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Marek's disease virus-host interplay: novel insights into lymphocyte infections of an oncogenic avian herpesvirus.

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Success is not final, failure is not fatal: it is the courage to continue that counts.

Not from Winston Churchill,
but from a 1930s Budweiser advertising campaign.

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2 List of figures

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4 List of abbreviations

AnHV-1	Duck plague virus
APC	Antigen presenting cell
BAC	Bacterial artificial chromosome
BoHV	Bovine herpesvirus
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
Cas9	CRISPR associated protein 9
CD	Cluster of differentiation
CoHV-1	Pigeon herpesvirus
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL	CXC ligand
CXCR	CXC receptor
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dpi	days post infection
EBER	Epstein-Barr virus-encoded RNA
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FFE	Feather follicle epithelium
FFPE	Formalin-fixed paraffin embedded
FHV	Feline herpesvirus
FISH	Fluorescence *in situ* hybridization
GaHV	Gallid herpesvirus
gC	MDV glycoprotein C
GO	Gene ontology
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HVT	Herpesvirus of turkey
ICP4	Infected cell protein 4
ICTV	International Committee on Taxonomy of Viruses
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
ILTV	Infectious laryngotracheitis virus
IMS	Imaging mass spectrometry
iNOS	Inducible nitric oxide synthase
IRES	Intronic internal ribosome entry site
IR_L	Internal repeat long
IR_S	Internal repeat short
JH-KO	JH knockout
kbp	Kilo base pairs
kDa	kilo Dalton
KSHV	Kaposi’s sarcoma-associated herpesvirus

LAT	Latency associated transcripts
LC-MALDI TOF/TOF	Matrix-assisted laser desorption/ionization time of flight
MD	Marek's disease
MDV	Marek's disease virus
MeHV	Meleagrid herpesvirus
MEM	Minimal essential medium
meq	MDV Eco Q-encoded protein
MHC	Major histocompatibility complex
miRNA	micro RNA
mTMR	multiple telomeric repeats
MΦ	Macrophage
n	sample size
NK cells	Natural killer cells
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming units
PGC	Primordial germ cells
pp14	Phosphoprotein 14
pp38	Phosphoprotein 38
PsHV-1	Pacheco's parrot disease virus
qPCR	quantitative PCR
RLORF4	Repeat long open reading frame 4
RLORF5a	Repeat long open reading frame 5a
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
sTMR	short telomeric repeats
TALEN	Transcription activator-like effector nuclease
TERT	Telomerase reverse transcriptase
TMR	Telomeric repeat region
TR	Telomerase RNA
TR_L	Terminal repeat long
TR_S	Terminal repeat short
U_L	Unique long
UL49.5p	MDV transmembrane protein UL49.5
U_S	Unique short
US3	MDV viral serine/threonine protein kinase
vIL8	Viral Interleukin 8
vLIP	MDV viral lipase homolog
VP5	Virus protein 5
vTR	Viral telomerase RNA
VZV	Varicella zoster virus

5 Prologue

“Really? – a chicken herpesvirus? I didn’t even know that herpes exist in chickens”.

During my time as a PhD student, I often had to convince people of the importance and relevance of our work and how it contributes to a better understanding not only of the virus-host interplay in avian species, but also of virus-induced cancers in general.

Chickens have served as model organisms for centuries: The field of embryology is based on discoveries made in chickens [1], a species that also vastly contributed to our understanding of the major concepts in immunology, genetics, cell biology, and, last but not least, virology and cancer [2]. Moreover, poultry (and especially chicken) production and consumption will likely continue to grow and strengthen its dominant position within the meat complex. Poultry meat already accounts for almost 45% of all meat that is consumed worldwide [3] and is one of the most important sources of animal protein. Research into poultry diseases will therefore improve the quality of poultry farming particularly in developing countries.

Viruses are incredible little pathogens– there is much more to add to this. But just consider this: there are approximately 1 million virus particles per milliliter of seawater [4]. Isn’t that fascinating enough already? As one of nine virus orders that have been identified yet and classified by the International Committee on Taxonomy of Viruses (ICTV) [5], herpesviruses have many very intriguing features including the establishment of lifelong infections termed latency, infections of both vertebrate and non-vertebrate species and associations with various cancers [6-8].

One of those herpesviruses is Marek’s disease virus, an important poultry pathogen that is able to rapidly transform host cells into cancer cells [9].

My PhD thesis, entitled “Marek’s disease virus-host interplay: novel insights into lymphocyte infections of an oncogenic avian herpesvirus” combines the aforementioned research areas and kept me active and curious during my PhD the last 4 years.

6 Introduction

6.1 Herpesviruses

Herpes is forever! This expression is based on the fact that herpesviruses have the ability to remain in the host for life by establishing a latent phase of infection. This hallmark of herpesvirus infections ensures lifelong virus persistence and escape from the host immune system. The latent virus can occasionally reactivate resulting in disease and virus spread. Other common features of herpesviruses are their host specificity and a long evolutionary history of coevolution with their host species, and they are enveloped viruses that possess a large DNA genome [10].

The classification of herpesviruses is complex and herpesviruses are found in mammals and birds, but also in fish, frogs, reptiles, and so far a single herpesvirus in bivalves (molluscs) [11]. All herpesviruses are assigned to the order *Herpesvirales* with three distinct families: the *Herpesviridae* (that infect mammals, birds, and reptiles), the *Alloherpesviridae* in fish and amphibians, and the *Malacoherpesviridae* in invertebrates. Furthermore, the family of *Herpesviridae* is divided into the subfamilies *Alpha*-, *Beta*- and *Gammaherpesvirinae* with prominent members like the human herpesviruses such as herpes simplex and Epstein-Barr virus. With steadily increasing numbers of identified virus species, the *Herpesviridae* family consists of more than 200 members [12]. More and more full genome sequences are available for a tremendous number of herpesviruses that need to be classified. For example, the International Committee on Taxonomy of Viruses (ICTV) Herpesvirales Study Group currently proposes the classification of 18 new species into existing genera [13].

To date, 9 human herpesviruses exist [10] and among those are representatives from each of the three subfamilies: Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) varicella-zoster virus (VZV), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6A and 6B (HHV-6A and HHV-6B), human herpesvirus 7 (HHV-7), and Kaposi's sarcoma-associated herpesvirus (KSHV). Among the veterinary herpesviruses, the most prominent members are viruses that infect pigs (pseudorabies), cattle (BoHV), horses (EHV), cats (FHV) and avian species (see below).

The most important avian herpesviruses that causes dramatic losses in poultry industry worldwide of up to 1-2 billion US-dollar annually, is Marek’s disease virus (MDV) [14]. Besides MDV, other avian herpesviruses like infectious laryngotracheitis virus (ILTV), herpesvirus of turkeys (HVT), Pacheco's parrot disease virus (PsHV-1), pigeon herpesvirus (CoHV-1), and

duck plague virus (AnHV-1) are of importance to veterinarians, poultry industry and bird keepers [15].

Herpesviruses are enveloped viruses and their shape is described as spherical to pleomorphic. They possess an icosahedral symmetry and usually have a diameter of 150 – 200nm in size. The lipid envelope bilayer is obtained by budding at an intracellular membrane. It embeds several different protruding glycosylated envelope proteins that form spike structures on the virus surface. The envelope surrounds an outer and inner amorphous protein coat, the tegument. The next layer, the nucleocapsid, consists of 162 capsomers of which 150 are hexameric and 12 are pentameric. The nucleocapsid protects the virus core that contains a linear double stranded DNA, which is monopartite and 120 – 240 kpb in size depending on the herpesvirus species [10-12].

