immune responses were shown to play an important role in the regulation of infectious bursal disease virus (IBDV) replication and development of lesions in the bursa of Fabricius (BF) (bursal lesions) but also in the recovery from the infection. Studies suggested that the host-genotype influences T-cell responses during the acute phase of infection. Genotype-related differences in the recovery phase were not investigated so far. The present study used commercial broiler- (BT), layer- (LT), dual-purpose type (DT) chicken lines as well as a specific pathogen free (SPF) LT chicken as a reference for comparison of T-cell related differences in IBDV-immunopathogenesis not only in the early phase post inoculation (pi) but also in the recovery phase. The Deventer formula was used to determine the optimal time point of inoculation with an intermediate plus IBDV strain when maternally derived antibody (MDA) titers were below the calculated breakthrough level of the virus for all genotypes. Differences in the bursal lesion development, intrabursal CD4+ and CD8+ T-cell accumulation and numbers of IBDV-positive cells were determined. In addition, anti-IBDV antibody development and the relative amount of anti-inflammatory cytokine mRNA were recorded until 28 days post IBDV inoculation. Differences between the genotypes were observed in the duration and magnitude of bursal lesions, CD4+ and CD8+ T-cell infiltration as well as the presence of anti-inflammatory Interleukin (IL)-10 and Transforming growth factor (TGF) β4 cytokine mRNA ($P < 0.05$). While the investigated immune parameters were comparable between the genotypes at seven days pi, during 14, 21 and 28 days pi a delayed recovery process in LT and DT chickens compared to BT chickens was observed ($P < 0.05$). Furthermore, the age and residual MDA levels had a genotype-dependent influence on the onset of the anti-IBDV specific humoral and T-cell mediated immune responses. This study suggests, that the impact of T-cell immunity on the recovery process after IBDV infection may need to be considered further for the development of new breeding programs for disease resistant chicken lines.

7.2 Introduction

Gumboro disease, caused by the infectious bursal disease virus (IBDV), is a worldwide distributed, highly contagious immunosuppressive disease in chickens, which can lead to great economic losses in the poultry industry. Currently, besides biosecurity measures vaccination programs are important tools to control IBD outbreaks in commercial chicken flocks (Müller et al., 2012; Ingrao et al., 2013).

Several studies demonstrated the impact of the chicken genotype and specifically the major-histocompatibilty complex (MHC)-haplotype on the resistance and susceptibility to viral, bacterial and also parasitic infections (Caron et al., 1997; Guillot et al., 1995; Lavi et al., 2005; White et al., 1994). Concerning IBDV infection, it was reported that some genotypes might be more resistant than others. The mortality rates can differ significantly between chicken lines and it was suggested that the MHC-haplotype might be associated with differences in the anti-IBDV antibody production after IBDV vaccination (Bumstead et al., 1993; Juul-Madsen et al., 2002, 2006). Experimental studies as well as field observations indicated that layer-type chickens may develop a more severe clinical disease and higher mortality rates compared to broiler-type chickens (Winterfield and Thacker, 1978; van den Berg et al., 1991; Bumstead et al., 1993).

The severity of IBDV infection might be associated with the intensity of the innate immune response during the acute phase of the infection (van den Berg, 2000; Rauw et al., 2007; Rauf et al., 2011). Direct and indirect activation of T-cells and macrophages may lead to the release of different pro-inflammatory cytokines. The composition of this so-called “cytokine storm”, is suggested to determine the outcome of the disease (Rauw et al., 2007). T-helper 1 (TH-1) cytokines, such as interferon (IFN)-γ, released by bursal associated T-cells, can stimulate macrophages to further produce pro-inflammatory cytokines, such as interleukin (IL)-6, IL-1β and CXCLi2 (Eldaghayes et al., 2006). Therefore, infiltrating T-cells seem to play an important role in the inflammatory process, virus clearance and bursa lesion development but also the recovery process after IBDV infection (Kim et al., 1999; Kim et al., 2000; Rauf et al., 2011; Rautenschlein et al., 2002). Studies demonstrated differences between

chicken lines in the onset and extent of virus replication, in the magnitude of immune cell infiltration and also in the expression of pro-inflammatory cytokines (Moody et al., 2000; Aricibasi et al., 2010; Ruby et al., 2006). Therefore, we may speculate that these differences would be related to genotype-associated variations in the T-cell response and its regulation (Tippenhauer et al., 2013).

Specific subsets of T-cells, the regulatory T-cells (Tregs), which possess immune regulatory properties, have been well studied in mammals (Dieckmann et al., 2001; Jonuleit et al., 2001; Hori et al., 2003). Tregs act as suppressor cells by controlling activated immune cells during infections to protect the organism against an inflammatory overreaction (Workman et al., 2009). Specifically the expression of IL-10 and also transforming growth factor β (TGFβ) were correlated with the suppressor function of Tregs (Dieckmann et al., 2001; Shanmugasundaram and Selvaraj, 2011). In murine models, it was demonstrated that IL-10 plays a role as an immune regulatory cytokine in viral, bacterial and parasitic infections (Moore et al. 2001; Couper et al., 2008; Rojas et al., 2017). Wu et al. (2016) demonstrated that chicken IL-10 is an effective inhibitor of IFNγ expression and nitric oxide (NO) production by macrophages, as it was demonstrated before in mammals (Couper et al., 2008). Furthermore, IL-10 has also been associated with disease resistance and susceptibility to different infectious diseases in mammals as well as protozoal infections in chickens (Moore et al., 2001; Rothwell et al., 2004).

Many studies mainly addressed the role of T-cells in IBDV pathogenesis during the acute phase of infection. But not much information is available on genotype-related differences in the recovery phase and on the role of anti-inflammatory, Treg-related cytokines, which may determine the duration of bursal lesions and possibly immunosuppression.

Therefore, the goal of this study was to determine genotype-associated differences specifically during the IBD-recovery phase after inoculation with an intermediate plus IBDV strain. We compared three different chicken genotypes, a layer-type (LT), boiler-type (BT) and a slow-growing dual-purpose type (DT) chicken up to 28 days post IBDV inoculation in bursal lesion development and virus clearance. Furthermore, the

development of humoral as well as T-cell related immune responses, such as intrabursal infiltration of T-cell subsets and the expression of Treg-related anti-inflammatory cytokines (IL-10 and TGFβ4) were investigated. SPF LT chickens served as a control, as these birds are known to be highly susceptible to IBDV-Infection and development of bursal lesions (Bumstead et al., 1993).

7.3 Material and Methods

Experimental chickens

Embryonated eggs of three different commercially available chicken genotypes (broiler-type (BT) = Ross 308, layer-type (LT) = Lohmann Brown+, dual-purpose type (DT) = Lohmann Dual) were provided by the BWE Brüterei Weser-Ems (BT), the Geflügelzuchtbetrieb Gudendorf-Ankum (LT) and Lohmann Tierzucht GmbH, Cuxhaven (DT), respectively. Specific pathogen free (SPF) White Leghorn chickens were used as a reference genotype (VALO Biomedia GmbH, Osterholz-Scharmbek, Germany). Birds were hatched and raised together at the Clinic for Poultry in groups of 60 birds per genotype. Later on, each genotype group was divided into two groups of 24 birds each with one group serving as the experimental and the other group serving as the respective control group. The chickens were housed in floor pens with wood shavings and had free access to food and water from the first day post hatch on. All animals were fed with the same commercial chicken diet (allmash-A, deuka, Deutsche Tierernährung Cremer GmbH). All experiments and procedures were authorized by the State Office for Consumer Protection and Food Quality of Lower Saxony (LAVES; approval number: 33.12-42502-04-15/1827) in Germany.

Experimental setup

Two experiments were conducted. At seven days of age, blood samples of ten birds per genotype were collected to determine the maternally derived anti-IBD antibodies (MDA) by a commercially available ELISA system. The Deventer formula was used to calculate the time point for IBDV-inoculation, when IBD MDA levels of all genotype groups had decreased below the respective breakthrough level of the virus strain used (Eterradossi and Saif, 2013). One day before the calculated time point of inoculation, blood samples were tested again for IBD MDA levels to confirm the inoculation time point. At 28 days post hatch (dph) in Exp. 1 and at 35 dph in Exp. 2, the groups were then divided into sub-groups of n=24 birds per genotype. A commercially available intermediate plus IBDV strain was used to orally inoculate the birds of the experimental groups (iIBDV groups) with one vaccine dose per bird. The vaccine was prepared

following the manufacturer's instructions and was administered with an irrigation/blunt cannula directly into the crop of the birds. Control groups received only the diluent of the vaccine (water). Birds were checked daily for clinical signs after vaccination. At seven, 14, 21 and 28 days post infection (pi) necropsy was conducted (n=6 birds/group) to investigate for the presence of macroscopic lesions and to collect tissue samples for both histological and immunohistochemical examinations as well as quantitative real-time (qRT)-PCR analysis.

Histological examinations

During the necropsies, tissue samples of the bursa of Fabricius (BF) were collected and fixed in 10% phosphate buffered formalin and stored at 4°C until use. Tissues were cut into 2-3 µm thick sections and stained with haematoxylin and eosin (H&E) following standard procedures. Bursal samples were microscopically evaluated for virus induced bursal lesions, using the following scoring system: 0=no lesions, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100% of follicles showing 50% of cell depletion) (Sharma et al., 1989). In addition, 100 bursal follicles of each bird of each genotype (either control or infected) were screened microscopically at a magnification of 400x for the quality of bursal lesions, such as lymphocyte depletion, destruction of bursal tissue structure, the presence of cystic cavities in the bursal medulla, the presence of cellular debris and heterophil infiltration.

Detection of IBDV by immunohistochemistry

The center of the tissue samples was cut into 2-3 µm thick sections covering a representative cross section of the tissue and subsequently stained for IBDV-antigen by using the Universal Vectastain® *Elite* HRP Kit (Vector Laboratories Inc., LINARIS Biologische Produkte GmbH, Dossenheim, Germany), which provided the secondary horse anti-mouse/rabbit IgG antibody and the biotinylated horseradish peroxidase (HRP) enzyme. An in-house polyclonal rabbit anti-IBDV serum was used to detect the virus antigen (Rautenschlein et al., 2007a, b) and the (3,3'-diaminobenzidine) DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) was used for visualization of the binding of the antibodies. IBDV-antigen positive cells were counted in three randomly

chosen microscopic fields at a magnification of 400x and the results are presented as group means of IBDV-antigen positive cells of six birds per group and time point ± standard deviation (SD) (Rautenschlein and Haase, 2005; Rautenschlein et al., 2007b; Tippenhauer et al., 2013).

Immunohistochemical detection of immune cells

Tissue samples of the BF were collected at necropsy in tissue freezing medium (Leica Biosystems, Tissue Freezing Medium, Wetzlar, Germany) and immediately snap frozen in liquid nitrogen and stored at -80°C until use. The tissue samples were cut into 4-5 µm thick sections to achieve a representative cross-section of the organ at -28°C and processed as previously described (Dobner et al., 2019; Han et al., 2016). Monoclonal mouse anti-chicken primary antibodies for the detection of intrabursal CD4+ (clone CT4) and CD8+ (clone EP42) T-cells as well as B-cells (Bu-1) were purchased from Southern Biotech (Birmingham, AL, USA) and used at a dilution of 1:500. The Vectastain® *Elite* ABC HRP Kit (Vector Laboratories Inc.) provided the secondary, biotinylated goat-anti-mouse IgG Fc and biotinylated horseradish peroxidase (HRP) enzyme. In a last step the (3,3'-diaminobenzidine) DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) was used for visualization. Mouse IgG_1 and mouse $IgG_{2\alpha}$ antibodies (Southern Biotech) were used as isotype controls to check the specificity of the primary antibodies. CD4+ and CD8+ positively stained cells were counted in three randomly chosen microscopic fields per bird at a magnification of 400x. Data are expressed as the means of six birds per group and time point ± SD. For the quantification of B-cell depletion and repopulation of the BF 100 follicles were investigated for the percentage of Bu-1 positive cells (Kim et al., 1999). By determining the percentage of Bu-1 positively stained cells for the 100 investigated follicles, a score was assigned to each bursal sample (0=100%, 1=99-75%, 2=74-50%, 3=49-25%, 4=24-0% Bu-1 positively stained cells in 100 microscopically investigated follicles per bird at a magnification of 100x.

Anti-IBDV antibody detection by ELISA

For the detection of circulating IgY specific anti-IBD antibodies, serum samples were investigated with a commercially available ELISA kit (Synbiotics IBD ProFLOK+®,

Synbiotics Corporation, San Diego, CA, USA). Following the manufacturer's instructions of the ELISA kit, sera were tested for anti-IBDV specific antibody levels per group. In a next step the mean antibody titer and ± SD were calculated. The "optimal time of vaccination" was calculated by the Deventer formula for each genotype (de Wit, 2001). The half-life time of chicken antibodies for LT and DT chickens was set at 5.5 days and for 3.5 days for BT chickens.

Detection of intrabursal cytokine mRNA expression

BF samples were collected in RNAlater solution (Merck KGaA, Darmstadt, Germany). RNA isolation was performed using the Masterpure™ RNA Isolation Kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) following the manufacturer's instructions. Purified RNA was resuspended in 100µl Tris-EDTA (TE) -buffer and the RNA concentration and quality for each sample was measured by the Nanodrop ND-1000 (PeqLab, Biotechnologie GmbH). RNA samples were stored at -80°C until use. Each sample was thawed once and diluted to a concentration of 50-80 ng/µl with RNAse/DNAse free water immediately before the qRT-PCR.

The qRT-PCR was performed in 10µl of TaqMan® RNA-to-C_T™ 1-Step Master Mix (Thermo Fisher Scientific, Darmstadt, Germany) with the QuantStudio 3 Cycler (Thermo Fisher Scientific, Darmstadt, Germany). The primer and probe sequences for the detection of IL-10, TGFβ4 and 60S ribosomal protein L13a (RPL-13, housekeeping gene) are shown in Table 1. In preliminary experiments the mRNA expression of RPL-13 was tested for its stable and comparable expression in IBDV-inoculated and control bursal samples (data not shown). All samples were tested in duplicates in 96-well plates (MicroAmp® Optical 96-well Reaction Plate, Thermo Fisher Scientific, Darmstadt, Germany). For each sample all three target genes were analyzed in the same plate. Negative and internal controls were also tested in duplicates and integrated on every plate. The cycling parameters were the following: 48°C for 15 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The passive reference dye 6-carboxy-x-rhodamine (ROX) was used for the normalization of fluctuations in fluorescence levels. Standard curves for each target gene were

generated by regression analysis of the mean values of four replicates per dilution ranging from 1:5-1:625. For each sample the obtained Ct-value was normalized to the housekeeping gene RPL-13. The data are expressed as the mean 40-Ct_{norm} values of six birds per group and time point ±SD.

