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FOWL ADENOVIRUS CAPSID PROTEINS AS BROAD-PROTECTIVE SUBUNIT VACCINES AGAINST INCLUSION BODY HEPATITIS (IBH) AND HEPATITIS-HYDROPERICARDIUM SYNDROME (HHS)

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Submitted by

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1. Abbreviations

aa: amino acid

Abs: antibodies

AGE: adenoviral gizzard erosions

AGPT: agar gel precipitation test

BW: body weight

CAV: chicken anaemia virus

CD: cluster of differentiation

CEL cells: chicken embryo liver cells

CELO: chicken embryo lethal orphan

crecFib: chimeric fiber protein

DIA: data-independent acquisition

dpb: days post booster

dpc: days post challenge

dpv: days post vaccination

EDS: egg drop syndrome

ELISA: enzyme-linked immunosorbent assay

FAdVs: fowl aviadenoviruses

FAdV-A to FAdV-E: species *Fowl aviadenovirus A* to *Fowl aviadenovirus E*

FAdV-1 to -8a and -8b to -11: Fowl adenovirus serotype 1 to 8a and 8b to 11

FCM: flow cytometry

fib: fiber

Fib-8a: FAdV-8a fiber

Fib-8b: FAdV-8b fiber

HAdVs: human adenoviruses

HEV: haemorrhagic enteritis virus

HHS: hepatitis-hydropericardium syndrome

hpi: hours post infection

IBDV: infectious bursa disease virus

IBH: inclusion body hepatitis

LMH: leghorn male hepatoma

mAbs: monoclonal antibodies

MS: mass spectrometry

nAbs: neutralizing antibodies

OD: optical density

ORF: open reading frame

Pb: penton base

Pb-Dd: penton-dodecahedron

Pb-7: FAdV-7 penton base

Pb-8a: FAdV-8a penton base

PBMCs: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFU: plaque-forming units

SPF: specific pathogen-free

$TCID_{50}$: 50% tissue culture infective dose

TCR: T cell receptor

VLPs: virus-like particles

VNT: virus-neutralization test

wpv: weeks post vaccination

2. Publications included in the thesis

2.1. Manuscript 1

Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH) provides type-specific protection guided by humoral immunity and regulation of B and T cell response

De Luca C, Schachner A, Mitra T, Heidl S, Liebhart D, Hess M

Veterinary Research, 2020, 51:143

2.2. Manuscript 2

Recombinantly expressed chimeric fibers demonstrate discrete type-specific neutralizing epitopes in the *Fowl aviadenovirus E* (FAdV-E) fiber, promoting the optimization of FAdV fiber subunit vaccines towards cross-protection *in vivo*

Schachner A, De Luca C, Heidl S, Hess M

Microbiology Spectrum, 2022, 10:e02123-21

2.3. Manuscript 3

Vaccination with a fowl adenovirus chimeric fiber protein (crecFib-4/11) simultaneously protects chickens against hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH)

De Luca C, Schachner A, Heidl S, Hess M

Vaccine, 2022, 40:1837-1845

2.4. Manuscript 4

Local cellular immune response plays a key role in protecting chickens against hepatitis-hydropericardium syndrome (HHS) by vaccination with a recombinant fowl adenovirus (FAdV) chimeric fiber protein

De Luca C, Schachner A, Heidl S, Hess M, Liebhart D, Mitra T

Frontiers in Immunology, 2022, 13:1026233

2.5. Oral presentations

- **Divergent expression profiles in a genomically conserved virulent/attenuated fowl adenovirus serotype 4 (FAdV-4) strain pair identifies possible biomarkers for virulence modulation**

  De Luca C, Nöbauer K, Hummel K, Hess M, Schachner A

  14th International Adenovirus Meeting, 18th-21st May 2021, Online Meeting

- **Local immune response provided by fowl adenovirus (FAdV) fiber vaccine plays a key role in protecting chickens from hepatitis-hydropericardium syndrome (HHS)**

  De Luca C, Mitra T, Schachner A, Heidl S, Liebhart D, Hess M

  7th European Veterinary Immunology Workshop, 29th-31st August 2021, Online Meeting

- **Fowl adenovirus (FAdV) induced diseases: vaccination strategies**

  De Luca C, Schachner A, Hess M

  11. Tagung des VET Arbeitskreises Geflügelforschung, 1st-2nd April 2022, Rust, Austria

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4. Declaration

I hereby confirm that the work within the present PhD thesis “FOWL ADENOVIRUS CAPSID PROTEINS AS BROAD-PROTECTIVE SUBUNIT VACCINES AGAINST INCLUSION BODY HEPATITIS (IBH) AND HEPATITIS-HYDROPERICARDIUM SYNDROME (HHS)” was carried out during my PhD study at the University Clinic for Poultry and Fish Medicine, University of Veterinary Medicine, Vienna, Austria.

I certify that I have followed the rules of Good Scientific Practice in all aspects.

Carlotta De Luca

Vienna, August 2022

5. Summary

Fowl adenoviruses (FAdVs) are associated with three disease complexes affecting the poultry industry: adenoviral gizzard erosion (AGE), caused by FAdV serotype 1 (FAdV-1), hepatitis-hydropericardium syndrome (HHS), caused by FAdV-4, and inclusion body hepatitis (IBH), caused by FAdV-2, -8a, -8b and -11. Despite the increasing burden that these diseases represent for the poultry industry, a comprehensive immunization strategy available in the field is still lacking. The objective of this thesis was to develop a broad-protective immunization strategy to protect chickens against IBH and, subsequently, HHS, using subunit vaccines based on FAdV recombinant capsid proteins, which are favourable for their efficient cost- and time-production. In the first study, specific pathogen-free (SPF) chickens were vaccinated with a recombinant FAdV-8a fiber protein and challenged with FAdV field isolates belonging to both a homotypic (FAdV-8a) or heterotypic (-8b) IBH-causing strain. Results showed that single fiber-based immunity was only able to protect the birds from homologous challenge, possibly due to the strictly type-specific neutralizing activity of fiber immune sera determined by virus neutralization test (VNT). In protected birds, vaccination prevented a post-challenge drop of peripheral B cells in blood, stimulated helper T lymphocyte proliferation while moderating the cytotoxic T cell response, and prevented challenge-induced changes in systemic monocytes/macrophages and $\gamma\delta^+$ T cell subpopulations. In order to overcome the lack of cross-protection linked to single fiber vaccines, the novel concept of recombinant chimeric fiber proteins (crecFibs) retaining epitopes from different FAdV serotypes was developed merging two consecutive segments in the fibers of FAdV-8a and -8b, swapped reciprocally to result in novel chimeras, crecFib-8a/8b and crecFib-8b/8a. The constructs showed the same reactivity as monospecific recombinant fibers in western blot against different FAdV antisera, and crecFib-8b/8a was able to induce cross-neutralizing antibodies against