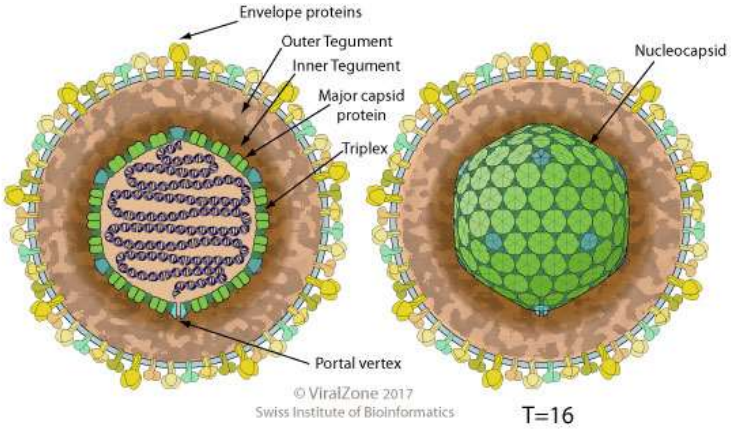

Figure 1: Structure of the alphaherpesvirinae virion

Herpesviruses are enveloped, spherical to pleomorphic viruses with an icosahedral symmetry and a diameter of 150 – 200nm. The lipid envelope embeds several different glycoprotein complexes (envelope proteins) and surrounds an outer and inner amorphous tegument. The nucleocapsid consists of 162 capsomers (150 are hexameric and 12 pentameric) and protects the linear double stranded DNA, which is monopartite and has a size of 120 – 240 kpb [10]. (Image from https://viralzone.expasy.org)

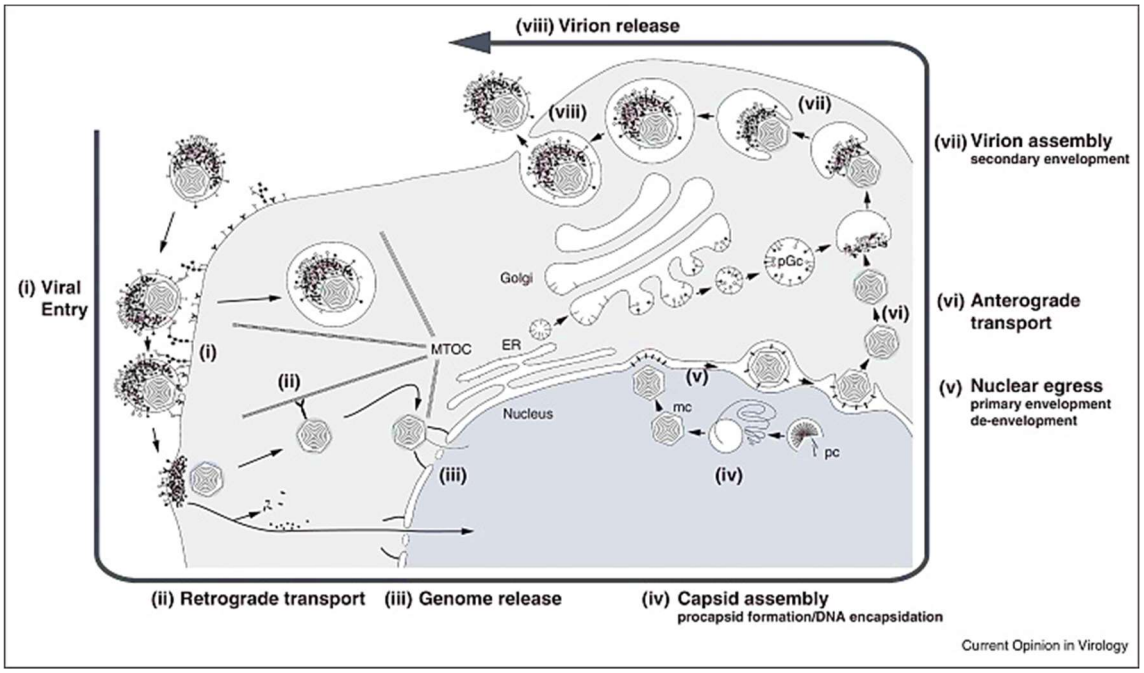

Figure 2: The herpesvirus life cycle

A herpesvirus infection (in this case exemplarily depicted for HSV) starts with entry of the virus by fusion of the viral envelope with the host membrane (i). In the host cell, the capsid is then transported to the nucleus along microtubules (ii) and the viral genome is released into the nucleus through nuclear pores (iii). Upon circularization of the viral DNA, the virus genome is replicated in the nucleus as concatamers, followed by the procapsid (pc) formation and subsequent encapsidation of cleaved DNA (iv). Capsids exit the host cell nucleus by an envelopment and de-envelopment mechanism in the nuclear membrane (v). Capsids are then transported to the locus of virion assembly (vi). Assembly includes a secondary envelopment and tegumentation and occurs close to the cell surface by budding into cellular vesicles. These vesicles originate from the Golgi complex (vii). Virions are released from the cell by vesicle fusion with the host cell plasma membrane (viii). MTOC = microtubule organizing center, ER = endoplasmic reticulum, mc = mature capsid. (Image and legend modified from [16])

6.2 Herpesvirus replication

Herpesvirus replication has been most extensively studied for the prototype members of the human herpesviruses, especially in HSV-1 [17]. The replication cycle of herpesviruses starts with cell entry by attachment of viral envelope glycoproteins to the host cell membrane and subsequent fusion. Upon fusion, the nucleocapsid penetrates the host plasma membrane. It subsequently enters the cytoplasm and is transported to the nucleus. There, the viral genome is released into the nucleus through the nuclear pore. Upon circularization of the DNA, the virus genome is transcribed in the nucleus as concatamers, a process that is followed by the procapsid formation and encapsidation of cleaved DNA. Herpesvirus procapsids are assembled in the nucleus of infected cells and final maturation of the virion occurs in the

cytosol. Capsids exit the nucleus by a primary envelopment and de-envelopment mechanism at the inner and outer nuclear membrane. The mature capsids are then transported to the site of virion assembly where it acquires the final tegument and the secondary envelopment. These steps of the virus life cycle occur close to the cell surface by budding into cellular vesicles originating from the Golgi complex. Virions are released from the cell by fusion of these cellular vesicles with the plasma membrane [16, 18].

The virus of interest in this thesis is MDV, an important avian herpesvirus that causes a devastating malignant tumor disease. The MDV story began in the early years of the 20th century…:

6.3 Marek's disease virus history

A Hungarian veterinary doctor, József Marek, discovered a disease in chickens in 1907. He examined chickens that suffered from severe paralysis of the legs and wings and described it as a polyneuritis after *post mortem* observation of thickened plexus and sciatic nerves [19]. His findings were published in the German journal "Deutsche Tierärztliche Wochenschrift" (Fig. 3) and set the basis for more than 100 years of MDV research with all its success stories and pitfalls. Similar reports were published in the following years by Kaupp (USA) and van der Walle (The Netherlands) [20, 21]. However, it took more than 20 years to link this polyneuritis disease to tumors. This link was confirmed by Pappenheimer *et al.* in 1929 [22, 23]. Following their findings, the disease terms that were previously used became unsatisfactory. A new term that Pappenheimer *et al.* suggested was "neurolymphomatosis gallinarum", which described both the neuronal lesions and the lymphoma [22, 23].

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Landestierarzt Prof. Dr. Edelmann, Medizinalrat in Dresden, Landestierarzt Feist, Regierungsrat in Strassburg i. E., Veterinärrat Dr. Garth in Darmstadt, Bezirkstierarzt Dr. Görig in Buchen, Oberamtstierarzt E. Theurer in Ludwigsburg und Prof. Dr. Vogel in Stuttgart

redigiert von Prof. Dr. Malkmus in Hannover.

№ 30. Ausgegeben am 27. Juli 1907. 15. Jahrgang.

Multiple Nervenentzündung (Polyneuritis) bei Hühnern.

(Aus der medizinischen Klinik der tierärztlichen Hochschule in Budapest.)

Von Prof. Dr. J. Marek.

(Mit 2 Abbildungen.)

Unsere Kenntnisse bezüglich der nervösen Erkrankungen des Geflügels sind derzeit noch recht bescheiden. In der Literatur findet man nämlich nur in sehr spärlicher Zahl von Nervenkrankheiten bei Vögeln beschrieben, deren anatomische Grundlage zudem gewöhnlich nicht näher untersucht wurde. Eine Ausnahme hiervon bildet die Beri Beri-ähnliche Polyneuritis der Hühner in Niederländisch-Indien, welche von Eykmann[1]) beschrieben und hin-

Unter dem Einfluss des von Eykmann supponierten Giftes entsteht nun in den peripherischen Nerven eine Degeneration, welche allmählich zum Zerfall der Markscheide der Nervenfasern führt, infolgedessen dann die Schwannsche Scheide eine Art Emulsion von Myelin-Kügelchen enthält und schliesslich die ganze Nervenfaser in einen dünnen, marklosen Faden sich verwandelt. Dabei verfallen die zugehörigen Muskeln der einfachen Atrophie.

Klinisch kennzeichnet sich die Krankheit durch sowohl an In- als an Extensität rasch zunehmende Lähmungserscheinungen seitens der Beine, so dass die letzteren alsbald gespreizt und im Knie- und Mittelfussgelenk gebeugt gehalten werden und das Tier beim Laufen öfters sinkt oder auch umfällt. Schliesslich wird das Belasten der Füsse überhaupt unmöglich und in diesem Stadium

Figure 3: First MDV publication (J. Marek, 1907)

"Multiple Nervenentzündung (Polyneuritis) bei Hühnern" – published in the *Deutsche Tierärztliche Wochenschrift* in 1907 by the veterinary clinician and pathologist Dr. Josef Marek. He was professor and head of the Department of Veterinary Medicine at the Royal Hungarian Veterinary School in Budapest, Hungary. (Image from [24])

MDV research developed and faced different challenges: for example, there were extensive discussions about the correct diagnosis of different lymphomas of MD and lymphoid leucosis, such as avian leucosis that is a retroviral disease and causes a variety of neoplastic conditions in chickens.