Statistical analysis

All statistical analyses were carried out with the program Statistix 10.0 (Analytical software, Thallahassee, FL, USA). A normal distribution of all data was considered before starting the analyses of the data. Differences between the genotype groups for each time point were analyzed using One-way analysis of variance (Completely Randomized Design) or Kruskal Wallis all-pairwise comparisons test. The Two Sample *t*-test was used for the comparison of IBDV-inoculated (iIBDV) and the corresponding control groups (c) for each genotype group and time point. Bursal lesion scores and B-cell depletion scores were determined for each time point and compared using the Chi-Square Test. Generally, $P < 0.05$ was considered as statistically significant. For the comparison of the numbers of T-cell populations in the BF between IBDV-infected and control groups $P < 0.01$ was considered as statistically significant.

7.4 Results

Anti-IBD antibody development

All non-inoculated control groups showed a decrease of anti-IBDV-MDA throughout both experiments. SPF chickens were anti-IBDV-MDA negative at the time of virus inoculation and the SPF control groups stayed anti-IBDV antibody negative throughout both experiments. Groups of iIBDV SPF chickens showed an increase in anti-IBDV antibody titers starting at 7 dpi in both conducted experiments. The titers increased until the end of the experiments at 28 dpi (Fig. 1).

Due to a different age of the parent flocks, anti-IBDV-MDA titers in BT, DT and LT chickens differed between Exp. 1 and Exp. 2 and were lower for all genotypes in Exp. 2 (S1 Table). The Deventer formula was used to calculate the optimal time of vaccination when anti-IBDV-MDA reached the calculated breakthrough level of the vaccine for all genotypes (S1 Table). The calculated time points were different for the genotypes, therefore day 28 and 35 were selected for Exp. 1 and 2, respectively to inoculate all birds at the same age (S1 Table). Serology at the day of inoculation indicated that in Exp. 1, only anti-IBDV-MDA levels of BT chickens were below the breakthrough level of the vaccine (S1 Table). LT and DT chickens still had mean MDA-anti-IBDV levels above the breakthrough level of 2500 at 28 dph (S1 Table). The titers ranged from 2,952 – 14,753 in DT chickens and 1,659 – 11,177 in LT chickens. In the second experiment the calculated time points for inoculation based on serum samples collected at 7 dph were 12 dph, 16 dph and 18 dph for BT, LT and DT chickens, respectively. Serum samples were tested again for anti-IBDV MDAs at 27 dph. Again DT chickens still had mean anti-IBDV MDA levels between 207 – 8053. Therefore, the inoculation time point was postponed to 35 dph in Exp. 2. At the day of inoculation at 35 dph the mean anti-IBDV-MDA levels of BT and LT chickens were below the breakthrough level of the vaccine (Fig. 1). In DT chickens the mean anti -IBDV-MDA levels were lower than in Exp. 1 but still higher in average than the breakthrough level with titers ranging from 161 – 8366.

The onset of anti-IBDV antibody responses of BT and LT groups differed between the experiments (Fig. 1). Inoculated IBDV BT and LT groups in Exp. 2 showed an earlier anti-IBDV antibody response at 7 dpi compared to the iIBDV BT and LT groups in Exp. 1, which showed seroconversion at 14 dpi (Fig. 1 a, b, c, d). Inoculated IBDV DT groups showed seroconversion at 14 dpi in both experiments. Nevertheless, the anti-IBDV antibody response in iIBDV DT groups of Exp. 2 was much higher compared to the antibody response of the respective group in Exp. 1 (Fig. 1 g, h). The mean anti-IBDV antibody titers of iIBDV LT chickens were significantly higher compared to iIBDV BT and DT chickens at 14 dpi in Exp. 1 ($P < 0.05$). At 28 dpi in Exp. 1, mean anti-IBDV antibody titers of iIBDV LT groups were significantly higher compared to the mean titers of iIBDV DT chickens ($P < 0.05$). In Exp. 2 no significant differences were observed in the magnitude of the anti-IBDV antibody development between the iIBDV genotype groups ($P > 0.05$). iIBDV BT and LT groups developed significantly higher anti-IBDV antibody titers ($P < 0.05$) compared to their control groups at 7 dpi, which was differentto the iIBDV DT groups, which didn't show a seroconversion at this time point (Fig. 1 d, f, h).

Overall, we did see a significant influence of the genotype on the decline of maternally derived antibodies, which is additionally influenced by the vaccination protocol of the parent flocks. Furthermore, also the seroconversion was variable between the tested groups, again genotype but also maternally derived antibodies may have modified the course of antibody development.

IBDV antigen load in the BF

In iIBDV SPF groups the highest IBDV antigen load was observed at 7 dpi and subsequently decreased over time in both experiments. At 28 dpi high individual variations were observed within the SPF group (Table 2, Fig. 2).

Interestingly the antigen load and clearance of IBDV, as indicated by the absence of a signal, in the BF was comparable for most genotypes between the two conducted experiments. Interestingly, in the groups of the DT chickens more variation was observed with respect to the peak of antigen load and overall numbers of antigen-

positive cells. Over time, a similar decrease in the IBDV antigen load was observed between LT and BT chickens, whereas iIBDV LT and DT birds had still a higher IBDV antigen load in the BF compared to the iIBDV BT chickens at 28 dpi, which was significant in Exp. 1 (Table 2, $P < 0.05$).

In Exp. 1, iIBDV DT birds showed a delayed increase in the IBDV antigen load in the BF at 14 dpi compared to Exp. 2 and the other inoculated groups (Fig. 2, Table 2). In Exp. 2 the highest IBDV antigen load in iIBDV DT chickens was observed at 7 dpi, which was comparable to the other iIBDV genotypes and the subsequent decrease of IBDV antigen was comparable to the iIBDV LT chickens (Fig. 2).

Clinical signs and macroscopical examinations

No clinical signs were observed in either of the iIBDV groups throughout both experiments. The onset of gross lesions, such as bursal edema and mottled spleens were observed in all investigated chickens of the iIBDV groups at 7 dpi. Only one out of six iIBDV DT chickens in Exp. 1 showed a mottled spleen at 7 dpi compared to six out of six DT birds in Exp. 2 (data not shown).

An increase in the relative spleen weights was observed in all iIBDV genotype groups but was only significantly differing in iIBDV SPF and LT birds compared to the corresponding control groups at 7 dpi ($P < 0.05$, data not shown). All iIBDV genotype groups showed a significant bursal atrophy starting at 7 dpi ($P < 0.05$, Fig. 3), except the DT chickens in Exp. 1, which showed a delayed bursal atrophy starting at 14 dpi (Fig. 3).

Development of bursal lesions and recovery

The bursal samples were investigated for virus induced bursal lesions. At 7 dpi in Exp.1 and 2, all birds of all iIBDV genotypes, except DT chickens in Exp. 1, showed severe bursal lesions, such as lymphocyte depletion, destruction of the normal bursal tissue structure, cystic cavities in the bursal medullary area, presence of cellular debris and infiltration of heterophils (data not shown). In iIBDV SPF groups bursal lesions peaked at 7 dpi and continuously decreased over time in both of the conducted experiments

(Table 3, Fig. 2). Histological bursal lesion development of iIBDV BT and LT chicken groups was comparable for both conducted experiments. Chickens of the iIBDV BT group showed a lower bursal lesion score (BLS) with a higher variation within the group compared to iIBDV LT and SPF chickens at 7 dpi. The bursal lesions in iIBDV BT birds stayed at a lower level compared to iIBDV LT groups throughout the experiment. iIBDV LT groups had a comparable bursal lesion development to iIBDV SPF groups at the early time points after inoculation (7 dpi), but the bursal lesions stayed at a higher level compared to all the other iIBDV genotype groups (Table 3, Fig. 2). At 21 dpi the iIBDV LT group had significantly higher BLS compared to the iIBDV SPF group ($P < 0.05$, Table 3). In Exp. 1 iIBDV DT birds showed a delayed start of bursal lesions at 14 dpi but the further development was comparable to the iIBDV LT genotype groups. In Exp. 2 iIBDV DT birds also showed the highest BLS at 7 dpi compared to the other inoculated genotypes. The bursal lesions in iIBDV DT chickens decreased slightly faster compared to the lesions in LT chickens but decreased slower compared to the lesions in BT chicken groups.

Chickens of the iIBDV SPF groups showed a significant decrease of B-cell numbers at 7 dpi in both conducted experiments. Coinciding with the histological examinations of the study, B-cell numbers increased again with all SPF birds showing fully repopulated bursal follicles at 28 dpi (Table 3, S1 Figure).

The decrease of B-cell numbers in the BF of iIBDV BT and LT groups was comparable between Exp. 1 and 2. BT birds showed a faster B-cell repopulation compared to LT birds starting at 14 dpi. At 14, 21 and 28 dpi in both experiments, iIBDV BT groups had lower B-cell depletion scores compared to iIBDV LT groups (Table 3; S1 Figure). Chickens of the iIBDV DT groups showed a delayed decrease in B-cell numbers in Exp. 1 starting at 14 dpi. In Exp. 2, iIBDV DT birds took an intermediate position between BT and LT birds since the B-cell numbers were lower compared to the iIBDV BT groups but higher compared to the iIBDV LT groups at 14 and 28 dpi (Table 3; S1 Figure). Daily variations between individual birds of one genotype group were noted as indicated by variable standard deviations. Whereas three out of six LT birds in Exp.

2 showed low B-cell depletion scores at 21 dpi (mean score 0.4), at 28 dpi five out six LT birds had high B-cell depletion scores (mean score 2.0).

Overall, our data demonstrate that the development of bursa lesions and the depletion of B cells in the bursal follicles coincide with the IBDV-antigen load (Table 2+3, Fig. 2). The recovery phase, which is described by the resolution of lesions and the repopulation of the follicles with B cells, may not fully correlate with the clearance of the IBDV-antigen depending on the genotype (Table 3, Fig. 2).

Infiltration of T-cells into the BF

The infiltration of CD4+ and CD8+ T-cells into the BF was determined by immunohistochemical staining. At all investigated time points no significant differences in T-cell numbers were observed between the control groups of the four genotypes (Fig. 4 + 5). The highest CD4+ T-cell numbers in iIBDV SPF birds were detected at 7 dpi and decreased continuously until 28 dpi in both conducted experiments (Fig. 4). Although total numbers varied slightly between experiments, the overall pattern was comparable. The intrabursal CD4+ T-cell infiltration was comparable over time between Exp. 1 and 2 for iIBDV LT and BT groups. Chickens of the iIBDV BT groups showed a continuous decrease in CD4+ T-cell numbers after seven dpi, while LT groups still showed significantly higher CD4+ T-cell numbers in the BF compared to BT groups at 14 (Exp. 2), 21 and 28 dpi (Exp. 1) (Fig. 4, $P < 0.05$; S2 Figure). While in Exp. 1, iIBDV DT groups showed a delayed increase in CD4+ T-cell numbers at 14 dpi (Fig. 4), in Exp. 2 the highest infiltration was observed at 7 dpi, which was comparable to the other groups. At 21 dpi intrabursal CD4+ T-cell numbers were comparable between iIBDV LT and DT chicken and were higher compared to iIBDV SPF and BT chickens.

A similar pattern of CD8+ T-cell infiltration dynamics was observed. No differences between the control groups were observed at either of the investigated time points (Fig. 5). In all control groups of all genotypes only a few CD8+ T-cells were counted in the BF, ranging from one to 15 positively stained cell per microscopic field (400x). The intrabursal infiltration of CD8+ T-cells peaked at 7 dpi in iIBDV SPF groups and

decreased afterwards again. The decrease in CD8+ T-cell numbers was not as precipitous as observed for CD4+ T-cell numbers up to 28 dpi.

The CD8+ T-cell infiltration pattern was comparable between Exp. 1 and 2 for both iIBDV LT and BT groups. The intrabursal numbers of CD8+ T-cell of iIBDV BT birds continuously decreased over time compared to iIBDV LT groups, where the numbers stayed at a constant level until 28 dpi (Fig. 5, S3 Figure). At 21 dpi iIBDV LT and DT groups showed significantly higher intrabursal CD8+ T-cell numbers compared to iIBDV BT chickens. Samples from chickens of the iIBDV DT groups in Exp. 1 showed a delayed increase of CD8+ T-cell numbers at 14 dpi (Fig. 5). At 7 dpi (Exp. 1) significantly higher numbers of CD8+ T-cells were determined in iIBDV BT and SPF groups compared to iIBDV DT groups (Fig. 5). In Exp. 2 CD8+ T-cell numbers in iIBDV DT chickens had already increased at 7 dpi, which was comparable to the other groups with respect to time point and numbers. Comparable to iIBDV LT birds, the CD8+ T-cell numbers of DT birds stayed at a higher level until 28 dpi (Fig. 5; S3 Figure).

Interestingly, after calculating the fold increase of CD4+ and CD8+ T-cell numbers between iIBDV groups and the corresponding control groups, it was observed after comparison that the CD8+ T-cell numbers increased much more than the CD4+ T-cell numbers. Depending on the time point and genotype, the CD4+ T-cell numbers increased by four to eleven times after iIBDV inoculation compared to the non-inoculated controls. CD8+ T-cells on the other hand increased by 11 – 38 times after iIBDV inoculation.

Correlating IBDV-antigen detection, bursal lesion development and T-cell infiltration, it becomes clear that the persistence of T-cells in the BF varies between genotypes and coincided with the persistence of bursal lesions but not the presence of IBDV antigen.

IL-10 and TGFβ4 mRNA expression in the BF

Only minor differences in IL-10 mRNA levels were observed between the non-inoculated control groups of all genotypes. iIBDV SPF groups showed significantly increased IL-10 mRNA levels until 21 dpi compared to the corresponding controls ($P < 0.05$). The mRNA expression pattern of IL-10 was comparable between the two

conducted experiments for iIBDV BT and LT chicken genotypes. IL-10 mRNA was significantly increased in iIBDV BT and LT groups at 7 dpi in both experiments (Fig. 6). In iIBDV LT groups IL-10 mRNA levels were significantly increased until 28 dpi, whereas iIBDV BT groups only had increased levels until 14 dpi (only Exp. 1). Inoculated DT chickens showed a delayed increase of IL-10 mRNA in Exp. 1 at 14 dpi (Fig. 6). Similar to iIBDV LT groups, DT groups showed increased IL-10 mRNA levels until 21 dpi.

No differences in the presence of TGFβ4 mRNA were observed between the non-inoculated control groups of all genotypes. At most of the investigated time points pi, no differences between iIBDV and control genotype groups were observed (Fig. 7). Samples from chickens of the iIBDV SPF groups showed a significant TGFβ4 mRNA transcription up-regulation at 21 dpi in Exp. 2. Samples from chickens of the iIBDV BT groups showed a significant TGFb4 up-regulation of transcription at 14 dpi and down-regulation of transcription at 21 dpi in Exp. 1. iIBDV LT groups showed a significant up-regulation of TGFβ4 mRNA transcription at seven and 28 dpi in Exp. 1.