both serotypes in chickens. This highlights distinct epitopes in these fibers: the conserved one detected in western blot and at least two type-specific epitopes participating in neutralization. When administered as single-antigen component, crecFib-8b/8a protected chickens against IBH-causing serotypes, promoting the advancement of broadly protective subunit vaccination strategies against FAdVs. This concept was therefore extended to achieve simultaneous protection against IBH and HHS, with the design of a chimeric construct retaining epitopes from FAdV-4 and -11 fibers (crecFib-4/11). The construct was used to vaccinate SPF chickens before challenge with either FAdV-4 or -11, and was able to protect the birds from both HHS and IBH. Clinical protection was associated with high levels of pre-challenge antibodies measured on enzyme-linked immunosorbent assay (ELISA) plates coated with the vaccination antigen, although the development of neutralizing antibodies was limited against FAdV-11 and absent against FAdV-4, indicating that protection granted by such antigen may be linked to different immunization pathways compared to crecFib-8b/8a. Nevertheless, birds immunized with crecFib-4/11 and challenged with FAdV-4, experienced a significant increase of hepatic B cells and the proliferation of circulating cytotoxic T lymphocytes at an earlier time point compared to the challenge control, with subsequent increase in liver and spleen. Overall, these findings imply a potent local response in the target and lymphoid organs as crucial component for the protection from HHS after crecFib-4/11 vaccination, even more so than the development of neutralizing antibodies. In conclusion, it was proven that the concept of chimeric fiber vaccines represents the first single-component FAdV subunit vaccine providing comprehensive protection against different FAdV-associated diseases.

6. Introduction to Fowl adenoviruses (FAdVs) and related prevention strategies

A *fowl aviadenovirus* was first isolated accidentally in 1949 from embryonated chicken eggs (Van Den Ende *et al.*, 1949). From that moment, FAdVs have been isolated from a number of different avian hosts, in either healthy or diseased birds (Hess, 2020). In 1963, an inclusion body hepatitis (IBH) was described with unresolved aetiology but later on attributed as the first FAdV-associated disease in chickens (Helmboldt & Frazier, 1963). The role of FAdVs as primary pathogens was initially questioned, given the frequent concomitant isolation of other pathogens such as chicken anaemia virus (CAV) and infectious bursal disease virus (IBDV) in diseased flocks (Hess, 2017). Later on, the identification of adenoviral gizzard erosion (AGE) and hepatitis-hydropericardium syndrome (HHS), additional diseases caused by FAdV, and various experimental infection studies, revealed that strains from specific serotypes are able to cause clinical affection in healthy chickens without the presence of other pathogens (Mazaheri *et al*., 1998; Okuda *et al*., 2001a; Schachner *et al*., 2018). To this date, FAdVs are globally widespread, and their related disease complexes represent an increasing burden for the poultry industry (Schachner *et al*., 2021).

6.1. FAdV taxonomy and classification

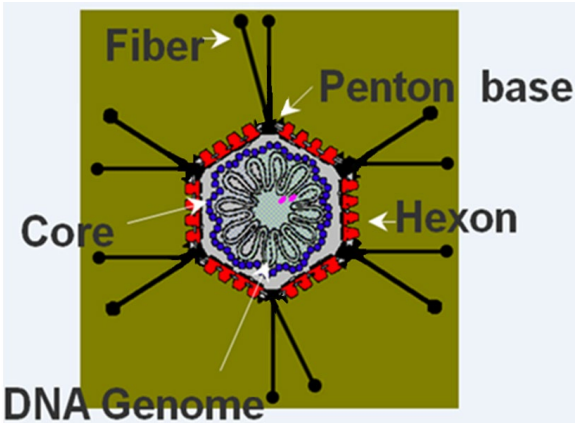
FAdVs belong to the family *Adenoviridae*, genus *Aviadenovirus*, and are currently classified into five species (FAdV-A to -E) according to their full genome sequences (Benkő *et al*., 2022). They are further divided into twelve types (FAdV-1 to -8a, and from -8b to -11), which were first determined by serological neutralization test and, subsequently, through molecular phenotyping (McFerran & Adair, 1977; Hess, 2000; Marek *et al*., 2010; Benkő *et al*., 2022). The major structural protein of FAdVs, the

hexon (and in particular its loop 1 region), has represented the main target of molecular phenotyping for decades since its first description (Raue & Hess, 1998). However, in recent years, an increased focus has developed over whole-genome sequencing, which not only allowed to fully characterize the FAdV genome, including fiber-coding genes and terminal open reading frames (ORFs), but also revealed the circulation of intertypic hexon/fiber recombinant FAdV-D and -E strains in the field (Schachner *et al*., 2019).

6.2. FAdV structure and identified epitopes

FAdVs are icosahedral, non-enveloped double-stranded DNA viruses, measuring 70-90 nm in size (Fig. 1) (Laver *et al*., 1971). The major structural proteins compose the capsid, which consists in 252 capsomers: 240 hexons and 12 pentons, each one further structured by a penton base and two fibers (Laver *et al*., 1971; Gelderblom & Maichle-Lauppe, 1982; Tan *et al*., 2001). The fiber protein is divided into three regions: a small tail at the N-terminus, a shaft regulating the length, and a terminal domain named knob. Each FAdV species possess a single gene codifying for its fiber protein, with the exception of FAdV-A and -C, whose fibers are codified by distinct ORFs, and hereby referred as fiber-1 and fiber-2 (Hess *et al*., 1995; Marek *et al*., 2012).

Fig. 1. Schematic representation of a FAdV and its major structural proteins



Differently from the fiber, which has shown a certain degree of intra-species recombination potential, the penton base retains a more conserved genomic sequence across the FAdV species spectrum (Schachner *et al*., 2019).

Despite the antigenic importance of FAdV capsid proteins, not many studies have focused on the identification of epitopes on such antigens. To date, most of the investigations were centred around hexon, especially from FAdV-4. These studies led to the identification of a conserved epitope among all FAdV serotypes, as well as FAdV-C-specific B cell epitopes, although monoclonal antibodies (mAbs) derived from the latter did not reduce *in vitro* infection and replication of the virus in leghorn male hepatoma (LMH) cells (Pan *et al*., 2018; Liu *et al*., 2021). Therefore, it can be hypothesized that other components, such as the fiber and penton base proteins, are crucially involved in the binding to the host cell and subsequent entry, and are thus fundamental to define pathogenicity. In fact, a neutralizing epitope was identified in FAdV-4 fiber-2, and the mAb developed from it efficiently inhibited FAdV-4 replication *in vitro* (Wang *et al*., 2018a). Additionally, two non-neutralizing B cell epitopes were identified in the shaft of the FAdV-8b fiber (Lu *et al*., 2019). As for the penton base protein, one promiscuous B and T cell epitope region was identified in the FAdV-4 penton base, and provided 50% protection (defined by survival rate after challenge) in chickens against HHS (Aziz *et al*., 2019).