In the early 1960's, Sevoian and Chamberlain provided evidence of the transmissibility of MD between chickens. To do so, they inoculated healthy chickens with blood, tissue and tumor cell suspensions from infected birds [25]. A few years later, in 1967, Churchill and Biggs identified a herpesvirus as the causative agent of MD [26]. Only then, the virus was designated as Marek's disease virus [27]. Over time, Marek's disease developed from a rather mild disease into a highly contagious lymphoproliferative disorder of chickens with a clinical picture that has changed dramatically since its initial recognition. Additional to the neurological signs and tumors, very virulent plus strains can nowadays cause severe brain edemas and acute deaths, and tumor lesions even in vaccinated chickens [28-31]. There is comprehensive evidence that the increasing virulence of MDV stains emerged independently in North America and Eurasia [32]

6.4 General facts and MDV replication cycle

6.4.1 MDV

MDV belongs to the genus *Mardivirus* in the subfamily *Alphaherpesvirinae* in the order *Herpesvirales*. Five different serologically related but distinct virus species belong to this genus: Gallid alphaherpesvirus 2 (GaHV-2/MDV), Gallid alphaherpesvirus 3 (GaHV-3/MDV serotype 2), the Meleagrid alphaherpesvirus 1 (HVT), and two viruses of quail and pigeons (Anatid alphaherpesvirus 1 and Columbid alphaherpesvirus 1) (ICTV Virus Taxonomy: 2017 Release). MDV are highly contagious viruses that cause lymphoma and various other symptoms, whereas the natural occurring but non-pathogenic serotype 2-strains can be used as live vaccines to protect from Marek's disease virus infections (same as the also closely related HVT) [33]. Despite of the widespread use of live attenuated vaccines, MDV remains a major pathogen of poultry and causes approximately one to two billion euros loss worldwide every year [14]. Even though Marek's disease vaccines are highly effective in minimizing commercial losses due to tumor formations and neurological deficits, they do not provide sterilizing immunity and thereby allow a continued evolution of MDV strains in vaccinated chickens [34-37]. This gradual evolution towards a greater virulence allows MDV to overcome the protection of current vaccines and poses a serious threat to poultry production. Furthermore, MDV is important for biomedical research as it is used as a versatile and convenient small-animal model for virus-induced tumor formation [9].

6.4.2 Clinical symptoms

The clinical picture of the disease can be described as follows: chickens infected with MDV will likely show clinical signs with several appearances, including neurologic, visceral, ocular and cutaneous forms [38]. Neurological manifestations may vary according to the nerve(s) affected and paralysis of one or more of the extremities can be observed. The wings can also be affected. Torticollis can appear due to an inflammation of nerves controlling the neck and vagal involvement will lead to dilatation of the crop. Visceral lesions derive from T cell lymphoma metastases and can occur in nearly every visceral organ. However, mostly heart, liver, spleen, kidneys, proventriculus, testes/ovaries, and muscles are affected [37]. In the ocular and cutaneous forms, blindness may occur (caused by iridocyclitis) and lymphoid proliferation in skin and feather follicles can happen, respectively [14, 39]. In MDV infections, nonspecific signs such as weight loss, paleness, anorexia, and diarrhea are also observed. Of note is, that MDV infections can lead to an acute mortality syndrome, where affected birds die with an early acute cytolytic disease prior to tumor formation [38].

Figure 4: Clinical MDV signs

Clinical signs that are observed in MDV infected chickens are torticollis, ataxia, and paralysis of the legs and wings (due to an enlargement of peripheral nerves). MDV infections may also cause increased mortality in chicklets of 1–2 weeks of age and lymphomatous lesions can occur in multiple organs such as the ovary, liver, spleen, kidneys, lungs, heart, proventriculus and skin - depending on the strain of MDV.

6.4.3 MDV replication

MDV has a complex replication cycle that can be divided in a productive (lytic) replication stage and a latent stage of infection [14]. As other herpesviruses, MDV can establish latency, whereby latency is described as the ability of the virus to lie dormant (latent) within a cell [40]. The widely accepted *Cornell model* of the MDV life cycle starts with an early macrophage infection in the lung after inhalation of airborne cell-free virus particles from a contaminated environment [41]. These cells transport the virus to the primary lymphoid organs, the bursa of Fabricius, thymus and spleen. In these organs, the virus is transferred to B cells, which are the primary target cells for lytic replication in an infected chicken. The massive lytic replication in B cells (and later also in T cells) leads to an immunosuppression which increases the susceptibility of infected birds to other infectious agents [42]. The infection of B cells results in the activation and infection of CD4+ T cells by direct cell-to-cell transfer [43-45]. In activated CD4+ T cells, the virus is able to establish a latent infection in which it integrates its genome into host telomeres. Virus integration is facilitated by viral telomeric repeats (TMRs) [46-48]. Consequently, those T cells can be transformed by the virus and MDV-transformed cells possess a regulatory T cell phenotype based on their cytokine and cell surface marker expression profiles [49, 50]. The latent virus can occasionally reactivate resulting in lytic replication of the virus. The efficiency of lymphoma formation is dependent on the virus strain and the genetic background of the chickens [51]. In order to be transmitted to other chickens, infected CD4+ T cells transport the virus to the skin where it replicates in the feather follicle

epithelial (FFE) cells. Those FFE cells release keratin-encased virus into the environment by desquamation of chicken dander [43, 52].

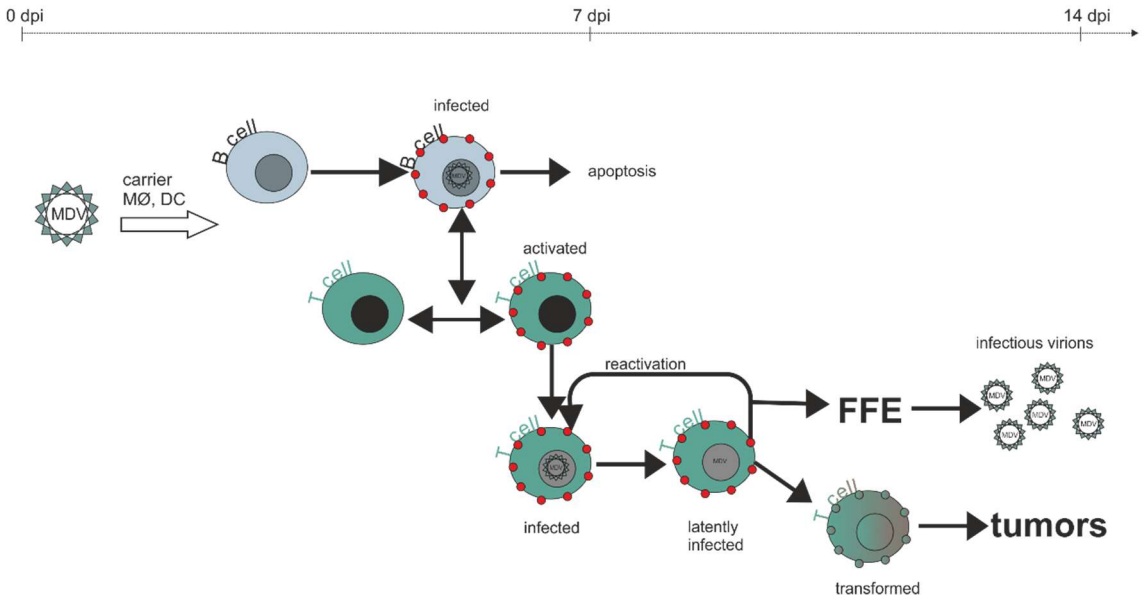

Figure 5: The MDV life cycle

Infection is initiated by the inhalation of cell-free MDV. Macrophages and dendritic cells are thought to transfer the virus to lymphoid organs such as spleen, thymus and bursa, where the virus infects and replicates in B cells. This leads to either B cell apoptosis or a subsequent infection of activated T cells. MDV is able to establish latency in infected T cells with virus reactivation or T cell transformation as two possible consequences. Latently infected T cells can transport the virus to the skin and the feather follicle epithelia (FFE), where cell free MDV is generated. MØ: macrophages; DC: dendritic cells; FFE: feather follicle epithelium; MDV: Marek's disease virus. (Image was kindly provided from Dr. A. Greco and modified from [53])

6.5 MDV genome structure

MDV possesses a rather large genome size of approximately 180kbp, (from https://www.ncbi.nlm.nih.gov/genome/viruses/, effective July 2018). The MDV genome is a class E genome that consists of a unique long (U_L) and a unique short (U_S) segment, each flanked by inverted terminal (TR_L and TR_S) and internal (IR_L and IR_S) repeats [54]. MDV and the closely related GaHV-3 and HVT share significant sequence homology throughout the genome except within the repeat-long regions. Both unique regions mainly encode for genes that are conserved amongst alphaherpesviruses and are involved in DNA replication and production of progeny virus. The four repeat regions contain most of the MDV-specific genes

that encode for proteins or RNAs that are important for pathogenesis, cellular tropism, tumorigenesis and latency [55, 56].