Overall, increased IL-10 mRNA levels were detected in birds which still had high numbers of T-cells and bursal lesions in the BF, therefore indicating the involvement of IL-10 in the bursal recovery phase after IBDV infection and genotype-associated differences.

7.5 Discussion

Several studies showed that differences in immune responses between chicken lines highly influence the susceptibility to different diseases (Kramer et al., 2001; Swinkels et al., 2007; Swaggerty et al., 2011). It was suggested that IBDV pathogenesis is influenced by genotype-associated differences in the modulation of T-cell responses during the acute phase of infection (Aricibasi et al., 2010; Tippenhauer et al., 2013). The importance of T-cell immunity during IBDV infection including the recovery phase was demonstrated before (Kim et al., 2000; Rautenschlein et al., 2002). Nevertheless, studies comparing T-cell related differences between genotypes beyond the acute phase (recovery phase) of IBDV infection haven't been conducted so far. For the present study, three different commercially available chicken lines (BT, LT and DT) were selected, which differ in their production performance and in their selection for different production traits. The DT chicken is a newly developed dual-purpose line and shows a more moderate production performance in egg- and meat production traits compared to the high-performing LT and BT chickens (Icken and Schmutz, 2013). A previously conducted study showed that these chicken lines differed in their innate and adaptive immune responses after inoculation with a recombinant herpesvirus of turkeys (HVT) IBDV (HVT-IBD) vaccine (Dobner et al., 2019). This study indicated that these genotypes might vary in their T-cell responses after vaccination. Therefore, two experiments were conducted to compare genotype-associated differences in IBDV pathogenesis with a specific focus on the role of T-cells and related anti-inflammatory cytokine expression in the BF during the recovery phase until 28 dpi.

Besides hygiene management standards, vaccination against IBDV plays a major role in the control of the disease by active or passive immunization (Müller et al., 2012). The timing of vaccine administration depends on different factors, such as the magnitude of residual MDA, the IBDV antibody breakthrough levels for the vaccine, the vaccine strain to be used and the field pressure (de Wit, 1998, 2001). The ELISA can be used to determine MDA levels and predict the optimal time of vaccination by collecting blood samples during the first week post hatch (Kouwenhoven and Van den Bos, 1992). The Deventer formula was developed to estimate the optimal time of

vaccination based on the half-life time of antibodies, which is depending on the genetic background of the chickens (de Wit, 1998, 2001). Furthermore the age of chickens at sampling, the antibody breakthrough level of the vaccine and the percentage of the birds having antibody levels below the breakthrough level for the vaccine at a possible time have to be considered (de Wit, 1998, 2001). Studies indicated that vaccination with IBDV live vaccines in the presence of anti-IBDV-MDA delays IBDV replication and the anti-IBDV specific antibody response (McCarty et al., 2005; Rautenschlein et al., 2005; Block et al., 2007).

Our objective in the present study was to vaccinate the birds of the different genotypes at the same age, when in all groups anti-IBDV-MDA decreased below the breakthrough level for an intermediate plus vaccine. Despite the appropriate use of the Deventer formula the prediction of the optimal vaccination time was less accurate as expected. Residual anti-IBDV-MDA in DT chickens decreased slower than in the BT and LT groups. Subsequently, residual levels interfered with virus replication in Exp. 1, when IBDV inoculation was done at 28 dph. As shown previously, IBDV replication and lesion development was delayed (Rautenschlein et al., 2005). In addition, our study showed that IL-10 mRNA transcription upregulation was detected earliest one week later than in the other groups coinciding with B-cell depletion. A delayed and lower anti-IBDV antibody response in DT chickens was detected. Furthermore, we observed high individual variations in the decrease of anti-IBDV-MDA in the DT chicken groups. This high variation may have been occurred due to the fact that the DT birds were not as long-bred as the LT and BT, which have been under selection for more than 30 years (Hunton, 2006).

Although LT chickens showed anti-IBDV-MDA above the breakthrough level for the vaccine in Exp. 1, the results for the other investigated immune parameters were comparable between both experiments in LT and BT chickens, suggesting that both experiments were comparable with respect to these parameters. Nevertheless, we could also observe differences in the magnitude and onset of the anti-IBDV antibody response between Exp. 1 and 2 in BT and SPF chickens, which had anti-IBDV-MDA levels below the breakthrough for the vaccine at the time of inoculation in both

experiments. This might indicate a difference in the maturation of the immune response at the time of inoculation (Fadly et al., 1976; Eterradossi and Saif, 2013).

Different studies indicated that the presence of viral antigen in the BF is associated with the onset of microscopic bursal lesions. It was suggested that the amount of the virus and the course of viral clearance might differ between chicken lines (Moody et al., 2000; Jain et al., 2013; Smith et al., 2015). In the present study, we confirmed that the highest numbers of bursal lesions were detectable when the IBDV antigen loads were the highest during the acute phase of infection at 7 dpi irrespective of the investigated genotype. By monitoring the bursal lesions and IBDV antigen loads until 28 dpi, we noticed differences between the chicken lines in the clearance of the virus and in the decrease of bursal lesions independent of the anti-IBDV-MDA levels at the time of inoculation. In all iIBDV groups the numbers of IBDV antigen positive cells decreased during the course of infection while iIBDV BT groups showed the fastest antigen decrease and bursal recovery. Chickens of the iIBDV LT groups showed a comparable IBDV antigen decline but still high bursal lesions at 21 and 28 dpi. This suggests a clear impact of the genotype on the recovery phase after IBDV infection, which may not be fully associated with the antigen load but may depend on other regulatory mechanisms (Aricibasi et al., 2010; Tippenhauer et al., 2013).

Genotype-associated differences in the virus clearance and outcome of the disease might be related to a varying modulation of T-cell responses (Tippenhauer et al., 2013). Studies in T-cell compromised SPF LT chickens showed that T-cells limit IBDV replication but also contribute to bursal lesion development and may delay recovery (Kim, 2000; Rautenschlein, 2002). A peak in CD4+ and CD8+ T-cell accumulation in the BF was demonstrated during the acute phase of infection at 7 dpi, which was comparable in absolute numbers between all iIBDV genotype groups and was accompanied by the highest bursal lesion scores and IBDV antigen loads in all iIBDV groups. Interestingly, iIBDV BT birds showed the highest fold-increase of CD8+ T-cells in relation to the corresponding control groups at 7 dpi. It was suggested that CD8+ cytotoxic T-cells (CTLs) contribute to IBDV clearance (Rauf et al., 2011, 2012) and that BT birds might be able to lower viral replication more efficiently due to a stronger CMI

during the acute phase of infection (Aricibasi et. al., 2010). We could confirm these findings in the present study and therefore speculate that the higher fold increase of CD8+ T-cells in BT chickens at 7 dpi might have accelerated the recovery process. Furthermore, we could see significant differences between the genotypes in the decrease of CD4+ and CD8+ T-cell numbers in the BF at later time points. Chickens of the iIBDV BT groups showed a significantly faster decrease in T-cell numbers, which was associated with a faster bursal lesion recovery until four weeks pi compared to LT birds. Again DT chickens might take an intermediate position due to their genotype associated mixed traits of LT and BT bird, as they in the case of CD4+ and CD8+ T-cells show a comparable decline to LT chickens. LT and DT as well as SPF chickens showed a less vigorous decline in CD4+ and CD8+ T-cell populations compared to BT chickens. But unexpectedly, the recovery was not fully associated with this observation. We may speculate that other cell types, such as macrophages may contribute to the recovery phase, which has to be investigated further in the future.

Cytokines and chemokines possess important regulatory and antiviral functions during immune responses against infectious diseases (Kaiser and Stäheli, 2014). Several studies demonstrated that the intensity of pro-inflammatory cytokine and chemokine responses during the acute phase of IBDV infection might define the individual outcome of the disease and that differences between genotypes exist (Eldaghayes et al., 2006; Rauw et al., 2007; Ingrao et al., 2013; Tippenhauer et al., 2013; Smith et al., 2015). Anti-inflammatory cytokines, such as IL-10 and TGFβ4 were also shown to be either up- or down-regulated after IBDV infection during the acute phase (Eldaghayes et al., 2006; Jain et al., 2013; Rasoli et al., 2015; Yu et al., 2015). Shanmugasundaram and Selvaraj (2011) demonstrated that the presence of IL-10 and TGFβ4 can be associated with a specific subset of suppressor T-cells, the regulatory T-cells. Tregs suppress immune responses mainly through IL-10 (Selvaraj et al., 2013). To the best of our knowledge the involvement and role of Tregs in the recovery process after IBDV infection hasn’t been addressed in any studies yet. In the present study we observed significantly increased IL-10 mRNA levels in the BF of all iIBDV groups at different time points pi. It was demonstrated that the genotypes differed significantly in the duration of elevated IL-10 mRNA levels. Chickens of the iIBDV LT and DT groups, which

showed a slower recovery from the infection still had signficantly increased IL-10 mRNA levels at later time points compared to BT chickens. This coincides with the number of intrabursal CD4+ T-cells in the BF of iIBDV LT and DT chickens. These findings suggest that IL-10 might play a significant direct or indirect role in the delay of recovery in LT and DT chickens. $CD4^+CD25^+$ T-cells are commonly considered as Tregs in mammalian and avian species. Yu et al. (2015) demonstrated that elevated levels of IL-10 in the BF could be associated with the accumulation of $CD4^+CD25^+$ Tregs. Therefore, our data provide circumstantial evidence of the involvement of Tregs in the recovery process after IBDV infection and that these cells might be modulated differently depending on the chicken genotype.

Besides $CD4^+CD25^+$ T-cells, $CD4^+TGF\beta^+$ T-cells were also defined as a Treg subset (Shanmugasundaram and Selvaraj, 2011; Gurung et al., 2017). Nevertheless, the existence of the unique Treg marker, the forkhead box protein 3 (*foxp3*) in avian species still has to be determined to be able to investigate the involvement of different Treg subsets in IBD pathogenesis further. Studies in mammals showed that other immune cell populations, such as macrophages, dendritic cells (DC), B-cells and other T-cell subsets can also be the source of IL-10 production (Moore et al., 2001; Couper et al., 2008). Therefore, this aspect has to be elucidated further in subsequent studies using for example flow cytometric analysis or FACS of different immune cell subsets in addition to Tregs for co-detection of IL-10 and TGFβ expression (Gurung et al., 2017).

The observed minor differences in the TGFβ4 mRNA transcription levels in the BF of the present study might indicate a less important role of this cytokine during the recovery of the BF due to an unclear expression pattern. Nevertheless, at different time points pi TGFβ4 mRNA levels were either significantly up- or downregulated in iIBDV groups, which was variable between genotypes, experiments and time points. Eldaghayes et al. (2006) observed a down-regulation of TGFβ4 levels after infection with a virulent and very virulent IBDV strain at two to three days pi. This indicates that the presence of TGFβ4 might preferably be regulated during the acute phase of

infection and that more virulent IBDV strains might lead to more obvious changes in the expression pattern. In our study we investigated an intermediate plus IBDV strain.

In conclusion, the study clearly demonstrated a genotype-dependent influence on the duration of the recovery process after IBDV inoculation. Differences between chicken lines were observed after inoculation with an intermediate plus virus strain in the clearance of IBDV antigen, which did not fully correlate with the clearance from bursal lesions. CD4+ and CD8+ T-cell numbers may provide an indicator for the progressing recovery. It was demonstrated that Treg related IL-10 mRNA transcription is involved in the recovery process after IBDV infection and that the expression pattern differed between genotypes during the recovery phase. These findings form an important basis for future investigations on regulatory T-cells and their role in infectious diseases to forward further the selection for immunological traits in chickens.

7.6 Acknowledgements

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7.7 Figures and Tables

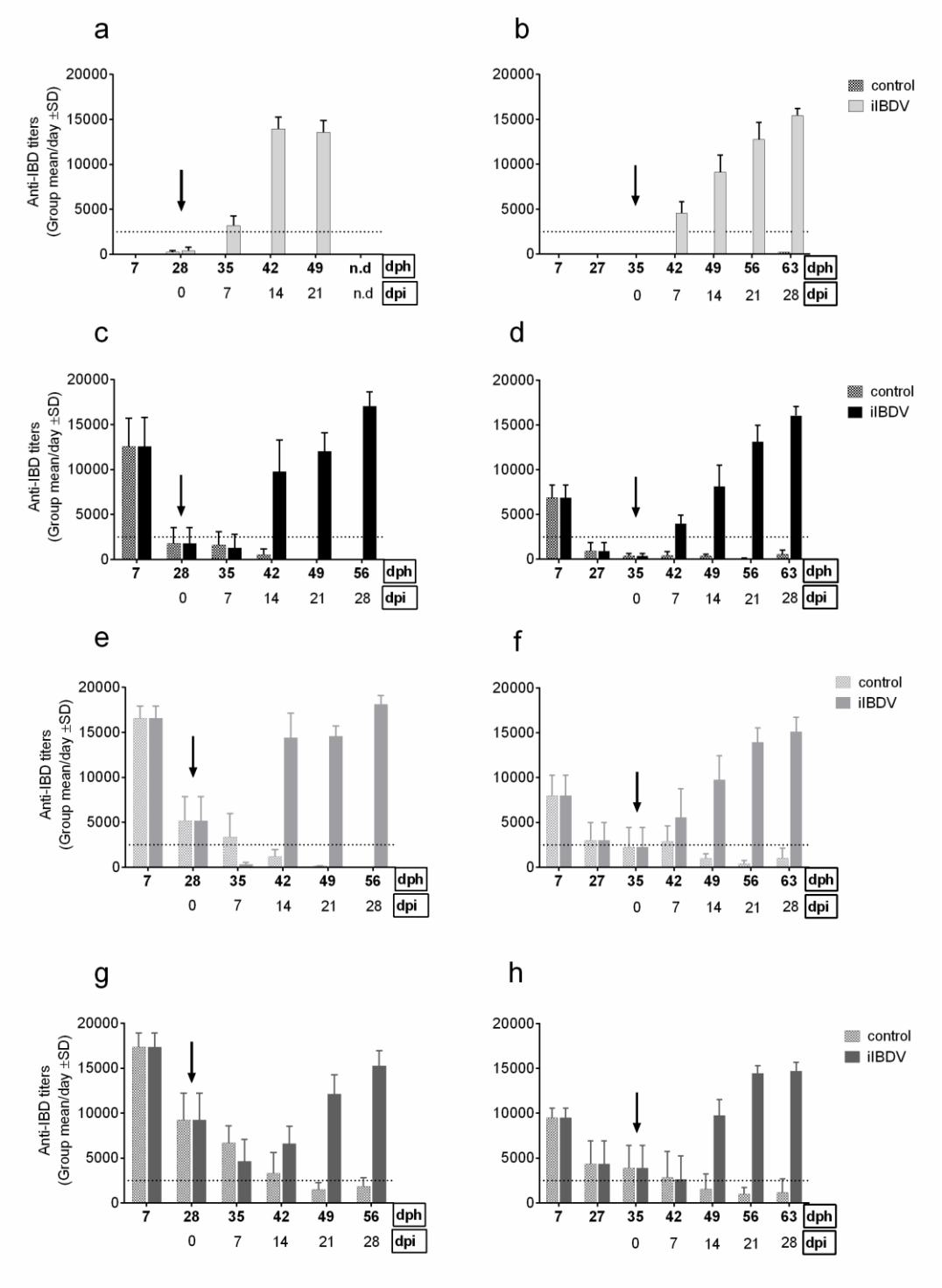


Fig. 1. IgG specific anti-IBDV antibody titers of all genotypes in Experiment 1 (a, c, e, g) and in Experiment 2 (b, d, f, h). a+b: SPF, c+d: BT, e+f: LT, g+h: DT. Shaded bars represent the respective control groups of each genotype and filled bars represent the IBDV inoculated genotype groups. Data are presented as the mean anti-IBDV specific

antibody titers of n=6 birds per group and time point ± SD. The time points before and at the day of inoculation show the mean anti-IBDV antibody titers of n=20 birds per genotype and time point ± SD. Arrows mark the day of inoculation at 28 (Exp. 1) or at 35 (Exp. 2) dph. n.d = not determined. The dotted line shows the breakthrough titer of the intermediate plus virus strain (y=2500).