6.3. FAdV-induced diseases

There are three different disease complexes affecting chickens caused by FAdVs: adenoviral gizzard erosion (AGE), hepatitis-hydropericardium syndrome (HHS), and inclusion body hepatitis (IBH) (Hess, 2020). AGE is associated to FAdV-A, serotype 1, and it is characterized by the affection of the gizzard of the host, whereas HHS (caused

by FAdV-C, serotype 4) and IBH (caused by FAdV-E, serotypes 8a and 8b, and FAdV-D, serotypes 2 and 11) are metabolic diseases that primarily target liver, spleen, heart (in case of HHS), lymphoid tissues, and may involve other organs such as pancreas, kidneys and muscles (Schachner *et al*., 2018). In fact, HHS and IBH share multiple similarities regarding their pathogenesis and immune mechanisms, overall causing severe damage to the poultry industry. On the other hand, protection against AGE seems to be bound to distinct pathways, as the onset of local immunity in the gastro enteric tract, rather than a systemic humoral response, is considered to be crucial (Okuda *et al*., 2001b; Ono *et al*., 2003a; 2003b; Grafl *et al*., 2013; 2014; 2020). For this reason, in the framework of elaborating a comprehensive immunization strategy aimed to protect chickens against FAdV-associated metabolic diseases, the present work focuses primarily on HHS and IBH.

6.3.1. Hepatitis-hydropericardium syndrome (HHS)

HHS was first described in the late 1980s in Pakistan, and has since then been observed in several Asian countries, as well as in Central and South America (Cheema *et al*., 1989; Schachner *et al*., 2018). Outbreaks are characterized by high mortality and severe clinical affection of the birds, with hydropericardium and hepatitis generally being the most prominent pathological findings, often accompanied by atrophy of the lymphoid organs and lymphocytic depletion (Schachner *et al*., 2018). HHS affects primarily broilers of 3-6 weeks of age, but can occasionally be observed in layers and broiler breeders (Cheema *et al*., 1989; Asrani *et al*., 1997; Mittal *et al*., 2014). Sporadic reports in other avian species such as quails, pigeons, ducks and ostriches have been described as well (Naeem & Akram, 1995; Hess *et al.*, 1998; Karunamoorthy & Manickam, 1998; Chen *et al*., 2015; Changjing *et al*., 2016).

6.3.2. Inclusion body hepatitis (IBH)

IBH was the first FAdV-associated disease ever reported, in 1963, and it has reached a worldwide distribution to this date (Helmboldt & Frazier, 1963; Schachner *et al*., 2018). Initially, IBH was believed to be caused by opportunistic pathogens, only becoming clinically relevant with the concurrent presence of immunosuppressive diseases such as IBDV and CAV (Hoffmann *et al*., 1975; Rosenberger *et al.*, 1975; Fadly *et al*.,1976). However, several reports of field outbreaks, as well as experimental infections, confirmed the role of IBH-associated FAdVs as primary pathogens (Schachner *et al.*, 2018). IBH induces a clinical picture that is very similar to the one associated to HHS, although less severe, with mortality reaching peaks of 30% and prominent pathological changes in the liver, which are frequently associated with the histological observation of intra-nuclear inclusion bodies in hepatocytes (Schachner *et al*., 2018). The disease induces metabolic disorders by frequently targeting further organs, including spleen, pancreas and kidneys (Cook, 1983; Reece *et al*., 1987; Saifuddin & Wilks, 1990; Steer *et al*., 2015; 2017; Matos *et al*., 2016a; 2016b; 2018; Chen *et al*., 2019). Because of these reasons, young broilers represent the main target host of the disease, although sporadic outbreaks in layers and broiler breeders were reported (Hess, 2020; Schachner *et al*., 2018). In fact, to induce experimental infections in broilers older than one week of age, a parenteral inoculation is needed (Hess, 2017). Recovery from the disease typically occurs within one week post infection, although shedding through the faecal route can persist for longer periods (Steer *et al.*, 2015; Matos *et al*., 2016b).

6.4. Vaccination strategies against HHS and IBH

Aside from biosecurity protocols, to date, there is a lack of comprehensive strategies to protect chickens against FAdV-related diseases. Commercial vaccines, consisting

in live and inactivated formulations against FAdV-4 and/or -8b, are only available in selected countries, whereas the rest of the world, including Europe, relies on autogenous vaccines. This led to the publications on numerous studies on experimental immunization strategies, either based on whole virus formulations (live or killed vaccines), or utilizing FAdV capsid proteins as immunizing antigens (subunit vaccines). An extensive list of these studies and the associated formulations, adapted from Schachner *et al*. (2018), is summarized in Table 1.

6.4.1. Inactivated vaccines

The first attempts to control FAdV-related diseases through the use of immunization strategies are dated back to the end of the Eighties/beginning of Nineties, with the inactivation of liver homogenates obtained from HHS outbreaks used as autogenous vaccines in Pakistan (Chishti *et al*., 1989; Afzal & Ahmad, 1990; Anjum, 1990). These preparations rely on the immunogenicity of the inactivated virus after treatment with various concentrations of formalin and are still in use in certain regions (Aziz *et al*., 2019). However, the quantity of administered antigen, as well as its characterization and quality, are difficult or impossible to standardize for such vaccines. As a consequence, cell or egg culture-purified and propagated virus were then used as antigen prior to inactivation (Naeem *et al*., 1995a; Kaur *et al*., 1997; Toro *et al*., 2002). When it comes to immunization strategies against IBH, however, the diverse aetiology of the disease has to be taken into account, and a broad-spectrum protection is necessary to efficiently control the spreading of the different FAdV species and serotypes associated with the disease. Inactivated vaccines have the potential to achieve a certain degree of heterologous protection across the different viral serotypes, and even species (Kim *et al.*, 2014; Steer-Cope *et al*., 2019). The formulation of bivalent vaccines containing different FAdV serotypes originating from

different species (FAdV-D and -E) was also shown to extend the protective spectrum to additional serotypes (Alvarado *et al*., 2007; Gupta *et al*., 2018). Furthermore, inactivated vaccines were demonstrated to grant vertical protection through transmission of maternal antibodies (Junnu *et al*., 2015; Kim *et al*., 2014; Gupta *et al*., 2018). More recently, a recombinant FAdV-4 expressing the FAdV-8b fiber was shown to protect chickens against both FAdV-4 and -8b, but did not provide coverage against FAdV-8a challenge (Lu *et al*., 2022). Nevertheless, this approach merges the protective properties of inactivated and subunit vaccines by using viral-based vectors expressing antigenic proteins from different parent strains. By this, it is possible to extend coverage against different poultry diseases, as demonstrated for a recombinant killed Newcastle disease virus (NDV) vaccine expressing FAdV-4 fiber-2, and a FAdV-4 whole virus vaccine expressing the IBDV protein VP2 (Tian *et al*., 2020; Zhang *et al*., 2022a).