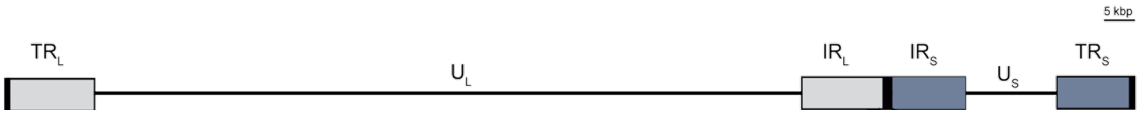

Figure 6: The MDV genome structure

The entire MDV genome is predicted to be about 180kbp in size. It is organized like a typical α-herpesvirus with unique long (U_L) and unique short (U_S) sequences flanked by terminal (TR_L and TR_S) and internal (IR_L and IR_S) inverted repeat regions.

MDV encodes for more than 100 annotated genes [54, 55]. Most of them annotated based on their homology to HSV-1 genes. However, MDV also carries a number of open reading frames (ORFs) that have not yet been investigated - neither regarding their coding potentials, nor regarding their transcriptional and translational products [57]. We and others are currently investigating these genes in order to better understand the complex MDV replication cycle, genome integration, and MDV-induced tumor formation.

6.6 MDV virulence factors

There are several viral factors that are involved in MDV pathogenesis (Table 1) and our knowledge of them is steadily increasing. Especially in the last years, MDV research made progress in terms of identification of mechanistic descriptions of these viral factors. The key player in MDV pathogenesis and probably the most studied protein in the MDV field is the major oncoprotein Meq. Meq is a basic leucine zipper protein (bZIP), which shows homology to the human proto-oncoproteins Fos and Jun [58]. Furthermore, MDV encodes for other proteins that influence disease progression at different stages: vIL8 [44, 59, 60], RLORF4 [61], and two phosphoproteins (pp14 and pp38) [62-70] (Table 1).

Virulence factor	Function
meq	Major oncogene, DNA-binding transcription factor related to bZIP proteins
RLORF4	Deletion results in attenuation *in vivo*
vIL-8	Secreted CXC chemokine involved in attraction of target cells
pp14	Neurovirulence factor
pp38	Deletion severely impairs tumor formation
miRNAs	Non-coding RNAs that regulate gene expression at the post-transcriptional level
vTR	Viral telomerase RNA homologue that is crucial for efficient MDV-induced lymphoma formation
TMR	Facilitate genome integration

Table 1: MDV virulence factors

Important MDV gene products involved in pathogenesis.

MDV also encodes for non-coding RNAs that are essential for pathogenesis and tumorigenesis. Besides several MDV-encoded micro RNAs that are located in the repeat regions of the virus genome [71, 72], the virus possesses a viral telomerase RNA (vTR) has an 88% sequence identity to the cellular TR in chickens (chTR) [73-75]. vTR was found to be the most abundant viral transcript detected in MDV tumor cells and is crucial for MDV-induced malignant transformation [75]. That might be due to an interaction with the chicken telomerase reverse transcriptase subunit (TERT) and that interaction could facilitate an enhanced telomerase activity [73], which almost always correlates with cell immortalization [76]. In order to maintain the virus genome in latently infected host cells and tumor cells, MDV integrates into the telomeres of host chromosomes using viral telomeric repeats (TMRs). Those TMRs (TTAGGG repeats) are present in the so-called a-like sequences that localize at both ends of the virus genome and at the junction between the IR_L-IR_S [46, 47, 77, 78]. Although much has been done to unravel underlying mechanisms and functions of MDV pathogenesis factors, all those factors, and probably more (maybe even unknown ones) have to be further characterized and studied to get a full overview of MDV infections as lytic and latent stages, and a more detailed knowledge of the cellular transformation.

6.7 Immunity and resistance to MDV

Several innate and adaptive immune responses are mounted after MDV infection or vaccination. B cells are involved in humoral immunity, which plays a minor role in the protection against MDV since the virus is highly cell-associated. Maternal antibodies provide little

protection in the first weeks of life [79] and only delay MD development in terms of clinical symptoms and tumor formation. On the other hand, maternal antibodies can also weaken immune responses to MDV vaccination, thereby decreasing vaccine efficiency [80]. A far stronger and more reliable immune response is mediated by cellular immunity: T cell-mediated immunity is mainly driven by CD8+ cytotoxic T cells, which are primed against late viral glycoproteins [81], but also against immediate early and early MDV proteins [82]. Additionally, CD4+ T cells are most likely involved and further work needs to confirm the exact role of CD4+ and CD8+ T cell subsets and their responses against MDV infections [80]. Macrophages and natural killer cells as major cellular components of the innate immune system are also thought to contribute to immunity against MDV [83-85].

The MDV vaccination history started with the launch of an HVT-based vaccine (FC126 strain) in the early 1970s [86, 87]. This was the first antiviral vaccine that efficiently prevented cancer in any species [35]. However, in the late 1970s, the HVT vaccine was no longer protective since more virulent emerging field strains emerged. Realizing the need for new MDV vaccines, Schat and Calnek isolated a non-pathogenic serotype 2 strain (SB1). A bivalent vaccine composed of HVT and SB1 [88] greatly improved protection from those new field strains and is referred to as the second generation of MDV vaccines. The third generation vaccine, CVI988, which still is the gold standard vaccine against MDV [87], was established and tested by Dr. Rispens of the Dutch Central Veterinary Institute [89, 90]. With the second generation and third generation vaccines, the virus was controlled and did usually not cause pathogenesis in vaccinated flocks (Fig. 7). CVI988 is currently used worldwide to protect long-lived chickens such as layers and breeders. Apparently, more doses of MDV vaccines are administered than any other, regardless of the species [91]. It is very efficient in the prevention of MD, but fails to provide sterilizing immunity and thereby allows virulent field strains to spread in infected chicken flocks [35]. The emergence of more virulent MDV field strains appears to coincide with the introduction of extensive vaccination programs (Fig 7). Considering this constant evolution of MDV strains towards a greater virulence, the disease remains a threat to poultry production [35-37, 92, 93] (Fig. 7). Besides the epidemiological data, this theory has also been investigated experimentally: it has been shown that MDV vaccines that do not prevent virus transmission can contribute to the development of highly pathogenic MDV that can cause more severe disease in unvaccinated chickens [93]. Hence, the development of new vaccines that inhibit virus spread and induce sterile immunity are an important goal for MDV research.

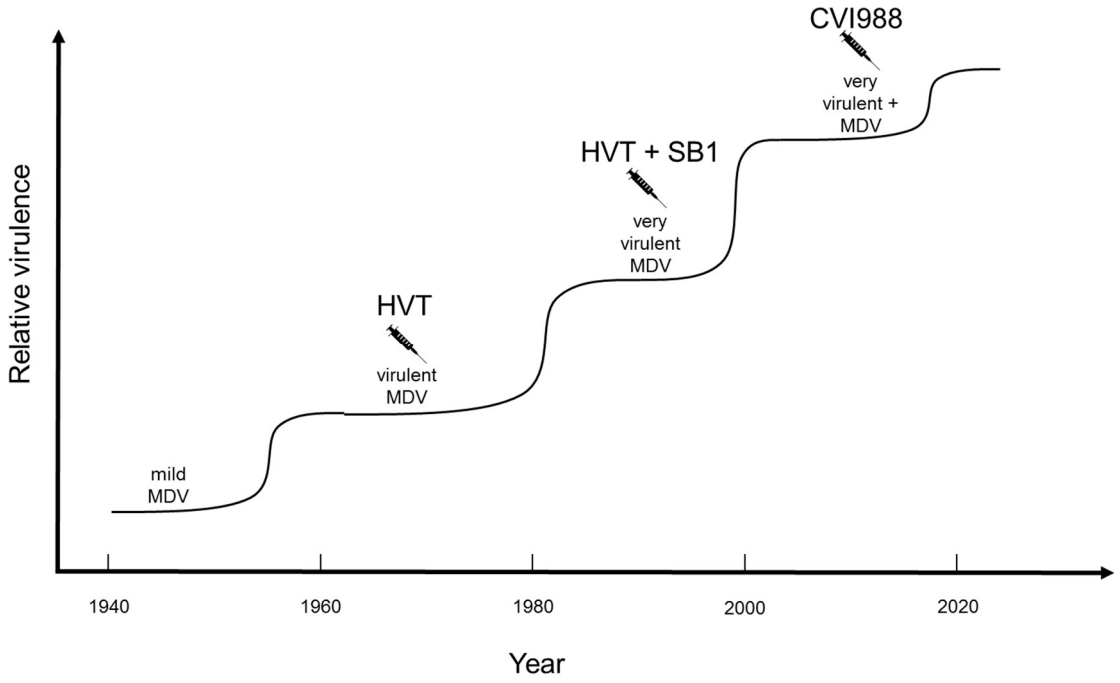

Figure 7: Evolution of MDV towards greater virulence

Increasing virulence of MDV field strains and introduction of the different MDV vaccines from 1940 to the present (Image adapted from [35]).