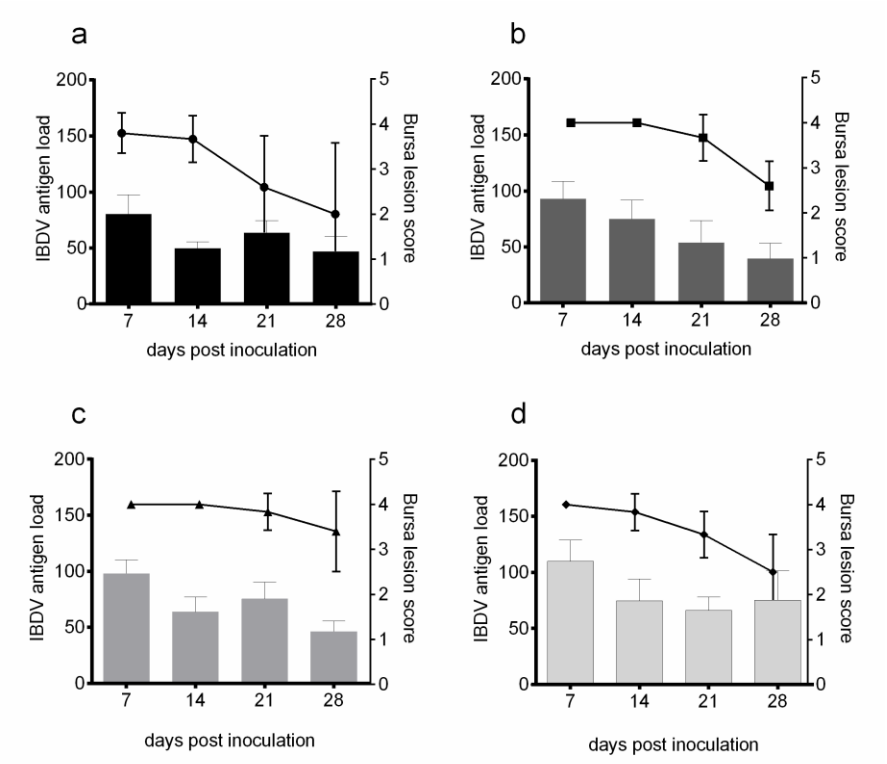


Fig. 2. Correlation between IBDV antigen loads and the histologically detected magnitude of bursal lesions in the BF of BT (a), DT (b), LT (c) and SPF (d) chickens (Exp. 2). Data are presented as mean IBDV antigen loads and bursal lesions scores per IBDV-inoculated genotype group and day ±SD (n=6/group). Bar graphs indicate the IBDV-antigen load, line graphs indicate the bursal lesion scores.

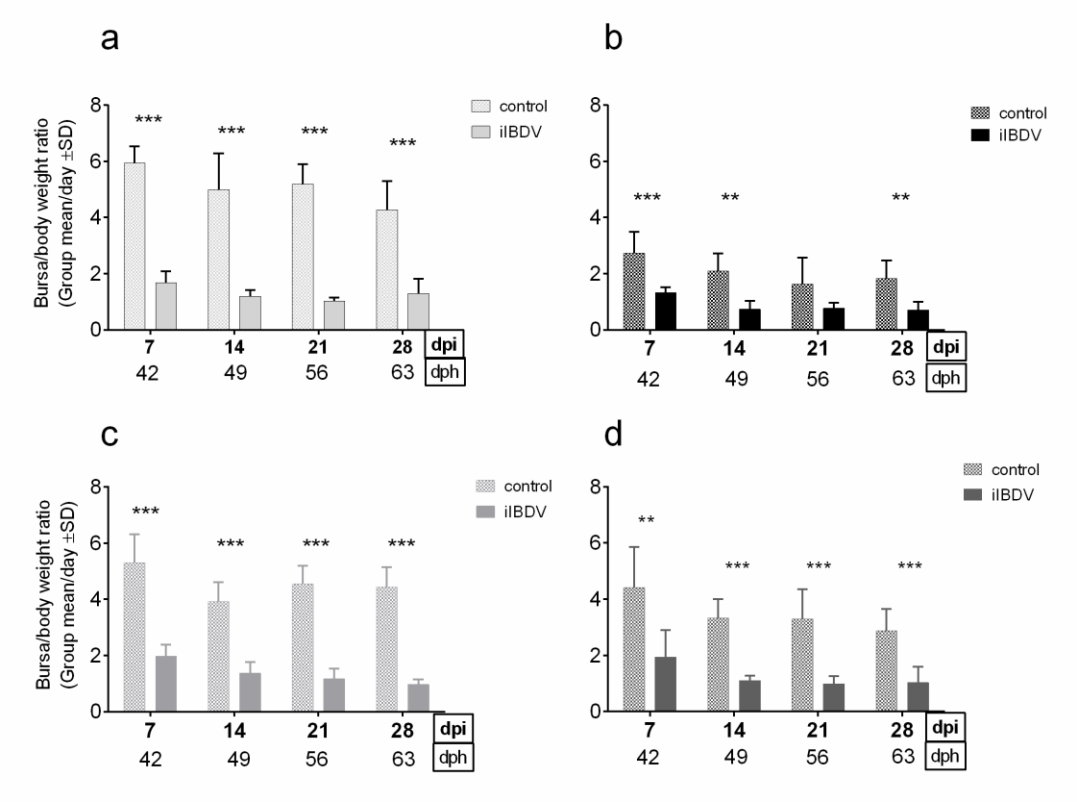


Fig. 3. Bursa/body weight ratios of SPF (a), BT (b), LT (c) and DT (d) chickens after 7, 14, 21 and 28 days after inoculation with an intermediate plus IBDV strain in Exp. 2. Data are presented as means of n=6 birds per group and time point ±SD. Asterisks (*) indicate significant differences between IBDV–inoculated (iIBDV) and control groups. (Wilcoxon Rank Sum Test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). dpi=days post infection; dph=days post hatch.

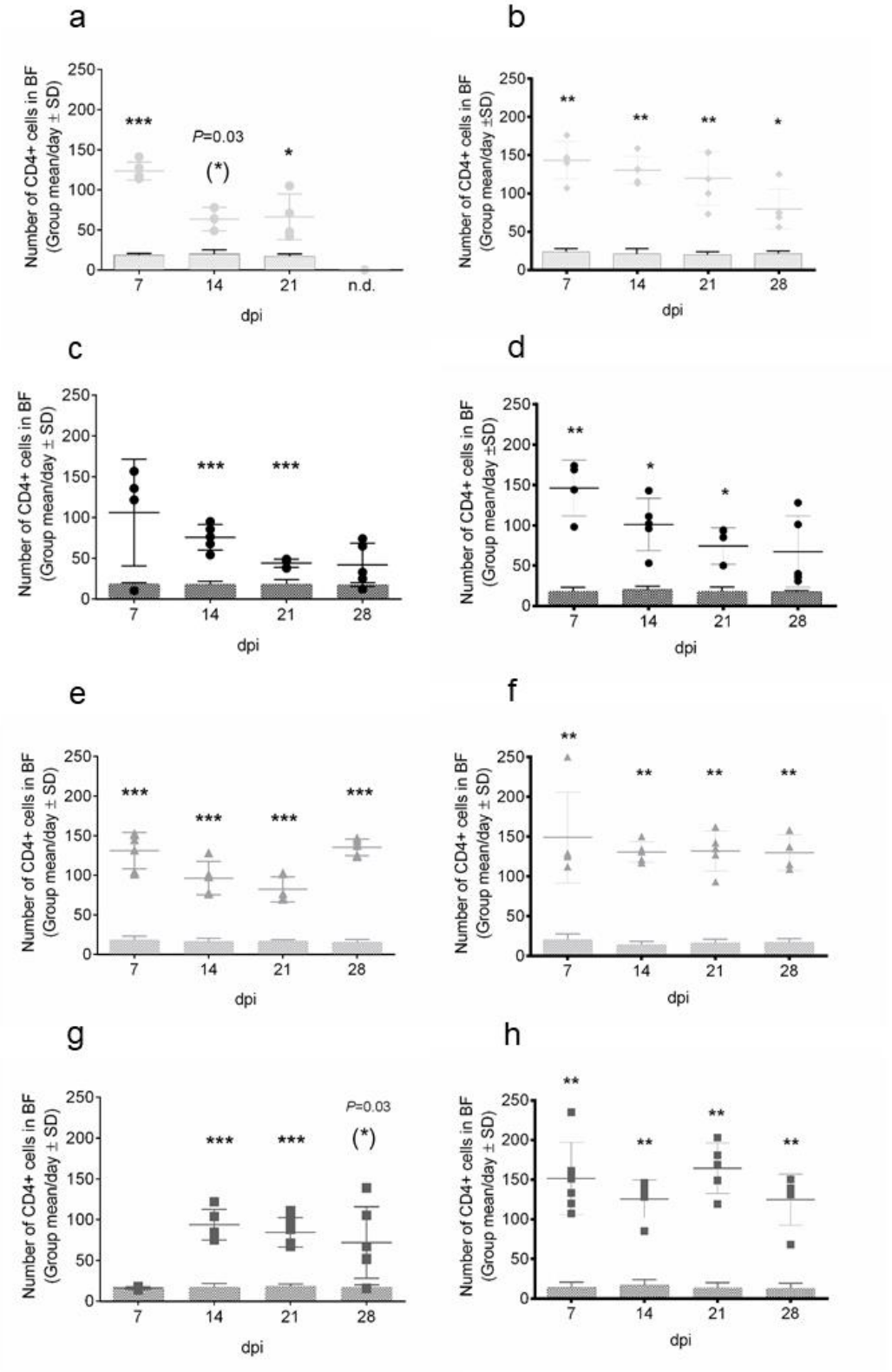


Fig. 4. Immunohistochemical determination of CD4+ T-cells in the BF of iIBDV (single-dotplot, which may overlap for some animals) and control groups (bars) of SPF (a, b),

BT (c, d), LT (e, f) and DT (g, h) chickens in Exp. 1 (a, c, e, g) and 2 (b, d, f, h). Data are presented as the mean numbers of CD4+ T-cells of n=6 birds per group and time point ±SD. Asterisks (*) indicate significant differences between the mean numbers of CD4+ T-cells of inoculated and control groups per group and day (Chi-Square Test; *$P < 0.01$; **$P < 0.001$; $P < 0.0001$). dpi=days post infection

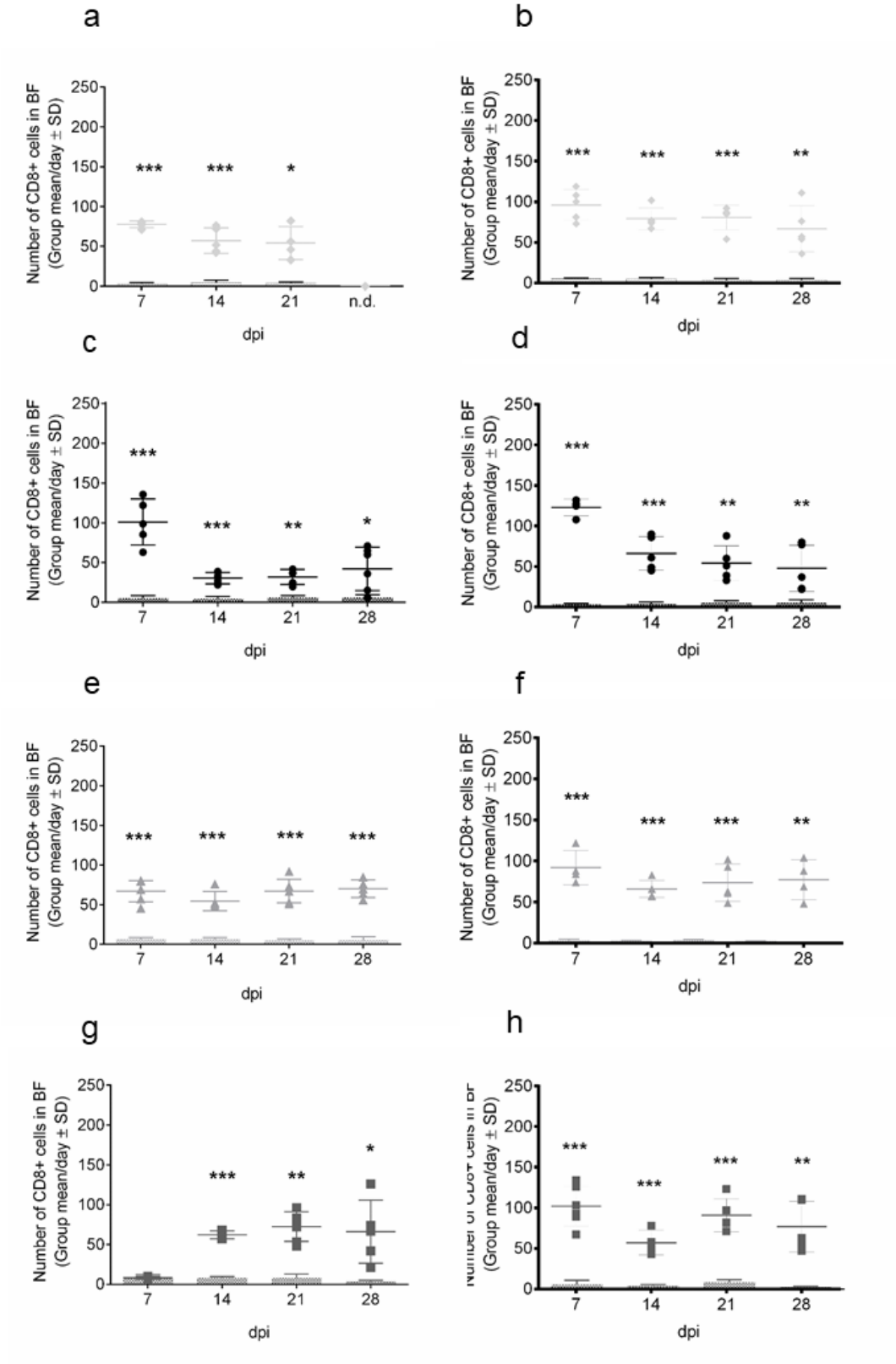


Fig. 5. Immunohistochemical detection of CD8+ T-cells in the BF of iIBDV (single-dotplot, which may overlap for some animals) and control groups (bars) of SPF (a, b),