Despite the fact that inactivated vaccines proved to be effective against FAdV-induced diseases, they require a parenteral route of administration, which makes them unsuitable for mass application in the field, a feature that is highly desirable in the poultry industry.

6.4.2. Live vaccines

FAdV vaccines based on live virus dates back to 1993, when a study from Pallister *et al.* (1993) described a successful spray-vaccination of specific pathogen-free (SPF) chickens with a FAdV-E strain isolated from an IBH outbreak in Australia, with birds being challenged with different isolates belonging to the same FAdV species but displaying different degrees of pathogenicity. Live vaccines against FAdV-8b are utilized in Australia to this date, although outbreaks of IBH caused by FAdV-8b and -11 are still reported, possibly due to the observed differences in vaccination

considering dose, timing and method of administration in the field, or the inability of such formulations to protect across diverse strains and serotypes (Steer *et al*., 2011). More recently, live vaccines against IBH were tested in bivalent formulations containing two FAdV species after virus propagation in cell culture, a concept also applied for inactivated vaccines, and they were able to achieve broad vertical protection against all IBH-causing serotypes by maternal antibody transfer to the progeny (Popowich *et al*., 2018). Embryo and cell culture played a pivotal role in the formulation of FAdV-4-based live-attenuated vaccines against HHS. In 2007, Schonewille *et al.* developed an attenuated strain from a virulent FAdV-4 field isolate by adapting it to a fibroblast cell line (QT-35), and the attenuated strain was successfully used to immunize chickens against HHS, a model confirmed later on by another live FAdV-4 vaccine attenuated through repeated passaging on chicken embryos (Schonewille *et al*., 2008; 2010; Mansoor *et al*., 2011). Similarly to the trend observed for inactivated vaccines, studies on live vaccination against HHS have recently focused on the development of recombinant viruses attenuated through the engineering of FAdV-4 capsid components. Examples include partial or complete deletion of fiber-2, substitution of the fiber with a CRISPR/Cas-generated fusion protein, or the replacement of the hexon protein with one from an apathogenic strain (Hu *et al*., 2021; Zhang *et al*., 2021; Mu *et al*., 2021; Pan *et al.*, 2021; Xie *et al.*, 2021a; 2021b; 2022).

Differently from inactivated vaccines, formulations containing live virus can be administered orally or through the respiratory route, and they are therefore suitable for mass application in the farm. However, live vaccines are associated with the risk of spillover to wild birds and subsequent dissemination of pathogens in the field, an eventuality that has been observed for aviadenoviruses as well (Devlin *et al*., 2016; Das *et al*., 2017). Furthermore, and similar to inactivated whole virus vaccines, tissue culture is needed to amplify vaccine strains, an important subject for production.

6.4.3. Subunit vaccines

The concept of capsid proteins as subunit antigens to formulate vaccines against FAdVs derives from previous studies that focused on other adenoviruses targeting poultry. In fact, the immunogenicity of structural proteins, in particular hexon, fiber and fiber-knob, was already demonstrated for diseases caused by haemorrhagic enteritis virus (HEV), and egg drop syndrome virus (EDSV). These studies demonstrated that the administration of adenoviral structural proteins in birds, and especially hexon and fiber-knob, is able to elicit systemic neutralizing antibodies (nAbs) and limit the splenic viral load after HEV infection (Van den Hurk & Van Drunen Littel-van den Hurk, 1993; Fingerut *et al*., 2003; Pitcovski *et al*., 2005). This led to the experimentation on FAdV subunit vaccines, with a number of studies that investigated the protective efficacy of different structural proteins. Given its high degree of phylogenetic conservation, the FAdV-4 penton base was successfully employed to immunize chickens against homologous infection, reaching a surviving rate of 90% (vs. 10% of the challenge control) (Shah *et al.*, 2012). However, later studies reported only partial protection after immunization with recombinant penton base against HHS, with a survival rate of vaccinated birds ranging from 35% to 67% after challenge (Wang *et al*., 2018b; 2019; Aziz *et al*., 2019). Such inconsistencies might be due to different antigen concentrations, as demonstrated by Wang *et al*. (2018b), but also reflect variations with regard to the route of vaccine administration, type and age of the birds. So far, no studies have investigated the penton base as possible vaccination antigen against IBH. Similarly, vaccination with recombinant hexon loop-1 was only able to grant incomplete protection against FAdV-4 (Schachner *et al*., 2014; Wang *et al*., 2018b). The protective efficacy of recombinant 100K protein was also assessed in a vaccination study that led

to a 40% survival rate of immunized chickens (vs. 10% in the challenge control) (Shah *et al*., 2016).

To this date, the most efficient results, aside for more comprehensive antigens like penton-dodecahedron complexes (Pb-Dd) (Wang *et al*., 2019), were achieved with FAdV-4 fiber-2 vaccination, which was first established to grant full protection against HHS in 2014 (Schachner *et al*., 2014). After this, numerous studies confirmed this outcome by investigating different fiber-2 vaccine doses, expression systems, formulations and vaccination regimes (Wang *et al*., 2018b; Chen *et al*.; 2018; Ruan *et al*., 2018; Yin *et al*., 2021). Recently, a vaccine containing a truncated fiber-2 fused with a hexon epitope sequence was also proved to be protective against HHS (Hu *et al*., 2021). FAdV-4 fiber-1 was experimentally investigated as immunization antigen as well, leading to somewhat contradictory findings and a survival rate of the vaccinated and homologous-challenged chickens ranging from 62% to 100% (Schachner *et al*., 2014; Wang *et al*., 2018b; 2019). Only the fiber protein was investigated as vaccine antigen for IBH, with the recombinant FAdV-8b fiber tested for protection against a homologous IBH-causing strain, achieving a survival rate of 82.7% in progeny of vaccinated breeders (vs. ~52% in the challenge control) (Gupta *et al*., 2017).

In general, subunit vaccines retain the same disadvantages as inactivated vaccines, such as the unsuitability to mass application, which is desirable in the field. However, recent studies have shown that it may be possible to overcome this issue by improving the expression system and the delivering of recombinant immunogens (Lucero *et al*., 2021). Moreover, recombinant bacteria expressing FAdV-4 capsid proteins such as hexon and fiber-2 have been successfully utilized to orally immunize birds against HHS, with the fiber protein being once again superior to the hexon in regards to percentage of survival after vaccination (Jia *et al*., 2021; 2022; Cao *et al*., 2022).

Depending on the expression system, recombinant proteins can be produced in fermenters omitting the use of tissue culture with impact on production costs.