Recent findings in avian immunology suggested that the chicken major histocompatibility complex class 1 (MHC-1) gene plays a crucial role in the immune response of the infected chicken towards MDV [51, 94-97]. Of note is, that (in contrast to humans), chicken only express a single predominant MHC-1 haplotype [96]. This single predominant haplotype has a very strong association with resistance and susceptibility to MDV: for example, it has been observed, that the MHC-1 B21 haplotype is much more resistant to MDV infections than B19 [98]. It is believed that the MHC-1 haplotypes can be ranked in terms of MDV susceptibility and resistance, respectively [99, 100] and interestingly, MHC haplotypes also influence the efficacy of vaccination against MDV [101]. More data is needed to elucidate the way in which the chicken MHC-1 contributes to disease resistance.

6.8 Transgenic chickens

Since the first report of transgenic animals in 1981 [102, 103], the field has vastly expanded and transgenic animals have become indispensable in today's biomedical research. However, research and development of transgenic chickens has encountered difficulties and therefore

has lagged far behind that of mammalian species [104]. Several new methods that facilitated the establishment of transgenic avian species, especially chickens and quail, have been developed recently [105]. Besides direct DNA injection into oocysts or into the germinal disc of zygotes [106], retroviral gene transfer methods [107, 108] and transposons-based techniques [109, 110] have been established to randomly manipulate the germline mostly for inserting additional genes. The latest developments in avian transgenesis, including the use of transcription activator-like effector nuclease (TALEN)-mediated gene targeting [111], gene targeting by homologous recombination in primordial germ cells (PGCs) [112] and the use of the CRISPR-Cas9 system [113, 114] have been successfully used to generate targeted gene knockout chickens for the first time. With these techniques, the set of applications for transgenic avian species as models are expanding in basic scientific research, for novel biotechnology approaches as well as to improve productivity in poultry industry [105]. Targeted knockout chickens, however, have not been used in infection experiments until now. The use of targeted transgenic chickens would tremendously improve our understanding of host factors and host cells involved in disease progression and/or host responses that would help to further dissect the pathogenesis of various pathogens. Applications of those technologies in biomedical research therefore are no longer limited by technological methods and skills, but only by creativity and imagination.

6.9 MDV tumors, imaging mass spectrometry and proteome analysis

The onset of MDV-induced tumor development is relatively rapid. Within 3-4 weeks post infection, the virus is able to establish latency, integrate into the host cell chromosomes and transform target cells, which then ultimately leads to fatal lymphoma in visceral organs [115]. It has been shown that MDV-induced tumors are dominated by a highly restricted number of clonal CD4+ T cells [116]. More and more evidence point to a critical role of epigenetic regulation such as histone modifications and DNA methylation that facilitate the maintenance of viral latency [117, 118]. As described in “6.5 MDV virulence factors”, MDV transcribed sequences that are noncoding (vTR, miRNAs) also significantly influence MDV tumorigenesis. However, the majority of features that are conductive to the whole latency and transformation complex in MDV infections are yet to be deciphered.

Imaging mass spectrometry (IMS) techniques allow mass spectrometric measurements with high resolution. Protein mass spectra are registered in a grid pattern over the analyzed surface and the distribution of specific masses can be then visualized. Applied to tissue sections, this unique tool can directly link histological structures to mass spectrometric data [119]. The huge

advantage of an IMS-based workflow is that it is not restricted to analytes of interest and allows an unbiased view on sample material. Furthermore, this technique is not limited to the availability of detection probes such as antibodies, fluorescent chromophores or nucleic acids. The use of detection probes requires a strong binding affinity for particular targets that enable those targets to be detected in a complex sample. This is drastically limited by the availability of molecular markers and the issue does not occur in IMS based approaches. On the contrary, IMS is not only highly versatile, but also very specific and numerous distinct masses can be detected and discriminated at the same time [120]. The IMS-based approach is likely to be applicable for the detection of macroscopically undetectable MDV-induced lymphomas and, combined with microdissection techniques, allows proteome analyses of snap-frozen and formalin-fixed paraffin embedded (FFPE) tumor samples from MDV-infected chickens.

6.10 Project introductions

Despite of many years of MDV research, many critical questions remain unanswered. This is due to a lack of tools and targeted transgenic chickens. In this thesis, we used the first targeted knockout chickens to provide novel insights into lymphocyte infections of this oncogenic avian herpesvirus. Furthermore, it supplies an overview and a critical evaluation of recent MDV literature (particularly from the past 5 years) on MDV virulence factors. Finally, this thesis contributes findings from proteomic analyses of pure MDV-induced T cell lymphomas.

The first paper of this cumulative dissertation determined the role of peripheral and mature B cells in MDV pathogenesis. Thanks to recent advances in avian genetics and the use of genetically engineered chickens that lack peripheral and mature B cells but still harbor immature precursor B cells in the bursa, this data show that B cells are dispensable for disease onset, disease progression and viremia, as well as for tumor development. Furthermore, this data allows a further refinement of the current model of MDV pathogenesis. The second paper is a digest of latest literature that describes novel *in vitro* and *in vivo* findings on MDV pathogenesis, with an emphasis on viral virulence factors, in form of a systematic review. The review combines datasets and summarizes our current understanding of the mechanisms of viral factors that are involved in MDV pathogenesis and lymphomagenesis. A deeper knowledge of the virus will also provide new strategies for the ultimate goal of our field of research: the vaccine development against this deadly poultry pathogen. In the third manuscript, an IMS-based pipeline was implemented in order to eventually identify potential protein biomarkers in MDV-induced lymphomas. This is a collaborative work with the *Friedrich-Loeffler-Institut* (Riems) that provides new insights into the proteomic profile of MDV-

transformed T cells *ex vivo*. IMS and subsequent non-contact laser capture microdissection (LCM) followed by a proteomic workflow was used as an 'open view' tool for MDV-tumor protein mass spectrometry (MS). The major objective of that study was to identify protein biomarkers that characterize transformed T cells – both in solid MDV-induced tumors and in blood samples, but also to establish a technique that allows a proteomic analysis of pure MDV-induced T cell lymphomas without contaminating surrounding tissue.

6.11 References

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7 Unraveling the role of B cells in the pathogenesis of an oncogenic avian herpesvirus

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8 Viral Factors Involved in Marek's Disease Virus (MDV) Pathogenesis

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9 Imaging mass spectrometry and proteome analysis of Marek's disease virus-induced tumors

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10 Discussion

10.1 General discussion

Marek's disease is one of the most frequent virus-induced cancers in the animal kingdom and has an enormous economic impact on poultry industry worldwide. Like other herpesviruses, MDV establishes latency and thereby ensures a life-long infection in its natural host, the chicken. As a remarkable feature, MDV maintains its genome in latently infected cells by integrating it into the telomeres of host chromosomes. As already mentioned and discussed in the previous chapters, this integration event makes MDV such a special and intriguing pathogen. MDV shares features with human herpesvirus and serves as a model in biomedical science – not only for virus-induced tumor formation. MDV also serves as an animal model for research on the role of immune control in herpesvirus infections and on factors and mechanisms leading to virus latency [1].

The MDV infectious life cycle is initiated by the inhalation of cell-free MDV. Macrophages are thought to transfer the virus to lymphoid organs such as spleen, thymus and bursa, where the virus replicates in B cells and subsequently in T cells. MDV is able to establish latency in infected T cells, which then transport the virus to the skin and the feather follicle epithelia, where cell free MDV is generated. A hallmark of MDV pathogenesis is its ability to integrate into telomeres and thereby transform T cells, which frequently results in tumor formation [2]. MDV replicates very efficiently in B cells, which coincides with the initial amplification step during infection and leads to high viral titers in the lymphoid organs and to viremia [3-6]. B cells were thought to play a crucial role in the virus life cycle. The subsequent infection of T cells and an accompanying immunosuppression leads to an ensuing onset of clinical symptoms and is required for disease onset and tumor development [5, 7-10]. Even though B cells are the most frequent cell type infected in vivo, it remained unclear if these cells indeed contribute to MDV pathogenesis and a plethora of bursectomy studies failed to provide conclusive answers [3, 11-17]. Therefore, the exact role of B cells in the MDV life cycle was not elucidated yet. New tools in avian immunology and the development of targeted transgenic chickens allowed you to assess the exact role of B cells in MDV.