BT (c, d), LT (e, f) and DT (g, h) chickens in Exp. 1 (a, c, e, g) and 2 (b, d, f, h). Data are presented as the mean numbers of CD8+ T-cells of n=5-6 birds per group and time point ±SD. Asterisks (*) indicate significant differences between the mean numbers of CD8+ T-cells of inoculated and control groups per group and day (Chi-Square Test; *$P < 0.01$; **$P < 0.001$). dpi=days post infection

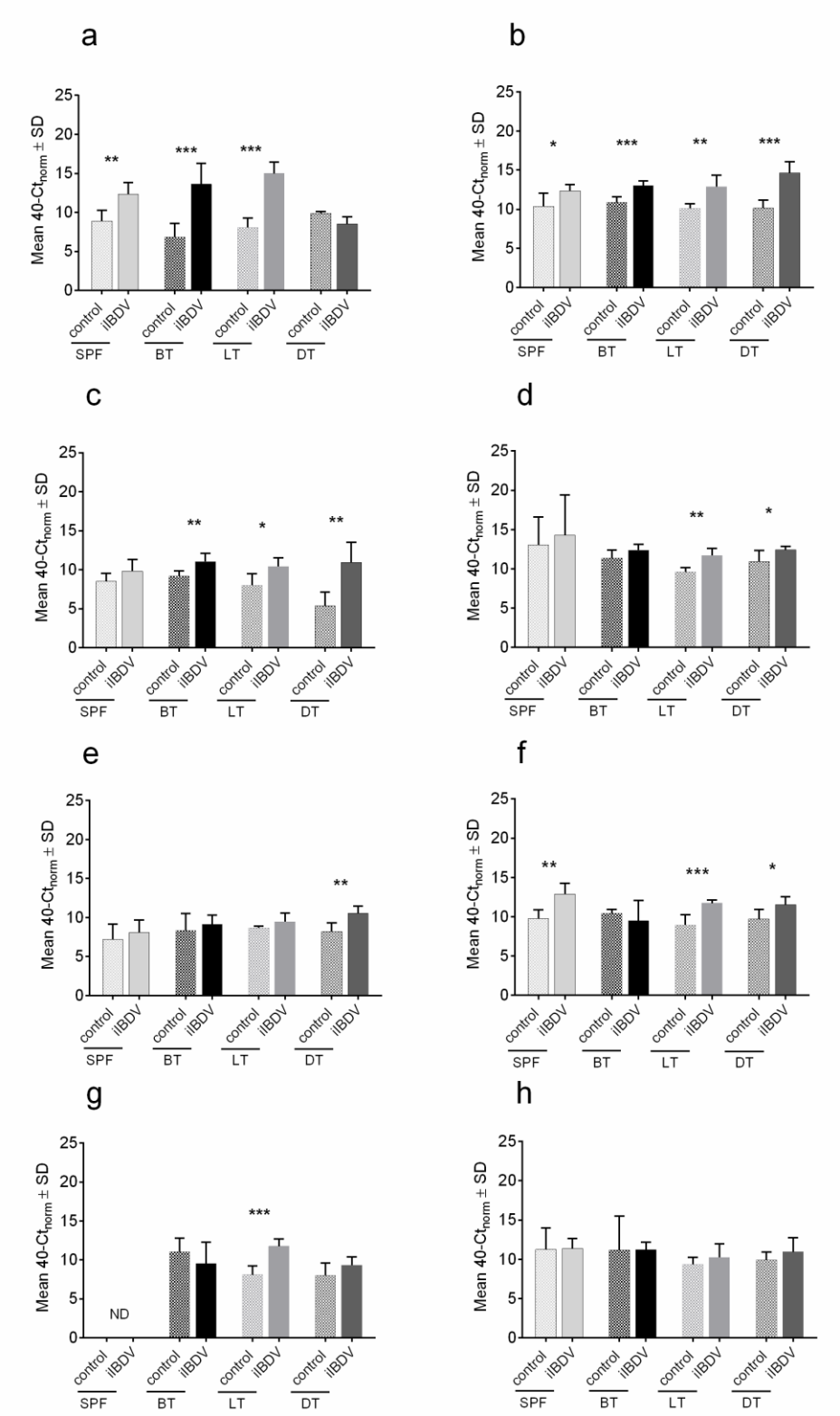


Fig. 6. IL-10 mRNA levels in the BF of control and iIBDV genotype groups at 7 (a, b), 14 (c, d), 21 (e, f) and 28 (g, h) days pi in Exp. 1 (a, c, e, g) and 2 (b, d, f, h). Data are

presented as the mean 40-Ct_{norm} values (Ct values normalized to RPL-13) per genotype group and time point ±SD (n=6). Asterisks (*) indicate significant differences between iIBDV and control groups of one genotype group per time point (Two Sample *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

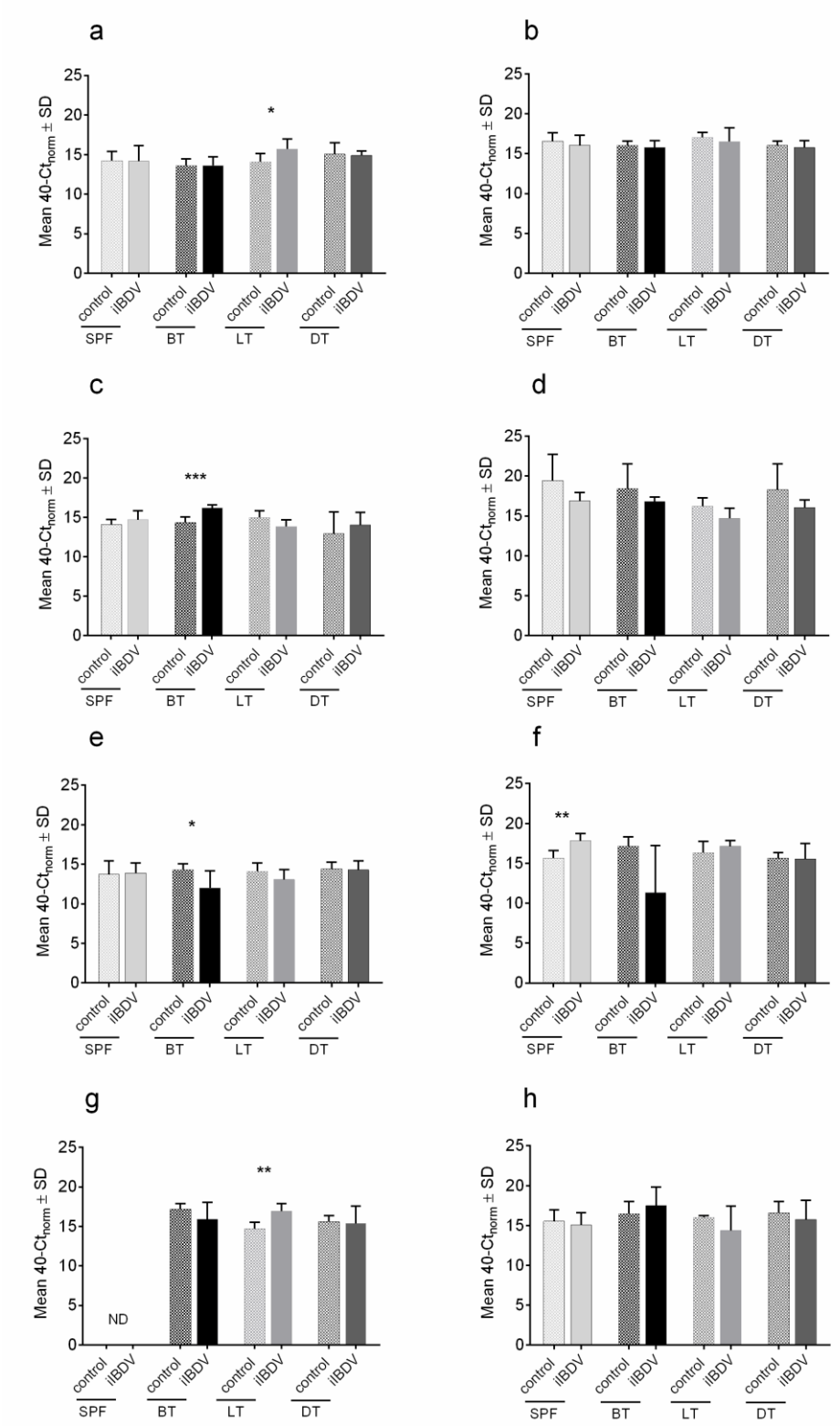


Fig. 7. TGFβ4 mRNA levels in the BF of control and iIBDV genotype groups at 7 (a, b), 14 (c, d), 21 (e, f) and 28 (g, h) days pi in Exp. 1 (a, c, e, g) and 2 (b, d, f, h). Data are

presented as the mean 40-Ct_{norm} values (Ct values normalized to RPL-13) per genotype group and day ±SD (n=6). Asterisks (*) indicate significant differences between iIBDV and control groups of one genotype group per time point (Two Sample *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 1. Respective primer and probe sequences for relative quantification of IL-10 and TGFβ4 in the BF.

RNA target		Primer / probe sequences (5'-3')	Accession number	Reference
IL-10	F-Primer	CAT GCT GCT GGG CCT GAA		
	R-Primer	CAT CAA GCA GAT CAA GGA GAC G	AJ621614	(Rothwell et al., 2004)
	Probe	(FAM)-CGA CGA TGC GGC GCT GTC A-(TAMRA)		
TGFβ4	F-Primer	AAG GAT CTG CAG TGG AAG TGG AT		
	R-Primer	CCC CGG GTT GTG TTG GT	M31160*	(Kogut et al., 2003; Swaggerty et al., 2004)
	Probe	(FAM)-ACC CAA AGG TTA TAT GGC CAA CTT CTG CAT-(TAMRA)		
RPL-13	F-Primer	GGA GGA GAA GAA CTT CAA GG C		
	R-Primer	CAA ACG CTC GTC TCT TTG G	NM_204999.1	
	Probe	(FAM)- CTT TGC CAG CCT GCG CAT G-(NQ)		
IL-10	F-Primer	CAT GCT GCT GGG CCT GAA		
	R-Primer	CGT CTC CTT GAT CTG CTT GAT G	AJ621614.1	(Rothwell et al., 2004)
	Probe	(FAM)-CGA CGA TGC GGC GCT GTC A-(TAMRA)		
TGFβ4	F-Primer	AAG ATC TGC AGT GGA AGT GGA T		
	R-Primer	CCC CGG GTT GTG TGT TGG T	M31160.1	(Kogut et al., 2003; Swaggerty et al., 2004)
	Probe	(FAM)-ACC CAA AGG TTA TAT GGC CAA CTT CTG CAT-(TAMRA)		

RPL-13	F-Primer	GGA GGA GAA GAA CTT CAA GG C		
	R-Primer	CCA AAG AGA CGA GCG TTT G	NM_204999.1	(Mitra et al., 2016)
	Probe	(HEX)- CTT TGC CAG CCT GCG CAT G-(BHQ-1)		

(*) refers to genomic DNA sequence

Table 2. Immunohistochemical detection of IBDV antigen in the BF of iIBDV BT, DT, LT and SPF groups in Exp. 1 and Exp. 2.

Genotype	Experiment	Average number of IBDV-positive cells in the BF of infected chickens/ 3 microscopic fields ± SD at days pi			
		7	14	21	28
BT	1	84.0±37.6 [a]	41.3±19.4 [b]	43.7±30.7	19.7±13.3 [b]
	2	80.3±17.3 [b]	49.9±5.8 [b]	63.7±10.6	38.4±22.6
DT	1	14.6±35.8 [b]	98.8±19.3 [a]	63.1±14.6	44.6±4.0 [a]
	2	93.0±15.6	75.0±16.8 [a]	54.1±19.5	39.9±11.9
LT	1	122.2±33.2 [a]	45.4±12.0 [b]	53.7±14.7	46.4±6.6 [a]
	2	97.9±12.2	63.7±13.2	75.3±14.9	46.2±9.4
SPF	1	121.1±5.8 [a]	71.6±33.2	62.1±17.3	ND
	2	109.8±19.2 [a]	74.7±19.5	66.0±12.0	68.5±27.2

[a, b] significant differences between genotype groups per day (One Way ANOVA, $P < 0.05$)

ND not determined

Table 3. Histopathological lesion and B-cell depletion development in different chicken genotypes after inoculation with an intermediate plus IBDV strain.