Table 1. Comprehensive list of studies on experimental vaccines for the control of FAdV-induced disease complexes

Vaccine type	Preparation/ formulation	Quantity of vaccination antigen	FAdV strain	Assessment of vaccine response	Protection (% survival rate)	Reference
Inactivated vaccine	Formalinized (0.5%) liver homogenate extract	0.5 ml of 30% liver homogenate	FAdV from an HHS field outbreak, n.c.[a]	Challenge test: homologous virus s.c.	80% (vs. 0% in challenge control group)	Chishti *et al*., 1989
Inactivated vaccine	Formalinized (0.1%) liver homogenates, s.c. administered	0.25 ml of 30% liver homogenate	FAdV field strains, n. c.	Field testing	Prophylactic administration: 98.92% (vs. 89.73% in unvaccinated birds) Administration during an outbreak: 97.67% (vs. 89.32% in unvaccinated birds)	Afzal & Ahmad, 1990
Inactivated vaccine	Formalinized (0.1%, 0.5%) liver homogenates	0.2-0.25 ml of liver homogenate	K31/89 (FAdV-4 field isolate)	Challenge test: homologous virus s.c.	≥85% (vs. 25-60% in challenge control groups)[b]	Anjum, 1990
Live vaccine	Mildly pathogenic strains, administered	10^5 $TCID_{50}$	FAdV-E field isolates	Challenge test: FAdV-E field isolates, spray-administered	Protection assessed through nAbs titers and virus re-isolation	Pallister *et al*., 1993

	through spray-vaccination				from coecal tonsils	
Inactivated vaccine	Cell cultured and egg-passaged formalinized virus, s.c. administered	$10^{3.5}$ LD_{50}	PARC-1 (FAdV-4 field isolate)	Serological response: AGPT Challenge test: homologous virus s.c.	100% (vs. 30% in challenge control groups)	Naeem *et al*., 1995a
Inactivated vaccine **Live vaccine**	Cell cultured formalinized (0.01%) virus Cell culture propagated virus, orally administered	Inactivated vaccine: 10^4 $TCID_{50}$ Live vaccine: 10^3 $TCID_{50}$	PL-1 (FAdV isolated from an IBH field outbreak)	Serological response: neutralization test in embryonated eggs Challenge test: homologous virus intraabdominally	n.c.	Kaur *et al*., 1997
Inactivated vaccine	Formalinized, oil-emulsified virus, s.c. administered	n.s.[c]	FAdV isolated from an HHS field outbreak, n.c.	Serological response: IHA Challenge test: homologous virus	100% (vs. 0% in challenge control group)	Zia *et al*., 2001
Commercial inactivated vaccine	Cell cultured inactivated, oil-emulsified virus	n.s.	FAdV-4	Serological response: VNT in vaccinated breeders	86.7% (vs. 73.3% in challenge control group)	Toro *et al.*, 2002

				Challenge test in progenies: FAdV-4 field isolate i.m.		
Commercial inactivated dual-serotype vaccine	Inactivated virus	n.s.	1047 (FAdV-11 field isolate) + 8565 (FAdV-8a field isolate)	Serological response: maternal antibody measurement of ELISA based on strain 8565 Challenge test in progenies: homologous strains and FAdV-8b s.c.	≥98% (vs. 0% in challenge control groups) for challenge with FAdV-8b[b] ≥92% for challenge with homologous strains[b,d]	Alvarado *et al.*, 2007
Live vaccine	*In vitro* attenuated vaccine, produced on QT35 cells, orally administered	0.5 ml of 10^5 $TCID_{50}$/ml	INT4 (FAdV-4) (MK572850)	Challenge test: virulent progenitor virus AG234 (MK572849) i.m.	100% (vs. 34.5% and 46.2% in challenge control groups)[b]	Schonewille *et al.*, 2008; 2010
Live vaccine	Chicken embryo-adapted vaccine, orally administered	reverse passive haemagglutination titer of $11log_2$	FAdV-4 field isolate (DQ264728)	Serological response: whole virus based ELISA with the	94.74% (vs. 30% in challenge control group)	Mansoor *et al.*, 2011

				homologous strain Challenge test: homologous virus orally & s.c.		
Passive immunization	Passive immunization by IgY from egg yolk, s.c. administered	15 ml of IgY suspension	FAdV-4 field isolate	Challenge test: homologous virus, orally	86.67% (vs. 33.34% in challenge control group)	Rani *et al*., 2012
Subunit vaccine	Recombinant Pb protein, expressed in *E. coli*	25 µg	FAdV-4 field isolate (HE653773)	Serological response: recombinant Pb ELISA Challenge test: homologous virus strain s.c.	90% (vs. 10% in challenge control group)	Shah et al., 2012
Live vaccine	Apathogenic strain, orally administered	10^8 $TCID_{50}$, $10^{8.8}$ $TCID_{50}$	FAdV-1 reference strain CELO	Serological response: VNT Challenge test: FAdV-1 field isolate orally	Protection confirmed by absence of pathological changes	Grafl *et al*., 2014; 2020
Inactivated vaccine	Formalinised (0.2%), oil-adjuvanted virus	$5x10^5$ $TCID_{50}$	FAdV-4 field isolate (HQ697593)	Serological response: AGPT,	80-85% for homologous challenge (vs. 0%	Kim *et al*., 2014

				commercial ELISA Challenge test in vaccinated and progeny chickens: homologous and serotype-heterologous virus strains (FAdV-5, -8a, -8b, -11) i.v. or i.m. (progeny)	in challenge control group) 95-100% for FAdV-5 challenge (vs. 60-70% in challenge control group) 100% for FAdV-8a challenge (vs. 60-80% in challenge control group) 90-100% for FAdV-8b challenge (vs. 10% in challenge control group) 100% for FAdV-11 challenge (vs. 30% in challenge control group)[b]	
Subunit vaccine	Recombinant fiber-1, fiber-2 and hexon loop-1 proteins, expressed in baculovirus system	50 µg	FAdV-4 reference strain KR5	Serological response: recombinant fiber-based ELISA, VNT Challenge test: FAdV-4 field isolate i.m.	fiber-1: 62% fiber-2: 96% hexon loop-1: 27% (vs. 22% in challenge control group)	Schachner *et al*., 2014

Live vaccine **Commercial inactivated vaccine**	*In vitro* attenuated vaccine, produced on Vero cells and lyophilized, orally administered Cell culture propagated virus, β-propiolactone inactivated and oil-emulsified vaccine Commercial inactivated vaccine (Bio-Angara, Sana Laboratories, Pakistan)	n.s.	FAdV-4 field isolate (DQ264728)	Serological and cellular response: commercial ELISA and phyto-haemagglutinin-P assay Challenge test: homologous virus strain orally	Live vaccine: 100% β-propiolactone inactivated: 70% oil-emulsified inactivated vaccine: 80% commercial inactivated vaccine: 60% (vs. 30% in challenge control group)	Ali *et al.*, 2015
Inactivated vaccine **Subunit vaccine**	Heat- and formalin (0.1%) treated virus, PCEP adjuvanted Recombinant hexon protein	Inactivated virus: 2 µg Recombinant hexon: 1 µg	SK (FAdV-8b field isolate) (JN112373)	Serological response: ELISA based on inactivated homologous whole virus	n.d.[e]	Dar *et al.*, 2015