MDV is a very efficient and reliable pathogen regarding disease initiation and tumor formation. Moreover, with annually 50 to more than 100 scientific papers on MDV, MDV research continually progresses. However, there are certain missing links that are needed to understand these processes in more detail. A lot is known for viral factors that contribute to MDV pathogenesis and especially tumorigenesis; however, there are many aspects that

have not been assessed yet and an update of the status quo combining latest findings helps to identify knowledge gaps in order to fill them.

Moreover, a closer look into MDV lymphomas and differentially regulated proteins in tumor tissues takes us a step closer to understanding MDV tumor biology. This was achieved using MALDI imaging and laser capture microdissection combined with mass spectrometry [18, 19] to visualize MDV tumors and identify tumor markers.

10.1.1 B cells are dispensable for efficient MDV pathogenesis and tumorigenesis

Our study on the role of B cells in MDV infections ("Unraveling the role of B cells in the pathogenesis of an oncogenic avian herpesvirus"), disease and tumor development describes the first infection experiment in transgenic knockout birds. I strongly believe that it provides an important insight into the role of B cells in MDV, since until now B cells were thought to play a vital role in MDV pathogenesis. Using the first targeted knockout chickens that lack peripheral and mature B cells, we could break this dogma and demonstrate that B cells are completely dispensable not only for lytic replication and spread in the host, but also for disease and tumor development. The genetic background of the chickens used to generate the JHKO birds were found to be more resistant to very virulent MDV. This explains the low MD and tumor incidences in naturally infected birds that were housed as sentinels with the experimentally infected JHKO chickens. Furthermore, an age-related resistance to MDV infections adds up to these data. Shedding of MDV only occurs after 14 dpi. and at that age, the JHKO chickens were even more resistant than directly post hatch. Of note is that the delayed arrival of virus in the spleen and the thymus could point to only a minor role of B cells in early MDV replication. However, our data will refine the current model of the MDV life cycle and furthermore pioneer the use of knockout chickens in infectious disease research. Furthermore, CD4+ and CD8+ T cells were also found to complement for the B cell loss in 4 dpi tissue samples. In these organs, however, the general infection rate was too low to make firm conclusions. Nevertheless, it is very clear that as long as enough T cells and/or the right T cell is infected, tumors are induced by MDV.

Follow-up questions for further research would definitely include the recruitment of those CD4+ and CD8+ T cells: how does the virus enter the T cells if B cells are not around? Does vIL-8 recruitment, that was shown to be effective for B cells and CD4+ CD25+ T cells [10], also apply to CD8 T cells? In an MDV *in vitro* integration assay (discussed below), we show that the virus can readily infect different chicken T cell lines. This *in vitro* assay, combined with binding, flow cytometry- and chemotaxis assays could elucidate vIL8 involvement in CD8 T cell infections. Furthermore, it would be more than interesting to evaluate the

contribution of immature bursal B cells to viremia. Since we found AV20+, MDV infected progenitor B cells in the bursal tissue samples of homozygous knockout chickens, these cells could very well facilitate productive infection and produce virus to be disseminated. However, the majority of infected cells in bursal tissue samples of homozygous knockout chickens were CD4+ and CD8+ T cells. This shows that the progenitor B cells are not as susceptible to MDV infection as compared to mature B cells in bursal tissues of heterozygous birds (Fig. 13).

The further use and applications of genetically modified chickens in both biomedical research and poultry production is not limited by techniques and technical skills anymore – only by creativity and innovativeness (and funding). Exemplarily, targeted knockouts in chickens have been used to study B cell development using light chain knockout chickens [20]. Furthermore, chickens expressing the Cre recombinase are used to study (trans-) gene expression [21] and of course, the CRISPR/Cas9 system is currently introducing a variety of applications for genetic modification in avian species [22-24].

10.1.2 The *Cornell model* revised

With the new insights into MDV pathogenesis and the MDV life cycle using B cell less chickens, we propose a refinement of the current model of the MDV life cycle (Fig. 18).

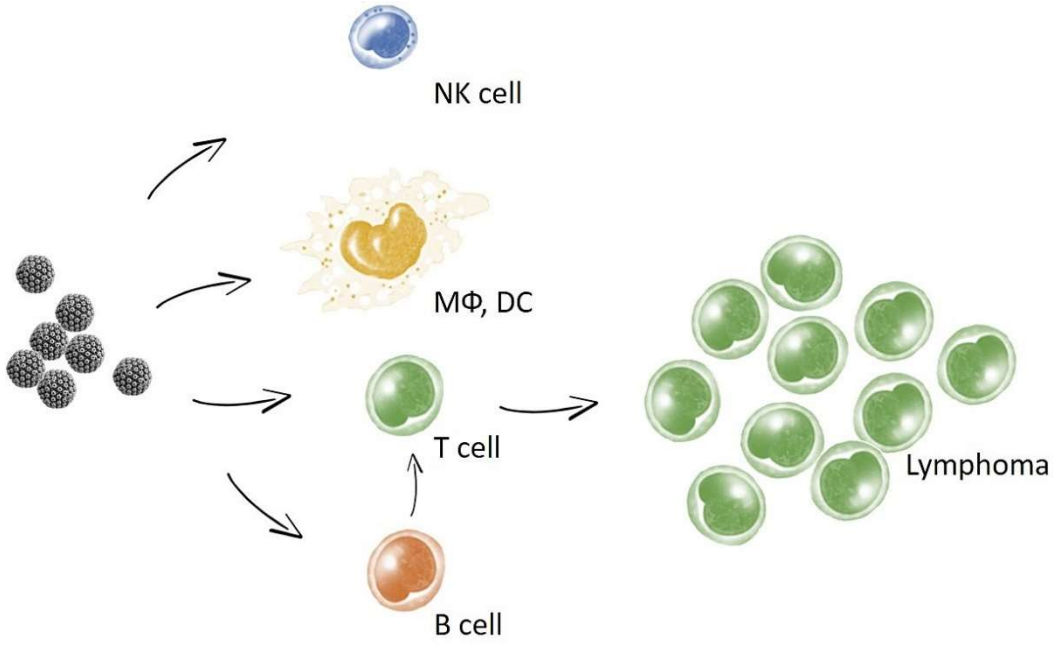

Figure 19: Proposed model of MDV infection

I propose that an MDV infection starts with the inhalation of cell-free MDV. Macrophages transfer the virus to lymphoid organs such as spleen, thymus and bursa, where the virus lytically replicates in those macrophages, but also in B cells, T cells and NK cells. MDV is able to establish latency in infected T cells, which then transport the virus to the skin and the feather follicle epithelia (FFE), where cell free MDV is generated.

It is true that there is a strong lytic infection of B cells [3, 25], but other immune cells are equally well infectable and could therefore contribute to initial viremia and high viral titers in the lymphoid organs. This hypothesis is supported by findings describing lytic infections in macrophages and dendritic cells [26]. Furthermore, NK cells support lytic MDV replication (Christine Jansen, unpublished data) and even T cells, the primary target cell for latent infections, are prone to the lytic stages [25]. A virus silencing through latency establishment and subsequent T cell transformation then leads to lymphoma development. However, to dissect the different target cells entirely, more *in vivo* data has to be generated and *in vivo* imaging tools could help to address these questions.

10.1.3 MDV encodes several genes involved in pathogenesis and tumorigenesis

Regarding MDV virulence factors, a lot is known (but a lot is not). In our review article “Viral factors involved in Marek’s disease virus (MDV) pathogenesis”, we describe and discuss the major players that orchestrate MDV pathogenesis. Most of the known MDV genes have homologues in other alphaherpesviruses, particularly in HSV-1. They often play important roles in DNA replication and various other processes essential for the virus lifecycle. MDV also encodes some specific genes, known to play central roles in the disease establishment and disease progression. In our review, we discuss MDV-encoded proteins, RNAs, and sequence elements that efficiently contribute to MDV pathogenesis. Factors that were not part of the manuscript, including vLIP, gC, US3p or UL49.5p, were not discussed because research data on those proteins is very limited and their contribution to MDV pathogenesis are of indirect nature or of minor interest to the field. The viral lipase homolog (vLIP) gene encodes for a secreted glycoprotein that was found to contribute to efficient viral replication in infected cells. vLIP mutant viruses caused a significantly lower disease incidence in experimentally infected chickens [27]. The MDV glycoprotein C (gC) has multiple splice variants and is essential for the horizontal transmission of the virus [28, 29]. The MDV-encoded serine/threonine protein kinase US3 involved in virus cell-to-cell spread [30] and a non-glycosylated transmembrane protein termed UL49.5 was shown to down-regulate surface expression of MHC class I [31] and cell-to-cell spread [32]. Since the MDV research community is rather small and performance of high throughput methods are only starting to be applied to the virus, I strongly believe that more virulence factors will be uncovered and that this could lead to recognition of new targets for vaccine development.