Genotype	Experiment	Group average bursal lesion score at days pi				Group average B-cell depletion score at days pi			
		7	14	21	28	7	14	21	28
BT	1	3.2 ± 1.6	3.3 ± 0.8	2.7 ± 1.2	1.4 ± 1.3	3.0 ± 0.7	1.0 ± 0.0	0.6 ± 0.5	0.4 ± 0.5
	2	3.7 ± 0.5	3.7± 0.5	2.6 ± 1.4	2.0 ± 1.6	3.4 ± 0.9	0.6 ± 0.9	0.0 ± 0.0[b]	0.4 ± 0.5[b]
DT	1	0.2 ± 0.4[b]	3.8 ± 0.4	3.7 ± 0.5	2.5 ± 1.0	0.0 ± 0.0[a]	2.6 ± 1.5	1.4 ± 0.6[a]	0.6 ± 0.6
	2	4.0 ± 0.0	4.0 ± 0.0	3.7 ± 0.5	2.6 ± 0.6	4.0 ± 0.0	1.0 ± 0.7	1.4 ± 0.9[a]	1.2 ± 0.8
LT	1	4.0 ± 0.0[a]	4.0 ± 0.0	3.8 ± 0.4[a]	3.8 ± 0.5	4.0 ± 0.0[b]	1.4 ± 0.5	1.2 ± 0.5	1.2 ± 0.5
	2	4.0 ± 0.0	4.0 ± 0.0	3.8 ± 0.4	3.2 ± 0.2	3.4 ± 0.9	1.2 ± 0.4	0.4 ± 0.5	2.0 ± 0.0[a]
SPF	1	4.0 ± 0.0[a]	3.6 ± 0.6	2.0 ± 1.0[b]	ND	3.8 ± 0.5[b]	1.2 ± 0.5	0.2 ± 0.5 [b]	ND
	2	4.0 ± 0.0	3.8 ± 0.4	3.3 ± 0.5	2.5 ± 0.8	4.0 ± 0.0	1.2 ± 0.4	0.8 ± 0.8	0.0 ± 0.0[b]

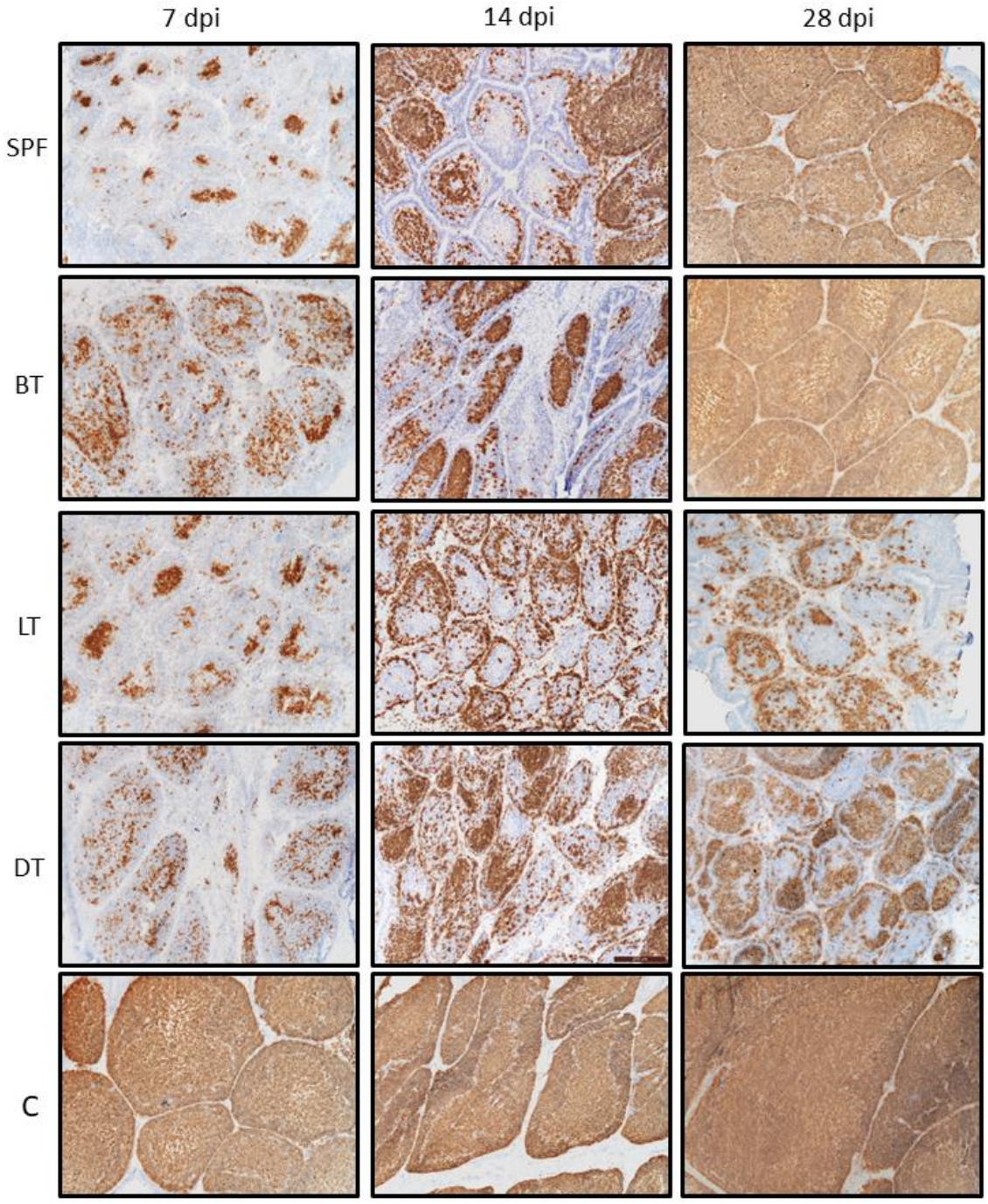
[a,b] significant differences between genotype groups per day (Chi-Square Test, $P < 0.05$)

Bursal lesion score: 0=no lesions, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100% lymphocyte depletion

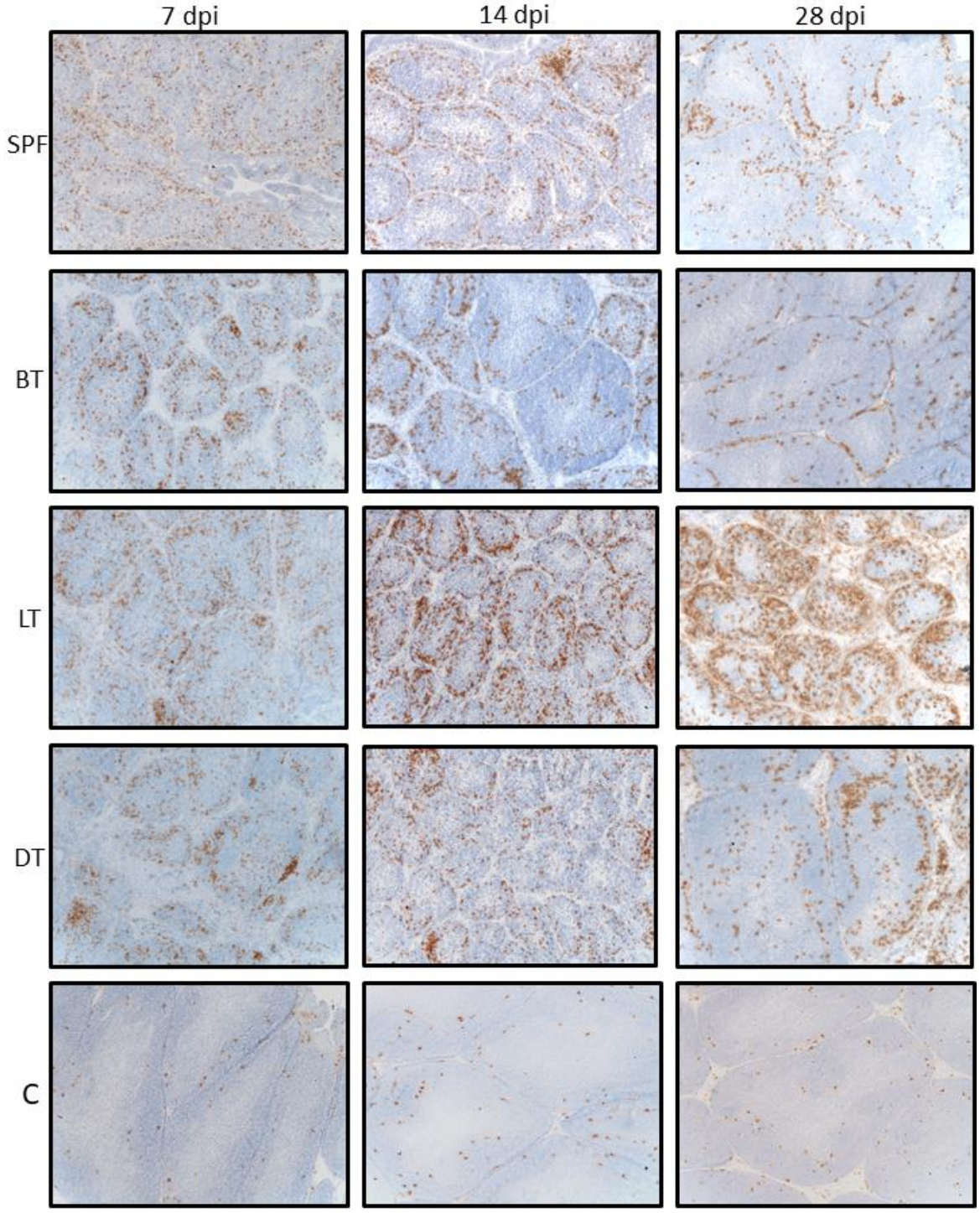
B-cell depletion score: 0=no depletion, 1=99-75%, 2=74-50%, 3=49-25%, 4=24-0% B-cells present in follicles

ND not detected

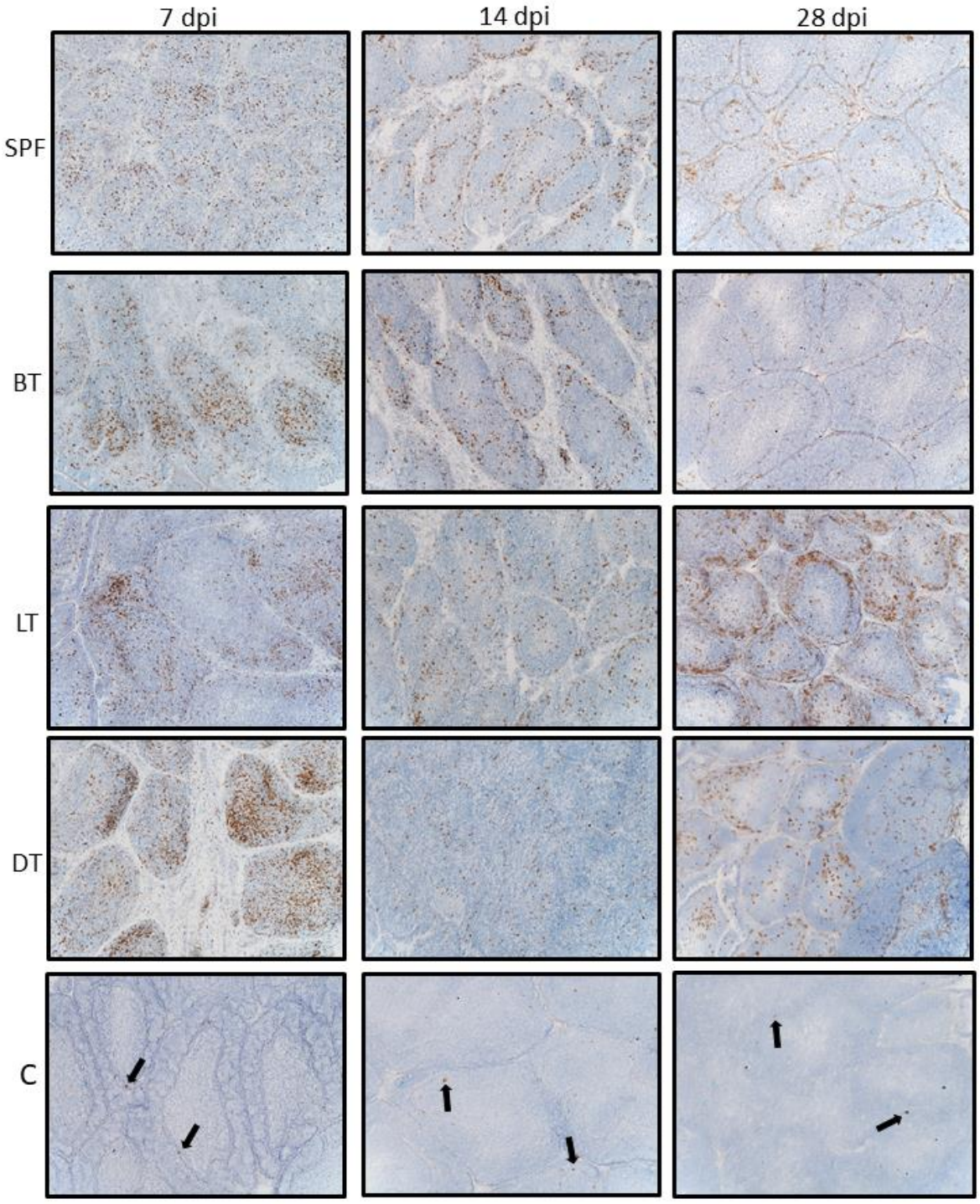
7.8 Supplementary Material



S1 Fig. Representative pictures of the immunohistochemical staining of B-cells in the BF of SPF, BT, LT, DT chickens and controls at 7, 14 and 28 days post IBDV inoculation. Positively stained cells are stained brown (DAB substrate). C = control



S2 Fig. Representative pictures of the immunohistochemical staining of CD4+ T-cells in the BF of SPF, BT, LT, DT chickens and controls at 7, 14 and 28 days post IBDV inoculation. Positively stained cells are stained brown (DAB substrate). C = control



S3 Fig. Representative pictures of the immunohistochemical staining of CD8+ T-cells in the BF of SPF, BT, LT, DT chickens and controls at 7, 14 and 28 days post IBDV inoculation. Positively stained cells are stained in brown (DAB-substrate). Black arrows indicate CD8+ T-cells. C = control

S5 Table. Deventer formula based time points for intermediate plus IBDV inoculation in BT, DT and LT chicken genotypes.

Genotype	Age of parent flocks in weeks		Mean titer at 7 dph (n=20/genotype)		Mean titer at day of inoculation (n=20/genotype)		Calculated time point of inoculation in dph	
	Exp.1	Exp.2	Exp. 1	Exp. 2	Exp. 1 (28dph)	Exp. 2 (35dph)	Exp.1	Exp.2
BT	37	43	12551 ± 3251	6867 ± 1405	1787 ± 1767	374 ± 269	17	13
DT	32	56	17377 ± 1610	9477 ± 1093	9247 ± 2988	3893 ± 2532	23	18
LT	36	56	16586 ± 1397	7995 ± 2267	5187 ± 2589	2237 ± 2217	23	18
SPF	57	22	-	-	-	-	-	-

dph = days post hatch

BT = broiler-type, DT = dual-purpose type, LT = layer-type, SPF = specific-pathogen-free, Exp.= experiment

7.9 References

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8. Discussion

IBDV is one of the most important immunosuppressive diseases in poultry and leads to great economic losses worldwide (MÜLLER et al. 2003; ETERRADOSSI and SAIF 2013; INGRAO et al. 2013). In the field, mortality rates and immunosuppression due to IBDV infection can vary depending on the virulence of the circulating virus strains, the secondary pathogen pressure, management factors but also the susceptibility of the flock itself (INGRAO et al. 2013). Besides hygiene management, vaccination plays the most important role for protecting chickens against the disease. Several studies showed that the genetic background of chickens influences IBDV-pathogenesis. By the use of newly developed chicken lines, differences in the immune responses upon IBDV infection can be expected and vaccination schedules might need to be adjusted.