	expressed in *E. coli*					
Inactivated vaccine	Binary ethyleneimine (BEI)-inactivated, oil-adjuvanted virus	10^8 $TCID_{50}$, 10^7 $TCID_{50}$, 10^6 $TCID_{50}$	FAdV-2 field isolate	Serological response: recombinant hexon-based ELISA, VNT Challenge test in embryonic eggs and progeny: homologous virus, allantoic, oral	90% (embryonic eggs) 100% (progeny of vaccinated broilers)[b]	Junnu *et al*., 2015
Subunit vaccine	Recombinant 100K, expressed in *E. coli*	25 µg	FAdV-4 field isolate	Serological response: recombinant 100K ELISA Challenge test: homologous virus s.c.	40% (vs. 10% in challenge control group)	Shah *et al*., 2016
Inactivated vaccine	Heat- and formalin (0.1%) treated virus, PCEP adjuvanted, *in ovo* administered	1-2 µg of inactivated virus	SK (FAdV-8b field isolate) (JN112373)	Serological response: ELISA based on inactivated homologous whole virus	n.d.	Sarfraz *et al*., 2017

				Cytokine expression analysis from spleen cells		
Inactivated vaccine	Cell-propagated or embryo-adapted, formalinized (0.03%), oil-emulsified virus	10^6 $TCID_{50}$	FAdV-HN (FAdV-4 field isolate)	Serological response: AGP and commercial ELISA Challenge test: homologous virus i.m.	100% (vs. 10% in challenge control group)	Du *et al*., 2017
Subunit vaccine	VLPs, recombinant fiber and fiber-knob proteins, expressed in *E. coli*	2x50 µg	SK (FAdV-8b field isolate) (JN112373)	Serological and cellular response: IgY assessment, VNT, FCM in PBMCs Challenge test in progeny: homologous virus i.m.	VLPs: 100% fiber: 82.7% fiber-knob: ~ 57% (vs. ~52% in challenge control group)	Gupta *et al*., 2017
Inactivated vaccine	Cell-propagated, formalinized (0.2%), oil-emulsified virus	10^6 $TCID_{50}$	HLJFAd15 (FAdV-4 isolate) (KU991797)	Serological response: commercial ELISA Cytokine expression	100% (vs. 10% in challenge control group)	Pan *et al.*, 2017

				analysis in serum Challenge test: homologous virus orally		
Inactivated vaccine	formalinized (0.1%), oil-emulsified virus	$6x10^4$ $TCID_{50}$	CH/GZXF/1602 (FAdV-4)	Challenge test: homologous FAdV-4 or FAdV-8a s.c.	100% (vs. 20-30% in FAdV-4 challenge control, and 80-90% in FAdV-8a challenge control group)	Xia *et al*., 2017
Subunit vaccine **Inactivated vaccine**	Recombinant fiber-2 protein, expressed in *E. coli* Formalinized (0.2%), adjuvant-emulsified virus	Recombinant fiber: 10 μg Inactivated vaccine: 10^6 $TCID_{50}$	FAdV-4 field isolate	Serological and cellular response: recombinant fiber-2 ELISA, VNT, FCM in PBMCs Cytokine expression analysis in serum Challenge test: homologous virus i.m.	fiber-2: 100% inactivated vaccine: 90% (vs. 0% in challenge control group)	Chen *et al*., 2018

Live dual-serotype vaccine **Inactivated dual-serotype vaccine**	Live viruses, orally administered Heat-inactivated viruses	Live vaccine: 10^4 $TCID_{50}$ for each serotype Inactivated vaccine: 10^6 $TCID_{50}$ for each serotype	SK (FAdV-8b field isolate) + 1047 (FAdV-11 field isolate)	Serological response: VNT Challenge test in progeny: homologous viruses, FAdV-2 reference strain 685, i.m.	98-100% (vs. 23% in challenge control group) for challenge with FAdV-8b 99% (vs. 80% in challenge control group) for challenge with FAdV-11 100% (vs. 95% in challenge control group) for challenge with FAdV-2	Gupta *et al*., 2018
Live single and dual-serotype vaccine	Cell culture propagated viruses, orally administered	Bivalent: 10^4 $TCID_{50}$ for each serotype Single: 10^6 $TCID_{50}$	Bivalent: FAdV-8a reference strain TR59 + 1047 (FAdV-11 field isolate) Single: SK (FAdV-8b field isolate) (JN112373)	Serological response: VNT Challenge test in progeny: homologous viruses, FAdV-2 reference strain 685, FAdV-7, i.m.	Bivalent live: ≥95% (vs. ~ 60% in challenge control group) for challenge with FAdV-2 ≥95% (vs. 80% in challenge control group) for challenge with FAdV-7 ≥90% (vs. ~ 70% in challenge	Popowich *et al*., 2018

					control group) for challenge with FAdV-8a 85% (vs. 10% in challenge control group) for challenge with FAdV-8b 100% (vs. ~ 68% in challenge control group) for challenge with FAdV-11[b,f]	
Subunit vaccine **Inactivated vaccine**	Recombinant fiber-2 protein, expressed in *E. coli* Inactivated oil-emulsion vaccine	Recombinant fiber: 0.5 µg, 1 µg, 2.5 µg, 5 µg, 10 µg Inactivated vaccine: 10^6 $TCID_{50}$	JSJ13 (FAdV-4 isolate) (KM096544.1) for recombinant fiber, SB15 (FAdV-4 isolate) for inactivated vaccine	Serological response: recombinant fiber-2 ELISA Challenge test: FAdV-4 isolate i.v.	100% with ≥2.5 µg dose of fiber-2 and inactivated vaccine (vs. 0% in challenge control group)[b]	Ruan *et al*., 2018
Subunit vaccine	Recombinant fiber-1, fiber-2, Pb and hexon loop-1 proteins, expressed in *E. coli*	n.s.	SXD15 (FAdV-4 isolate) (KU569296.1)	Serological response: recombinant proteins ELISA Challenge test: homologous virus i.m.	fiber-1 (50, 100, 200 µg dose): 75%, <75%, 100%, respectively fiber-2 (50, 100, 200 µg dose): 100%	Wang *et al*., 2018b

					Pb (50, 100, 200 µg dose): 35%, 50%, <100% hexon loop-1 (50, 100, 200 µg dose): 70%, 75%, <100%, respectively (vs. 45% in challenge control group)[b]	
Subunit vaccine **Commercial inactivated vaccine**	Recombinant Pb and Pb^{1-225} (aa 1-225) proteins, expressed in *E. coli*, s.c. administered Formalinized liver homogenate extract, s.c. administered	Recombinant Pb: 100 µg Inactivated vaccine: 300 µl of liver homogenate	FAdV-4 field isolate (HE653773.1)	Serological response: recombinant Pb and Pb^{1-225} ELISA Challenge test: FAdV-4 field isolate s.c.	50% (vs. 0% in challenge control group)	Aziz *et al.*, 2019
Inactivated vaccines	Formalinized (0.2%), oil adjuvant virus emulsion, i.m.	10^6 $TCID_{50}$	SDJN0105 (FAdV-4 field isolate) (MN102413)	Serological response: commercial ELISA	100% (vs. 20% in challenge control group)	Meng *et al*., 2019