10.1.4 Mass spectrometric techniques reveal potential MDV tumor markers

As a new tool in the MDV research toolbox, an IMS-based approach was used to visualize MDV tumors, identify changes in protein expression during the MDV transformation process and find possible tumor markers. For that, MDV-induced tumors from different recombinant viruses were subjected to a workflow that allows an *in situ* molecular mapping of characteristic protein mass signatures. These regions were micro-dissected and applied to a LC-MALDI TOF/TOF mass spectrometric pipeline. In total, we found 19 proteins that were up- or downregulated in MDV tumors as compared to primary chicken T cells and uninfected tissue controls. These proteins were not strongly regulated, suggesting that MDV is a rather silent invader. Nonetheless, the identified potential transformation markers were employed in a gene ontology (GO) term enrichment analysis and were found to be associated with five pathways: (i) nucleosome assembly, (ii) regulation of transcription, (iii) inflammatory response, (iv) immune response, and (v) oxidation-reduction process. As a confirmation,

RNAs of randomly selected transformation markers were validated by qPCR. To confirm that they are indeed markers for MDV-induced transformation, further functional analyses are needed. Additionally, the identification of protein profiles in cells of different stages of latency and transformation would unravel further details. For this, *in vivo* time-course experiments would be necessary. Considering that it is impossible to synchronize infections of CD4+ T cells in chickens this remains very challenging. As a follow-up on the tumor imaging and proteome analysis manuscript, we are also looking into the transcriptional and translational profile of MDVs target cells for lytic replication *in vivo*. Here, we made use of whole transcriptome shotgun sequencing, mass spectrometry and protein profiling, and microarray technologies to identify MDV transcripts and MDV proteins expressed in lytically infected B cells comparing the very virulent MDV field strain RB1B and the MDV vaccine CVI-988/Rispens. We are currently analyzing these data sets and will submit the manuscript soon.

10.2 Final remarks and outlook

During my PhD in the *Viral Integration and Tumorigenesis Group* at the Institute of Virology, I worked on different projects that all focused Marek's disease virus-host interplay with a special emphasis on lymphocyte infections. Exceeding the three manuscripts of this doctoral thesis, I have been working on several other projects that strongly link to these projects in terms of content: As one of many groups that work on MDV, we also work on the development of novel MDV vaccines. As MDV vaccines have been shown to induce mild immunosuppression and hence, reduce an immune response against other pathogens, this immunosuppression also leads to an increased susceptibility to E.coli and possibly other pathogens [33, 34]. Chickens are probably one of the most vaccinated animal species today and MDV-induced immunosuppression with a subsequent reduction of vaccine responses could reduce the efficacy of many vaccines that are applied to chickens early in life. Additionally, MDV field strains can still circulate in MDV-vaccinated birds and that might likewise induce immunosuppression in these animals, even if they do not develop Marek's disease symptoms. Therefore, there is a need for novel vaccines that do not suppress the chicken immune system. Recent work from our laboratory shows that the MDV-encoded chemokine vIL-8 facilitates the recruitment of B cells and CD4+ CD25+ T cells to the site of infection [10]. A productive and lytic virus replication in the recruited cells causes severe lymphocyte reduction [5]. Therefore, an abrogation of the chemokine expression by start codon mutation in CVI988, the gold standard vaccine strain, should reduce the number of lymphocytes that are recruited, infected, and killed by MDV. In addition, we made use of a

previously reported vaccine candidate that lacks the major oncogene meq (Δmeq) and has an enhanced vaccine protection against highly pathogenic MDV strains compared to other commercial vaccines. This Δmeq mutant still induces severe thymus atrophy [35]. Introduction of a vIL8 start codon mutation, which leads to abrogation of the protein in this virus background, likely reduces or eliminates the aforementioned immunosuppressive effect. That could result in a vaccine that provides an enhanced protection without the negative side effects. As a last set of vaccine candidates, two viruses that harbor a mutation in the template sequence of viral telomerase RNA (vTR) were generated in RB1B (by Kaufer *et al.* [36]) and in CVI. The RB1B vaccine candidate was tested *in vivo* and not only abrogates virus-induced tumor formation but also reduces the number of infected lymphocytes via the elimination of MDV infected cells. I constructed and successfully obtained virus stocks of the same mutation in the CVI background. These novel vaccine candidates will be tested *in vivo* soon. This work links to the review article “Viral factors involved in Marek’s disease virus (MDV) pathogenesis” with real life applications and could be a step forward in the prophylactic treatment of MDV in field conditions. Furthermore, some of the vaccine candidate viruses are currently under investigation regarding their ability to infect and activate NK cells in collaboration with the group of Dr. C. A. Jansen (Avian Immunology Group, Faculty of Veterinary Medicine, Utrecht University, NL). A detailed understanding of the contribution of other cell types, such as NK cells, to the MDV-induced immunosuppression can close knowledge gaps and thereby facilitate a more focused and goal-oriented development of novel MDV vaccines. As another data set that will contribute new insights, some of the vaccine viruses will be used in *in vitro* studies of cell death/cell survival in primary B cell cultures in collaboration with the group of PD Dr. Sonja Härtle (Institute for Animal Physiology, Ludwig-Maximilians-Universität München). In order to broaden the tools for *in vitro* studies in MDV research, we also set to establish an MDV *in vitro* integration assay. Until now, the investigation of the MDV integration mechanism required animal experiments due to the lack of an *in vitro* integration assay. With the establishment of an *in vitro* integration assay using immortalized chicken T cell lines, the latent infections, integration and transformation of MDV can be studied *in vitro*. Using quantitative PCR and the fluorescence *in situ* hybridization (FISH) technique will allow a quantitative evaluation of MDV maintenance and genome integration. With this, the mechanism that allows MDV to maintain its virus genome during latency and to induce deadly lymphomas can be further investigated. Potential viral and cellular factors that could be involved in these processes can be unraveled which would link this study to the review article on viral virulence factors. Potential viral factors in T cell transformation are the different MDV TMRs (as mentioned), but also the viral DNA polymerase UL30 [37], UL29 (a single strand DNA binding protein) and UL12, a 5’-3’ exonuclease [38]. We are currently also assessing

integration of the different MDV vaccines (CVI988/Rispens, SB-1, HVT) and the role of their TMRs at the end of their genomes in the integration process.

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11 Summary

Marek's disease virus-host interplay: novel insights into lymphocyte infections of an oncogenic avian herpesvirus.

Marek's disease is a highly contagious lymphoproliferative disorder of chickens caused by an oncogenic and strictly cell-associated alphaherpesvirus known as Marek's disease virus (MDV). MDV is prevalent worldwide and causes fatal lymphomas in the chicken, resulting in a high economic burden. Despite the widespread use of live attenuated vaccines, MDV remains a major pathogen of poultry and continues to be a threat to poultry health and welfare. It has been widely assumed that MDV initially infects B cells, which are the primary target cells in an infected chicken. MDV is then subsequently passed to T cells where it is able to establish a latent infection by integrating its genome into the host telomeres. This integration is a prerequisite for T cell transformation, tumorigenesis and a fatal outcome for the infected chicken.

The complex viral processes underlying MDV infections in poultry leading to T cell transformation and lymphomagenesis involve a plethora of viral factors ranging from viral proteins to non-coding RNAs. In order to further our understanding of MDV pathogenesis, I set out to define the exact contribution of specific lymphocytes towards MDV pathogenicity *in vivo* and uncover the proteomic makeup of MDV-transformed cells. Furthermore, this thesis presents an up-to-date review on the advances in MDV research with a specific focus on its virulence factors.

To directly assess the role of B cells in MDV pathogenesis, I utilized the first targeted knockout chickens (JH-KO) that lack mature and peripheral B cells in an *in vivo* MDV challenge study. These data broke the dogma regarding the vital role of B cells in MDV pathogenesis, and demonstrated that they are completely dispensable for virus replication, spread in the host, disease and tumor development. Moreover, it was shown that CD4+ and CD8+ T cells complement for the loss of B cells in JH-KO chickens in terms of virus amplification and virus spread in the host.

Secondly, advances in tumor imaging and mass spectrometry allowed acquisition of MDV-tumor proteomic data. This thesis describes the establishment and implementation of an imaging mass spectrometry (IMS)-based pipeline that was used to identify potential protein biomarkers of MDV-induced lymphomas. IMS and subsequent non-contact laser capture

microdissection of MDV lymphoma was followed by a proteomic workflow and provides an unbiased ‘open view’ tool for protein mass spectrometry of MDV-induced tumors.

Lastly, this thesis provides a review of all recent literature and advances in MDV research on virus virulence factors. This summarizes the current scientific consensus of how viral factors contribute to MDV-induced pathogenesis and tumor formation. Several important viral factors involved in MDV pathogenesis have been discussed, including the major oncoprotein Meq, the viral chemokine vIL-8, MDV-encoded microRNAs, RLORF4, RLORF5a, pp14, pp38, a virus-encoded telomerase RNA, and viral telomeric repeats.