The goal of this study was to determine the genotype-associated differences in the development of the immune system, innate and adaptive immune responses, and vaccine responses after vaccination with different IBDV vaccines. This study used common commercially available chicken lines (Lohmann Brown plus and Ross 308) and a newly developed dual-purpose breed chicken (Lohmann Dual) as well as a SPF LT chicken line in the second part of thesis.

The following discussion is based on the data presented in the two enclosed manuscripts.

Chapter 6: Immune responses upon *in ovo* HVT-IBD vaccination differ between different chicken lines.

Chapter 7: Genotype-associated differences in bursal recovery after infectious bursal disease virus (IBDV) inoculation.

In general, the structure of all studies was designed to compare variations in the development of the immune system and immune responses between the genotypes without vaccination (control groups) as well as after the administration of different IBDV vaccines. Therefore, the discussion will first focus on the genotype-influence itself and subsequently elaborate on the observed variations between the genotypes upon vaccination and end with a chapter about the importance of T-cell immunity in the recovery after IBDV infection.

8.1 Genotype effects

8.1.1 Variations in the development of the immune system

The immune system of chickens starts developing during embryogenesis (FELLAH et al. 2014). Several studies demonstrated that structural changes within the lymphoid organs and tissues in the first weeks post hatch are necessary for complete functionality JEURISSEN et al. 1994; MAST and GODDEERIS 1999). Immune organ weights, the distribution and numbers of immune cell populations as well as the development of important functional compartments can be used as indicators of maturation (JEURISSEN et al. 1994; MAST and GODDEERIS 1998, 1999; OLÁH et al. 2014). In both conducted studies, we measured the body weight development, the relative BF and spleen weights as well as intrabursal CD4+, CD8+ T-cell numbers at different time points ph. In the first study we also determined splenic CD4+, CD8+ T-cell, B-cell and macrophage numbers, which define the B-cell (PELS) and T-cell compartments (PALS) and finally the numbers of GC in the spleen and CT.

The body weight as well as the relative bursal and splenic weight development differed significantly between the genotypes in both conducted studies ($P < 0.05$). In all experiments, BT chickens showed significantly higher body weights compared to LT and DT chickens at all investigated time points ($P < 0.05$). In contrast, the relative bursal and spleen weights were significantly lower in BT chickens at all investigated time points of both studies compared to LT chickens ($P < 0.05$). DT chickens took an intermediate position in body and relative immune organ weight development. Interestingly, female DT chickens showed a comparable relative spleen weight

development to female BT chickens, while the relative spleen weight development of male DT chickens was more similar to male LT chickens. The introduction of a dwarf gene on the female side of DT chickens has an effect on the body size and weight of the hens and might therefore be related to the similarities in relative lymphoid organ weights to female BT chickens.

It was suggested that the selection towards a fast growth and high body weight in BT chickens led to a decrease in relative growth of the immune organs and might therefore indicate a loss of immunological function (PARMENTIER et al. 1995; CHEEMA et al. 2003). The data of our studies showed that BT chickens mounted the most vigorous immune responses compared to LT and DT chickens during the first five weeks ph, which will be discussed in the next chapter. Therefore, we suggest that the relative growth of immune organs might not be the only indication for immune responsiveness. As mentioned above, the numbers of immune cell populations and the structural lymphoid organ organization should be taken into account.

The spleen represents the most important secondary lymphoid organ, which harbors important structures, which are necessary for the development of humoral immune responses (OLÁH et al. 2014). The number and size of GC, the structural organization of the PELS and PALS indicate the developmental status of the spleen (JEURISSEN et al. 1994). A varying manifestation of these structural units was suggested for different chicken lines selected for a high or low antibody response to SRBC (PARMENTIER et al. 1995; KREUKNIET et al. 1996). We demonstrated that BT chickens developed significantly higher numbers of GC in the spleen and CT with increasing age compared to age-matched LT and DT chickens. The structural development of the PELS and PALS was comparable between all genotypes. No differences were observed in the size of the structures and in the numbers of immune cells (CD4+, CD8+, B-cells and macrophages) between the genotypes. Furthermore, the age had a significant impact on the intrabursal CD4+ T-cell and splenic macrophages numbers in all genotypes. Intrabursal CD4+ T-cell numbers decreased from 14 to 28 dph and stayed a consistent level until 63 dph, which was comparable between all genotypes in both conducted studies. The numbers of splenic

macrophages also decreased with the age from 14 to 28 dph in all genotypes. We may speculate that the development of the investigated immune cell populations and structures was already finished as early as 14 dph. Therefore, we strongly suggest including earlier time points during the first week after hatch in the future.

8.1.2 Variations in innate and adaptive immune responses

In the present study, isolated spleen leukocytes were used to investigate the *in vitro* immune responsiveness of macrophages and T-cells after stimulation with either LPS or ConA. Differences in the CMI between LT and BT birds were assessed, using various LT (White Leghorn, Brown Nick) and BT chicken lines (Avian x Avian, Hubbard x Hubbard, Ross) at different ages by *in vitro* ConA and PHA stimulation of spleen cell cultures or intradermal PHA injection (CORRIER 1990; LESHCHINSKY and KLASING 2001; KOENEN et al. 2002). These studies achieved opposing results. In our studies, we could confirm that the magnitude of the CMI was significantly influenced by the genotype ($P < 0.05$). BT chickens showed the highest CMI after *in vitro* ConA stimulation of spleen cell cultures followed by the spleen cells of DT and finally LT chickens at all investigated time points. Significant differences were only observed at 35 dph. Several studies showed that immune responses in general also vary within different BT and LT chicken lines and might even depend on the B-haplotype of the chickens (GEHAD et al. 1999; KRAMER et al. 2003; PARMENTIER et al. 2006; KJAERUP et al. 2017). Therefore, variations between experiments should be considered with respect to the used BT or LT chicken lines and the age of the birds.

Macrophages as cells of the innate immune system are important for the detection and phagocytosis of invading pathogens but also fulfill regulatory functions by initiating and directing the immune responses in the beginning of an infection (KLASING 1998; QURESHI 2003). The magnitude of macrophage responses was shown to depend on the genetic background of the chickens. Studies using chickens with different B-haplotypes showed that the magnitude of iNOS activity and NO production was associated with the respective B-haplotype of the chicken lines (HUSSAIN and QURESHI 1997, 1998; DIL and QURESHI 2002). It was demonstrated that macrophages, which derived from a BT chicken line (MQ-NSCU) and a LT chicken line

($B^{15}B^{15}$), were classified as hyper-responders to LPS compared to macrophages from two other layer-type strains (GB1 $B^{13}B^{13}$ and GB2 $B^{6}B^{6}$). In our studies, *ex vivo* NO production of spleen cell cultures differed significantly between BT, LT and DT chicken lines ($P < 0.05$). The chicken genotypes used in our studies might possess different B-haplotypes. BT chicken spleen cell cultures released the highest amounts of NO compared to the other genotypes, whereas LT and DT chickens showed a comparable NO release after LPS spleen cell stimulation. Again DT chickens took an intermediate position in the macrophage activity, measured by NO release after LPS stimulation.

The genotype-associated differences in the development of the immune system and innate and adapted immune responses provided the basis for the interpretation of differences in IBDV-specific vaccine immune responses between the chicken lines.

8.2 Genotype variations after IBDV vaccination

Vaccination represents the most important tool for the protection of poultry against a variety of different diseases. The development of new vaccines against important immunosuppressive diseases, such as IBDV, is therefore steadily ongoing and necessary (MÜLLER et al. 2012; ALKIE and RAUTENSCHLEIN 2016). In our studies we used two different IBDV vaccines, which were applied at different time points during embryogenesis or after hatch for comparison between chicken genotypes.

8.2.1 Anti-IBDV specific MDA

Initial anti-IBDV MDA levels differed between the genotypes in both conducted studies. DT and LT chickens had similar initial anti-IBDV MDA levels, which were higher compared to the BT chickens in all experiments. The differences in anti-IBDV specific MDA levels can be explained by different vaccination regimes of BT and LT breeder flocks. While BT breeder flocks usually receive a one-time live IBDV vaccination, LT breeder flocks receive an additional inactivated booster vaccination. The half-life time of LT chickens' antibodies is considered to be generally higher than the half-life time of BT chickens' antibodies. Different studies reported a varying half-life time of antibodies of LT chickens ranging from three to eight days (WYETH and CULLEN 1976; SKEELES et al. 1979; WOOD et al. 1981). BT chickens' half-life time of

antibodies were reported to range from three to 5.5 days (DE WIT 1998, 2001; AL-NATOUR et al. 2004; GHARAIBEH and MAHMOUD 2013).

We observed significant differences in the decrease of anti-IBDV specific MDA between the genotypes and variations between the conducted studies. As expected, BT chickens showed the lowest initial anti-IBDV specific MDA levels and the fastest decrease in all conducted studies. LT and DT chickens showed a comparable decrease of MDAs in the first study whereas the decrease of MDAs was varying between the two genotypes in the second study. Within both experiments of the second study we observed a large deviation in the decrease of the DT chickens' anti-IBDV MDA levels in contrast to the other genotypes. Furthermore, DT chickens also showed a higher variation in mean anti-IBDV antibody levels per group and investigated time point. As a newly developed chicken line, the DT chickens might generally show a higher heterogeneity compared to long-term selected chicken lines. This might be reflected by a higher variation in the magnitude and decrease of MDA as well as by inconsistent antibody levels of the DT parent flocks (SCHIJNS et al. 2014).

8.2.2 Anti-IBDV specific humoral immune responses

While the HVT-IBD vaccine was shown to be effective in the presence of anti-IBDV specific MDA (BUBLOT et al. 2007; LE GROS et al. 2009), the intermediate plus vaccine virus strain might be neutralized by residual MDA (MCCARTY et al. 2005; RAUTENSCHLEIN et al. 2005). The Deventer formula was used to calculate the optimal time of intermediate plus live IBDV vaccination based on the breakthrough level of the vaccine, the age of the chickens at sampling and the genotype-dependent half-life time of antibodies (DE WIT 1998, 2001; BLOCK et al. 2007).

We demonstrated significant genotype-associated differences in the onset and magnitude of the anti-IBDV antibody responses between the chicken lines after HVT-IBD vaccination. BT birds showed an earlier seroconversion and a significantly higher increase of anti-IBDV antibodies compared to LT and DT chickens ($P < 0.05$). We confirmed previously observed findings that the HVT-IBD vaccine induces an adaptive

humoral immune response without interference of high anti-IBDV specific MDA levels (BUBLOT et al. 2007; LE GROS et al. 2009; PRANDINI et al. 2016).

The second study revealed differences between the genotypes in the anti-IBDV specific humoral immune response depending on the age (Exp. 1: 28 dph, Exp. 2: 35 dph) and the residual anti-IBDV specific MDA levels at the time of intermediate plus IBDV inoculation. The highly susceptible SPF chickens, which were anti-IBDV MDA negative at the time of inoculation, showed an anti-IBDV antibody response at 7 dpi in both conducted experiments. Irrespective of MDA levels, BT, LT and DT chickens showed seroconversion at 14 dpi in Exp. 1. In Exp. 2, BT and LT birds showed an earlier seroconversion at 7 dpi, while DT chickens showed seroconversion again at 14 dpi. We may speculate that the age of the birds at the time of inoculation and therefore the developmental status of the BF might also play a role in the susceptibility of the birds to the vaccine. The anti-IBDV specific antibody response in DT chickens might have been delayed due to mean MDA levels above the breakthrough level of the vaccine. The magnitude of anti-IBDV specific antibody levels was only differing significantly between the genotypes in Exp. 1, where LT chickens had significantly higher antibody levels compared to BT and DT chickens at 14 dpi and compared to DT chickens at 28 dpi ($P < 0.05$). Based on the ELISA system, the present study only detected anti-IBDV specific IgG antibodies. Further differences might be determined in the future by the use of additional serological tests, such as the VNT. BT and LT chickens were also shown to differ in the ability to mount IgM and IgG specific responses (KOENEN et al. 2002), therefore IgM specific antibody responses could be included in future investigations.

8.2.3 IBDV vaccine-induced lesions

IBDV might cause gross lesions of different lymphoid organs as early as three dpi. We specifically looked for macroscopic changes of the BF, such as bursal edema, hemorrhagic foci and hemorrhagic discoloration. Furthermore, the spleen was investigated for splenomegaly and white pulp hyperplasia. HVT-IBD vaccination did not lead to any macroscopic lesions in any of the chicken genotypes. Intermediate plus inoculated chickens of the second study showed bursal edema and swollen spleens at

7 dpi in both conducted experiments, except DT chickens, which did not show any lesions in Exp. 1 of the second study.

Depending on the virulence of the vaccine strain, live IBDV vaccines might cause severe bursal atrophy (KIM et al. 1999). In the present study, the inoculation with an intermediate plus strain led to bursal atrophy in all inoculated genotypes. At 7 dpi all inoculated genotype groups showed a significant bursal atrophy, which lasted until the end of all experiments (28 dpi). However, DT chickens showed a delayed bursal atrophy at 14 dpi in Exp. 1 of the second study. Residual MDA levels might have prevented the induction of macroscopic lesions and bursal atrophy in DT chickens.

IBDV infection might lead to severe lymphocyte depletion in the bursal follicles, which mainly affects the target cells of IBDV, the IgM+ B-lymphocytes (KAUFER and WEISS 1980). Histological and immunohistochemical investigations were used to determine the bursal lesions and B-cell depletion and were evaluated by a specific scoring system. No bursal lesions and B-cell depletion was observed after HVT-IBD vaccination in any of the inoculated genotype groups. The intermediate plus IBDV inoculation led to severe bursal lesions and B-cell depletion, which peaked in all inoculated genotype groups at 7 dpi. Again DT chickens showed a delayed start of bursal lesions at 14 dpi (Exp. 1).

8.2.4 The HVT – “adjuvant” effect

During the first days ph, chickens get in contact with an environment full of new antigens, despite their still developing immune system. Therefore, *in ovo* vaccination might stimulate an early induction of innate and adaptive immune responses, which might lead to a better immunocompetence and protection against early ph pathogen exposure in young chickens (SHARMA and GRAHAM 1982; GIMENO et al. 2015). HVT was shown to be most suitable as a vector virus for the development of recombinant vaccines against different pathogens and can be applied by the *in ovo* route (DARTEIL et al. 1995). Several studies demonstrated that HVT might have a positive effect on the development of the immune system and vaccine efficacy of chickens. *In ovo* HVT inoculation of SPF LT chickens at EID 18 led to an increase of

MHC-I expression and lymphoproliferative responses of T-cells. In addition, they developed a stronger immune response against different unrelated antigens such as KLH, PHA and ConA at hatch compared to non-inoculated control groups (GIMENO et al. 2015). Furthermore, HVT was shown to have synergistic, protective effects when combined with other vaccines (WITTER 1992; NAZERIAN et al. 1996).