				Challenge test: homologous virus i.m.		
Inactivated vaccine	Formalinized (0.1%), water-in-oil virus emulsion	$10^{7.05}$ $TCID_{50}$	FAdV-8a, -8b (GU120267) and -11 (GU120269) field isolates	Serological response: commercial ELISA, VNT Challenge test: homologous and heterologous viruses (same used for vaccination) i.p.	Homologous and heterologous (FAdV-8a vaccine vs. -11 challenge) protection confirmed by absence of pathological changes	Steer-Cope *et al*., 2019
Subunit vaccine **Inactivated vaccine**	Recombinant Pb-Dd, fiber-1, fiber-2 and Pb proteins, expressed in *E. coli* Inactivated vaccine	Recombinant proteins: 0.5 µg Inactivated vaccine: n.s.	SXD15 (FAdV-4 isolate) (KU569296.1)	Serological response: recombinant proteins ELISA Cytokine expression analysis in PBMCs Challenge test: FAdV-4 isolate i.m.	Pb-Dd, fiber-1 and inactivated vaccine: 100% fiber-2: 80% Pb: 67% (vs. 26.7% in challenge control group)	Wang *et al*., 2019
Subunit vaccine	Recombinant fiber protein,	50 µg	FAdV-8a reference strain TR59	Serological and cellular	Homologous (but not heterologous)	Present study

	expressed in baculovirus system			response: recombinant fiber ELISA, VNT, FCM in PBMCs Challenge test: FAdV-8a and -8b field isolates i.m.	protection confirmed by absence of clinical signs and significantly reduced pathological changes	(De Luca *et al*., 2020)
Live recombinant vaccine **Inactivated recombinant vaccine**	NDV LaSota vaccine strain expressing FAdV-4 fiber-2 (rLaSota-fiber2) generated by reverse genetics, i.m. administered live or inactivated	10^7 EID_{50} (for both vaccines)	CH/HNJZ/2015 (FAdV-4 field isolate) (KU558760)	Serological response: fiber-2 ELISA (FAdV-4) and HI (NDV) Challenge test: homologous FAdV-4 or NDV velogenic strain i.m.	Live vaccine: 100% for both challenges Inactivated vaccine: 70% for challenge with FAdV-4 (vs. 0% in challenge control groups)	Tian *et al*., 2020
Subunit vaccine	Truncated region of fiber-2 protein fused with coding sequence of one hexon epitope, expressed in *E. coli*	2.5 µg, 5 µg, 2x5 µg (booster), 7.5 µg	HB1505 (FAdV-4 field isolate)	Challenge test: homologous virus, i.m., oral	100% with ≥5 µg dose (vs. 0% in challenge control group)[b]	Hu *et al*., 2021

Live recombinant vaccine	*Lactococcus lactis* expressing FAdV-4 hexon, and *Enterococcus faecalis* expressing hexon with dendritic cells targeting peptide, orally administered	*Lactococcus lactis*: 10^{10} CFU *Enterococcus faecalis*: $5x10^{9}$ CFU	GX01 (FAdV-4 field isolate)	Serological response: IgG from sera and sIgA from jejunal lavage on hexon ELISA Cytokine expression analysis in spleen Challenge test: homologous virus, i.m.	*L. lactis/*hex: 50% *L. lactis*/hex-DC: 60% *E. faecalis*/hex: 80% *E. faecalis*/hex-DC: 90% (vs. 0% in challenge control group)	Jia *et al.*, 2021
Live recombinant vaccine	Recombinant virus FAdV-4-RFP_F1 expressing RFP-fiber-1 fusion protein generated by the CRISPR/Cas9 technique, i.m. administered	$2x10^{5}$ $TCID_{50}$	SD2015 (FAdV-4 isolate)	Serological response: VNT Challenge test: homologous virus i.m.	100% (vs. 18% in challenge control group)	Mu *et al.*, 2021
Live recombinant vaccine	Recombinant chimeric viruses obtained by replacing their hexon gene	Live vaccine: n.s. Inactivated vaccine: 0.3 ml of 10^{7} PFU/ml	rHN20 (non-pathogenic chimeric virus derived from the combination of FAdV-4 field isolate HLJFAd15 with ON1 or	Serological response: VNT (for rHN20-vvIBDV-VP2 vaccine)	Live vaccine: 100% (vs. 0% in FAdV-4 challenge control group and 30% IBDV	Pan *et al.*, 2021 Zhang *et al.*, 2022a

Inactivated recombinant vaccine	with a non-pathogenic one, or different ORF combinations replaced by EGFP (rDL3-EGFP and rHN20-EGFP), or expressing a virulent IBDV VP2 protein (rHN20-vvIBDV-VP2), all expressed in *E. coli*, i.m. administration rHN20-vvIBDV-VP2 formalinized (0.1%) and oil-adjuvated		EGFP casettes) (KU991797, GU188428)	Challenge test: FAdV-4 i.m., or IBDV i.m. (inact.)/i.n. (live vaccine)	challenge control group)[b] Inactivated vaccine: 100% (vs. 10% in each challenge control group)	
Subunit vaccine **Inactivated commercial vaccine**	Three different purified epitopes of hexon protein Formalin-inactivated commercial	2x100 µg Inactivated vaccine: 300 µl of liver homogenate	FAdV-4	Serological response: hexon epitopes ELISA Challenge test: FAdV-4 field isolate	Hexon epitopes: 90%, 70%, 40%, depending on the epitope commercial inact. vaccine: 50% (vs. 50% in challenge control)	Tufail *et al*., 2021

	vaccine Angara NIAB					
Live recombinant vaccine **Inactivated vaccine**	Recombinant virus FA4-EGFP expressing EGFP-Fiber-2 fusion protein generated by the CRISPR/Cas9technique, i.m. administered Inactivated oil-emulsion formulation, i.m. administered	Live vaccine: 10^4 $TCID_{50}$, 10^5 $TCID_{50}$, 10^6 $TCID_{50}$ Inactivated vaccine: $5x10^6$ $TCID_{50}$	SD (FAdV-4 field strain)	Serological response: VNT Challenge test: homologous (wild type) virus i.m.	100% (vs. 0-20% in challenge control group)[b]	Xie *et al*., 2021a
Live recombinant vaccine	Recombinant fiber-2-edited FAdV-4 with a deletion of 7-40aa in Fiber-2, generated by CRISPR/Cas9 technology, i.m. administered	$2.5x10^4$ $TCID_{50}$	SD (FAdV-4 field strain)	Challenge test: homologous (wild type) virus i.m.	100% (vs. 20% in challenge control group)	Xie *et al*., 2021b
Subunit vaccine	Recombinant fiber-2 protein, recombinant	Recombinant fiber and DNA: 50 µg, 100 µg, 150 µg	GZ-QL (FAdV-4 field isolate)	Serological: commercial ELISA	Fiber: ≥80% Fiber DNA: 80%	Yin *et al.*, 2021