Overall, this thesis contributes towards a greater understanding of MDV pathogenesis, shedding light on the cell types involved in virus replication and spread *in vivo* and factors present in MDV-induced tumors.

12 Zusammenfassung

Das Zusammenspiel des Virus der Marek‘schen Krankheit mit dem Wirt: neue Einsichten in Lymphozyteninfektionen eines onkogenen aviären Herpesvirus‘.

Die Marek’sche Krankheit ist eine hochinfektiöse und lymphoproliferative Erkrankung der Hühner und wird durch eine Infektion mit dem onkogenen und strikt zellassoziierten Alphaherpesvirus der Marek’schen Krankheit (*Marek’s disease virus*, MDV) ausgelöst. MDV ist weltweit prävalent und verursacht tödliche Lymphome in Hühnern, was zu hohen wirtschaftlichen Verlusten führt. Trotz des weit verbreiteten Einsatzes von attenuierten Lebendimpfstoffen ist MDV weiterhin ein wichtiger Infektionserreger und ein Risiko für Tiergesundheit und Tierwohl in der Geflügelhaltung. *In vivo* infiziert MDV sehr effizient B Zellen, Zellen die lange als essentiell für eine Virusamplifikation im infizierten Wirt galten. MDV wird dann auf T Zellen übertragen, in welchen das Virus eine latente Infektion etablieren und das Virusgenom in die Telomere der Wirtszelle integrieren kann. Diese Integration des Genoms ist Voraussetzung für die T Zell Transformation, für die Tumorentstehung und für den tödlichen Verlauf der Erkrankung im infizierten Huhn.

Die komplexen viralen Mechanismen die zur MDV Pathogenese und Tumorgenese beitragen, beziehen eine Vielzahl viraler Faktoren von Proteinen bis hin zu nichtcodierenden Ribonukleinsäuren mit ein. Um das generelle Verständnis von der MDV Pathogenese voranzubringen, beschreibt diese Dissertation neue Einblicke in die Beteiligung von spezifischen Lymphozyten an der MDV Pathogenität *in vivo* und deckt das Proteom von MDV-transformierten Zellen auf. Zusätzlich beinhaltet diese Dissertation den neusten Stand der MDV Forschung in Form einer systematischen Übersichtsarbeit, mit einem speziellen Fokus auf die viralen Virulenzfaktoren.

Um die Rolle von B Zellen in der Krankheitsentstehung und -entwicklung zu beurteilen, konnte ich die ersten transgenen Knockouthühner (JHKO), welche keine ausgereiften und peripheren B Zellen mehr aufweisen, in einem *in vivo* Infektionsversuch nutzen. Diese Daten brechen mit dem Dogma der zentralen Rolle von B Zellen in der MDV Pathogenese und zeigen, dass B Zellen für die Virusreplikation, die Ausbreitung im infizierten Wirt und auch für Krankheits- und Tumorentstehung komplett entbehrlich sind. Darüber hinaus wurde gezeigt, das CD4+ und CD8+ T Zellen für die Inexistenz von B Zellen in JHKO Hühnern in Bezug auf Virusamplifikation und Ausbreitung des Virus im Wirt kompensieren.

Fortschritte in der Tumorbildgebung und der Massenspektrometrie erlauben die Erfassung von MDV Tumor-Proteomdaten. Die vorliegende Dissertation beschreibt die Etablierung und die Umsetzung einer auf der massenspektrometrischen Bildgebung (*imaging mass spectrometry*, IMS) basierenden Pipeline, die genutzt wurde um mögliche MDV Tumormarkerproteine zu identifizieren. IMS und subsequente berührungslose Laser Mikrodissektion (*non-contact laser capture microdissection*) von MDV Tumoren gefolgt von einem proteomischen Workflow stellt hier eine unvoreingenommene Möglichkeit proteinmassenspektrometrischer Untersuchungen an MDV Tumoren dar.

Zuletzt bietet diese Dissertation ein Review der gesamten Literatur und der Fortschritte bezüglich viraler Virulenzfaktoren in der MDV Forschung der letzten Jahre. Das Review fasst die derzeitige Lehrmeinung hinsichtlich viraler Faktoren in der MDV-induzierten Pathogenese und Tumorgenese zusammen. Mehrere wichtige virale Faktoren, die in die Krankheitsentstehung involviert sind, wurden hier diskutiert. Dazu gehören das Hauptonkogen Meq, das virale Chemokin vIL8, MDV-codierte microRNAs, RLORF4, RLORF5a, pp14, pp38, eine viruscodierte Telomerase RNA und virale, sich wiederholende Telomerregionen.

Zusammenfassend trägt diese Dissertation zu einem besseren Verständnis der MDV Pathogenese bei, indem sie neben der Rolle von Zellen, welche die Virusreplikation und die Virusausbreitung im Wirt *in vivo* ermöglichen, auch Proteinbiomarker in MVD-induzierten Tumoren beschreibt.

13 List of publications

13.1 Scientific publications

Bertzbach LD, Vychodil T, You Y, Previdelli RL, Kaufer BB, 2019. **A quantitative in vitro assay to assess telomere integration of an oncogenic avian alphaherpesvirus**. In preparation.

Conradie AM, Bertzbach LD, Parcells M, Kaufer BB, 2019. **Evolutionary changes in the major oncogene directly determine Marek's disease virus pathogenicity**. In preparation.

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13.2 Talks and poster presentations

Luca D. Bertzbach, Florian Pfaff, Viktoria I. Pauker, Sonja Härtle, Axel Karger, Benedikt B. Kaufer: Poster „**The transcriptional and translational landscape of the Marek's disease virus (MDV) genome in *in vitro* infected primary chicken B cells**" 03/2019, 29th Annual Meeting of the Society for Virology, Düsseldorf

Luca D. Bertzbach, Tereza Vychodil, Renato L. Previdelli, Benedikt B. Kaufer: Poster „**A quantitative assay to assess Marek's disease virus genome integration *in vitro***" 04/2018, ZIBI Graduate School Retreat, Rheinsberg

Luca D. Bertzbach, Tereza Vychodil, Renato L. Previdelli, Benedikt B. Kaufer: Poster „**A quantitative assay to assess Marek's disease virus genome integration *in vitro***" 03/2018, 28th Annual Meeting of the Society for Virology, Würzburg

Luca D. Bertzbach, Tereza Vychodil, Benedikt B. Kaufer: Talk "**A quantitative assay to assess Marek's disease virus genome integration**" 09/2017, DRS Biomedical Sciences Doctoral Symposium, Berlin

Luca D. Bertzbach, Maria Laparidou, Bernd Kaspers, Benjamin Schusser, Benedikt B. Kaufer: Talk "**Peripheral and Mature B Cells are Dispensable for Marek's Disease Virus Pathogenesis**", 03/2017, 27th Annual Meeting of the Society for Virology, Marburg

Luca D. Bertzbach, Maria Laparidou, Bernd Kaspers, Benjamin Schusser, Benedikt B. Kaufer: Poster "**Peripheral and Mature B Cells are Dispensable for Marek's Disease Virus Pathogenesis**", 03/2017, ZIBI Graduate School Retreat, Nauen

Luca D. Bertzbach, Maria Laparidou, Bernd Kaspers, Benjamin Schusser, Benedikt B. Kaufer: Talk "**Peripheral and Mature B Cells are Dispensable for Marek's Disease Virus Pathogenesis**", 09/2016, DRS Biomedical Sciences Doctoral Symposium, Berlin

Luca D. Bertzbach, Maria Laparidou, Bernd Kaspers, Benjamin Schusser, Benedikt B. Kaufer: Talk "**Peripheral and Mature B Cells are Dispensable for Marek's Disease Virus Pathogenesis**", 09/2016, XIVth Avian Immunology Research Group (AIRG) Meeting 2016, Herrsching

Luca D. Bertzbach, Maria Laparidou, Bernd Kaspers, Benjamin Schusser, Benedikt B. Kaufer: Talk "**Peripheral and Mature B Cells are Dispensable for Marek's Disease Virus Pathogenesis**" 07/2016, 11th International Symposium on Marek's Disease and Avian Herpesviruses, Tours (France)

Luca D. Bertzbach, Tereza Vychodil, Benedikt B. Kaufer: Poster "**Establishment of a Marek's disease virus (MDV) *in vitro* integration assay**", 03/2016 ZIBI Graduate School Retreat, Kremmen

Luca D. Bertzbach, Viktoria I. Pauker, Axel Karger, Benedikt B. Kaufer: Poster „**Determining Changes in Protein Expression Profiles of Infected B- and T Cells upon Marek's Disease Virus Infection**" 03/2015, ZIBI Graduate School Retreat, Potsdam

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15 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, am 10.04.2019

Luca Danilo Bertzbach