In the present study we therefore evaluated the impact of the *in ovo* HVT-IBD vaccination at EID 18 on secondary humoral immune responses against IBV and NDV vaccination at the first day ph and the development of the BF and spleen with their functional compartments and immune cell populations. In the field many vaccines are commonly applied during the first days after hatch to induce a protective immunity as early as possible.

We observed a significant influence of HVT-IBD vaccination on secondary antibody responses. A field study in commercial LT chickens demonstrated that the antibody development against other viral diseases, such as IBV, NDV and EDS was significantly higher in HVT-IBD vaccinated groups compared to other live IBDV vaccinated groups (PRANDINI et al. 2016). The live IBDV vaccines induced a higher B-cell depletion in the BF and in the blood. This may explain why the groups, which received the live IBDV vaccines, developed a lower antibody response against the other pathogens (PRANDINI et al. 2016; ROH et al. 2016). In the present study we detected significantly higher anti-IBV and –NDV antibody levels at some of the investigated time points in HVT-IBD vaccinated groups compared to non-HVT-IBD vaccinated control groups at different time points after vaccination. These data provide further evidence that HVT might be enhancing the immune responses against other pathogens.

Furthermore, we could see a genotype-dependent increase in the relative spleen weights (LT birds) and number of GCs in the spleens (BT birds) of HVT-IBD vaccinated groups compared to non-HVT-IBD vaccinated control groups. KREUKNIET et al (1996) reported that after immunization with SRBC, chickens selected for high antibody responses developed larger and more PELS and GC in the spleen compared to chickens selected for low antibody responses. Higher numbers of macrophages in the spleen, forming the outer ring of the PELS, were associated with the ability to mount

higher humoral immune responses (KREUKNIET et al. 1996). In the present study, we detected a significant increase of macrophage numbers in the spleen of BT and DT HVT-IBD vaccinated groups compared to the corresponding non-HVT-IBD vaccinated control groups ($P < 0.05$). BT chickens generated the highest anti-IBDV antibody response, while DT chickens showed a comparable anti-IBDV antibody response to LT chickens. GCs are important for the induction of humoral immune responses, Ig isotype switching, affinity maturation and B-cell memory development (YASUDA et al. 2003). Therefore, we may speculate that the higher numbers of GC and macrophages in the spleen after HVT-IBD immunization might be related to the magnitude of the antibody response in BT chickens.

Nevertheless, the mechanisms behind HVT immunomodulation are still unknown. Similar to MDV, HVT shows latency in MHC-II+ T-cells (SCHAT and NAIR 2013). The continuous antigen presentation during latency in lymphoid tissues might stimulate or modulate the host immune responses (TSUKAMOTO et al. 2002). Studies in murine infection models showed that some herpesviruses might induce cross-protection by up-regulation of specific cytokines during the latent phase of infection (BARTON et al. 2007). Experimental *in vivo* and *in vitro* studies showed that HVT modulates different gene expression pathways and up-regulates cytokine genes, such as IL-10 and IFNγ, which play an important role also during MDV pathogenesis (KARACA et al. 2004; ABDUL-CAREEM et al. 2007). Further investigations should focus on the potential activation of specific immune cells during HVT latency, which might contribute to the immunomodulatory effects.

8.3 The role of T-cell immunity in IBDV infection and bursal recovery

8.3.1 Genotype-variations in T-cell accumulation and virus clearance

T-cells are resistant to IBDV infection (MÜLLER et al. 1979b). They still play an important role in vaccine protection and the recovery process. After IBDV infection, high numbers of CD4+ and CD8+ T-cell infiltrate into the BF (TANIMURA and SHARMA 1997; KIM et al. 1998). T-cells not only seem to play an important role in inflammation, virus clearance and bursal lesion development but also in the recovery process (KIM et al. 2000; RAUTENSCHLEIN et al. 2002). The magnitude and onset of T-cell accumulation was shown to differ between chicken lines as well as the onset and extent of virus replication during the acute phase of IBDV infection (MOODY et al. 2000; RUBY et al. 2006; ARICIBASI et al. 2010; TIPPENHAUER et al. 2013). The varying virulence of the virus strains used might also lead to different results by comparing different chicken lines (TIPPENHAUER et al. 2013). We hypothesized that T-cell responses beyond the acute phase of infection during the recovery process might also vary between different chicken lines.

We saw a peak in IBDV antigen loads in all IBDV inoculated (iIBDV) groups at 7 dpi, which was accompanied by the highest numbers of infiltrating CD4+ and CD8+ T-cells and the highest bursal lesion scores. All iIBDV groups showed a decline in IBDV antigen positive cells overtime, but iIBDV BT groups had the lowest number of IBDV antigen positive cells at 28 dpi in comparison to all other iIBDV genotypes. In iIBDV BT groups the decrease in IBDV antigen was accompanied by a continuous decline in CD4+ and CD8+ T-cell numbers and bursal lesions. iIBDV LT and DT groups still had higher CD4+ and CD8+ T-cell numbers and bursal lesions until 21 and 28 dpi despite the decline in IBDV antigen positive cells. The fold increase of intrabursal CD8+ T-cells between iIBDV and control groups was much higher compared to the fold increase of intrabursal CD4+ T-cells. Cytotoxic CD8+ T-cells were shown to play an important role in the elimination of virus-infected cells, which was suggested to be mediated by Fas/Fas-L and PFN-Gzm-A pathways in IBV, RSV, AIV and also IBDV infection (HOFMANN et al. 2003; KAPCZYNSKI et al. 2011; RAUF et al. 2012; TAN et al. 2016). ARICIBASI et al (2010) demonstrated that BT chickens had significantly higher

numbers of intrabursal CD8+ T-cells at three and five dpi after virulent and vv IBDV inoculation compared to LT chickens, which might lead to a faster virus clearance and less clinical signs. We could not confirm this observation but may speculate that differences between studies might also be related to the varying virulence of IBDV strains. Therefore, the observed differences in the duration of intrabursal T-cell accumulation and bursal lesions between the genotypes could not be fully explained by the decline in IBDV antigen.

8.3.2 "Treg-related" cytokine mRNA expression

Inflammatory reactions during the acute phase of IBDV infection have been well studied and were proposed to be the dominating immune response during the acute phase of infection (ETERRADOSSI and SAIF 2013). CD4+, CD8+ T-cells and macrophages mainly contribute to the induction of inflammation through the release of pro-inflammatory cytokines during the acute phase of infection (ELDAGHAYES et al. 2006; RAUW et al. 2007; INGRAO et al. 2013). Effector CD4+ T-cells can differentiate into many T-helper (Th) subsets, which contribute to varying effector functions. In chickens, the Th1/Th2 effector cell polarization was demonstrated before (DEGEN et al. 2005) but has to be fully determined also at the molecular and cellular level (KAISER and STÄHELI 2014; DAI et al. 2019). In mammals, the natural Tregs are $CD4^+CD25^+$*foxp3*$^+$ (LI et al. 2008). Two subpopulations of chicken regulatory T-cells were described: the $CD4^+CD25^+$ and $CD4^+TGF\beta^+$ T-cells (SHANMUGASUNDARAM and SELVARAJ 2011; GURUNG et al. 2017). Despite the fact, that the avian *foxp3* orthologue gene has not been determined yet, several studies investigated the involvement of $CD4^+CD25^+$ T-cells as avian Tregs in different bacterial and viral diseases including IBDV (SELVARAJ 2013; SHANMUGASUNDARAM et al. 2015; YU et al. 2015; LEE et al. 2017). Avian $CD4^+CD25^+$ T-cells were shown to possess a similar cytokine expression profile to mammalian natural Tregs, which is characterized by the expression of IL-10 and TGFβ. After IBDV or *Salmonella* Enteritidis infection, elevated IL-10 mRNA expression levels were associated with significantly increased $CD4^+CD25^+$ Treg numbers in the BF or CT, respectively (SHANMUGASUNDARAM et al. 2015; YU et al. 2015).

During the acute phase of IBDV infection IL-10 and TGFβ4 were shown to be either up- or downregulated depending on the virulence of the virus strain (ELDAGHAYES et al. 2006; JAIN et al. 2013; RASOLI et al. 2015; YU et al. 2015). In our studies we compared the IL-10 and TGFβ4 mRNA expression in the BF between the genotypes after intermediate plus IBDV inoculation during the recovery phase until 28 dpi. We demonstrated that the duration of elevated IL-10 mRNA expression was significantly influenced by the chicken genotype. While fast recovering iIBDV BT groups had elevated IL-10 mRNA levels until 7 dpi (Exp. 2) and 14 dpi (Exp. 1), iIBDV LT and DT groups showed elevated IL-10 mRNA levels until 21 and 28 dpi. We may speculate that this increased intrabursal IL-10 expression in iIBDV LT and DT can be associated with the higher intrabursal T-cell accumulation.

The mRNA expression pattern of TGFβ4 was unclear. TGFβ4 was either up- or downregulated, which was variable between genotypes, time points and experiments. We suggest that TGFβ4 might be mostly regulated during the acute phase of infection and after infection with more virulent strains.

8.4 Conclusions

Innate and adapted immune responses as well as the development of the immune system were significantly influenced by the chicken genotype. Furthermore, the immune responses after application of different IBDV vaccines also differed significantly between the genotypes.

We demonstrated that DT chickens take an intermediate position between BT and LT chickens with respect to the investigated immune parameters but show more similar immune responses to the LT chickens. As a cross-breed of BT and LT chicken lines, DT chickens might possess mixed traits of both chicken lines. Nevertheless, the decrease of anti-IBDV specific MDA in DT chickens varied significantly from LT chickens and differed between the experiments. We therefore conclude and suggest that vaccination regimes in the field need to be modulated depending on the respective genotype in the flock.

Concerning the vaccination with conventional live IBDV strains, newly developed chicken lines might need to receive multiple doses to ensure the protection and uniformity of antibody levels in the flocks. *In ovo* HVT-IBD vaccination induced a detectable anti-IBDV specific humoral immune response in all genotypes, despite residual anti-IBDV MDA levels. The differences in the magnitude of anti-IBDV specific antibody development between the genotypes led to the conclusion that irrespective of the genotype, lower MDA levels at the time of inoculation might lead to stronger anti-IBDV specific humoral immune responses.

Our studies revealed a significant influence of the genotype on the IBDV recovery process, which was indicated by differences in the duration of intrabursal T-cell accumulation and bursal lesions but interestingly could not be fully explained by the decline in IBDV antigen. It can be suspected that Tregs might be involved in the recovery process after IBDV infection and that these cells might be modulated differently depending on the chicken genotype. Furthermore, the findings of the present study provide cicrumstantial evidence that IL-10 plays a major role in the recovery process after IBDV infection.

8.5 Future perspectives

8.5.1 IBDV vaccination and pathogenesis

The HVT-IBD vaccine can also be applied at the first day of life. Preliminary findings demonstrate that the magnitude of the anti-IBDV specific humoral immune response differs between *in ovo* or ph vaccinated genotypes (unpublished results). This might indicate differences in the developmental status of the immune system between LT and BT chickens, which should be investigated in the future. From our studies, we suggest that additional time points, at the time of HVT-IBD vaccination and during the first week after HVT-IBD vaccination need to be included in future studies.

Many studies indicated that the chicken MHC is associated with resistance or susceptibility to different infectious diseases. For IBDV infection, resistant and susceptible chicken lines with different MHC-B-haplotypes were determined based on the antibody response against inactivated as well as live attenuated IBDV vaccine and

based on the severity of bursal lesions (JUUL-MADSEN et al. 2002, 2006). The distribution of different MHC-B-haplotypes in commercial chicken lines is still scarcely known. Newly developed techniques, such as next generation sequencing (NGS) methods for typing all the classical MHC alleles present within birds should help to determine the MHC alleles of birds from commercial breeding companies (TREGASKES and KAUFMAN 2018). As a consequence, this information might provide the basis for future studies in disease resistance and susceptibility and might even be included in the selection and breeding of more resistant birds for the commercial poultry industry.

8.5.2 IBDV as a model to study Tregs

T-cell immune responses were shown to play an important role during IBDV infection. Our studies indicated that Tregs might be involved in the pathogenesis and bursal recovery by demonstrating significant up-regulation of Treg-related cytokines in the BF, specifically IL-10. We saw elevated levels of IL-10 accompanied by high numbers of infiltrating CD4+ and CD8+ T-cells. The specific marker for Tregs, the *foxp3*, still has to be determined to further investigate the role Tregs during IBDV infection. Studies in mice and humans showed that IL-10 is not only produced by Tregs, but also by macrophages, DCs, B-cells and other T-cell subsets (MOORE et al. 2001; COUPER et al. 2008). Therefore, further immune cell populations, such as macrophages or endothelial cells have to be considered to play a role in the recovery process and should be addressed in future studies. In the last years a lot of progress has been achieved in the development of techniques for the generation of targeted gene knockouts in chickens (transgenic chickens) (SCHUSSER et al. 2013; SCHUSSER et al. 2016; SID and SCHUSSER 2018). By modification of chicken primordial germ cells (PGC), gene knockout experiments targeting parts of the B-cell or T-cell system (γδ T-cells) have been developed or are currently under investigation (SCHUSSER et al. 2013; HELLMICH et al. 2018). In the future, targeted T-cell knockout studies might reveal new insights into the importance of different T-cell subsets during IBDV pathogeneses and bursal recovery.

8.5.3 Dual-purpose chickens (Lohmann Dual)

During the last years, animal welfare concerns constantly increased with respect to intensive livestock farming. In the poultry industry in Germany one major concern is the killing of one day male LT chickens. DT chickens, which can be used for both meat- and egg production might present one solution to this problem (ICKEN and SCHMUTZ 2013; DAMME et al. 2015). The use of DT chickens might be the only solution to avoid this practice, at least transitionally, especially when other approaches, such as the *in ovo* sex determination techniques are still not feasible for large-scale application in the commercial poultry production system (WEISSMANN et al. 2013; GALLI et al. 2016, 2017). Since, immunoprophylactic measures are one of the most important tools for the health management of commercial poultry flocks, studies were and are still needed, which address the immunocompetence of DT chickens in comparison to other commercially available chicken lines. For the use of DT chickens in the field, the results of this part of the “Integhof” project suggest that prophylactic strategies should be modified depending on the genotype to optimize the immunocompetence of the respective flocks. Further continuous selection of DT chickens might lead to a higher uniformity of these chickens in their performance parameters and immune responses in the future.

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