Commercial inactivated vaccine	fiber DNA plasmid, expressed in *E. coli* Inactivated virus	Inactivated vaccine: n.s.		Cytokine expression analysis in serum Challenge test: homologous virus i.m.	commercial inactivated vaccine: ≥50% (vs. 0% in challenge control group)[b]	
Live recombinant vaccine **Inactivated recombinant vaccine**	Recombinant virus obtained by replacing its hexon gene, either used live (administered i.n., s.c. or i.m.) or after inactivation with formalin (0.1%) and oil-adjuvanted	Live vaccine: 10^4, 10^5, 10^6 PFU Inactivated vaccine: 0.3 ml of 10^7 PFU/ml	rHN20 (non-pathogenic recombinant virus derived from FAdV-4 field isolates HLJFAd15 and ON1) (KU991797, GU188428)	Serological response: VNT Challenge test: homologous virus (HLJFAd15) i.m.	100% (vs. 0% in challenge control group)[b]	Zhang *et al*., 2021; 2022b
Live recombinant vaccine **Inactivated vaccine**	*S. cerevisiae* strain expressing FAdV-4 fiber-2 protein, orally administered Inactivated FAdV-4 vaccine	Live vaccine: 10^9 CFU Inactivated vaccine: n.s.	SX17 (FAdV-4 field isolate) (MF592716.1)	Serological response: FAdV-4 fiber-2 ELISA Cytokine and immune cells expression	100% (vs. 86% in challenge control groups)	Cao *et al*., 2022

	(Qingdao, China), i.m./s.c. administered			analysis in cecal tonsils Challenge test: homologous virus i.m.		
Subunit vaccine	Recombinant chimeric fiber protein retaining epitopes from FAdV-4 (fiber-2) and -11, expressed in baculovirus system	2x50 µg	FAdV-4 reference strain KR5 + 13/14796 (FAdV-11 field isolate)	Serological and cellular response: recombinant chimeric fiber ELISA, VNT, FCM in PBMC and target organs Challenge test: FAdV-4 and -11 field isolates i.m.	Homologous and heterologous protection confirmed by absence of clinical signs and significantly reduced pathological changes	Present study (De Luca *et al*., 2022a; 2022b)
Live recombinant vaccine	*Lactococcus lactis* and *Enterococcus faecalis* expressing FAdV-4 fiber-2, expressed in *E.coli*, orally administered	*Lactococcus lactis*: 10^{10} CFU *Enterococcus faecalis*: $5x10^9$ CFU	GS01 (FAdV-4 field isolate)	Serological and cellular response: IgG in serum and sIgA in jejunum via fiber ELISA, T cells quantification	*Lactococcus lactis*+fib-2: 60-80% *Enterococcus faecalis* +fib-2: 90-100% (vs. 0% in the challenge control group)	Jia *et al*., 2022

				from PBMCs via CCK-8 method Cytokine expression analysis in spleen Challenge test: FAdV-4 homologous		
Inactivated recombinant vaccine	Recombinant FAdV-4 expressing FAdV-8b fiber, generated by CRISPR/Cas9 technology, formalinized (0.3%) and oil-adjuvated	10^6 $TCID_{50}$	Recombinant FA4-F8b, obtained by merging isolates SD (FAdV-4) and JSSQ15 (FAdV-8b)	Serological response: VNT Challenge test: FAdV-4 and -8b homologous viruses, and FAdV-8a, parenteral route	100% (vs. 20% in challenge control group) for challenge with FAdV-4 Homologous protection for challenge with FAdV-8b confirmed by significant reduction of viral titers in target organs No heterologous protection observed for	Lu *et al*., 2022

					challenge with FAdV-8a	
Subunit vaccine	Recombinant chimeric fiber protein retaining epitopes from FAdV-8a and -8b, expressed in baculovirus system	50 µg, 2x50 µg	FAdV-8a and -8b reference strains TR59 and 764	Serological response: recombinant chimeric fiber ELISA, VNT Challenge test: FAdV-8a and -8b field isolates i.m.	Homologous and heterologous protection confirmed by absence of clinical signs and significantly reduced pathological changes[b]	Present study (Schachner *et al*., 2022)
Live recombinant vaccine	Recombinant FAdV4-EGFP expressing EGFP-Fiber-2 fusion protein, and FAdV4-EGFP-rF2 lacking Fiber-2, generated by CRISPR/Cas9 technology, i.m. administered	$2.5x10^4$ $TCID_{50}$	SD2015 (FAdV-4 field strain)	Serological response: VNT Challenge test: homologous (wild type) virus i.m.	100% (vs. 0% in challenge control group)	Xie *et al*., 2022
Subunit vaccine **Inactivated vaccine**	Recombinant WZ- and ON1-Fiber-2 proteins, expressed in *E. coli*	Recombinant fiber: 2 µg Inactivated vaccine: 10^6 $TCID_{50}$	WZ and ON1 (FAdV-4 field strains)	Serological response: homologous whole virus ELISA	WZ-Fiber-2 and inactivated vaccine: 100% (vs. 0% in challenge control group)	Zhao *et al.*, 2022

	Inactivated WZ, emulsified with oil-adjuvant			Challenge test: WZ strain (FAdV-4)	ON1-Fiber-2: 0% (vs. 0% in challenge control group)

[a]n.c., not characterized
[b]results summarized if more than one experiment was conducted
[c]n.s., not specified
[d]no values reported for challenge control group
[e]n.d., not done
[f]results for FAdV8b-SK vaccine not reported

6.5. FAdV immunological features

In chickens, FAdV infection triggers the development of type-specific nAbs, a feature that was used to outline the serological differentiation of FAdVs in 12 different serotypes (McFerran & Adair, 1977; Hess, 2000). The kinetics of the antibody development and the cellular immune response after FAdV vaccination and/or infection are tied to several factors such as the genetic lineage and age of chickens, the route of infection, and the possible presence of other concurrent pathological agents (Schachner *et al*., 2018).

Various clinical reports and experimental studies indicate that FAdVs are immunosuppressive, and consequently to their tropism for lymphoid tissues of the host, such as spleen, thymus and bursa of Fabricius, both HHS and IBH were shown to cause depletion of B and T lymphocytes (Saifuddin & Wilks, 1992; Naeem *et al*., 1995b; Singh *et al*., 1996; 2006; Shivachandra *et al*., 2003; Schonewille *et al*., 2008).

6.5.1. Humoral response

Protection studies on the different viral subunits demonstrated that it is possible to generate specific antibodies measured by enzyme-linked immunosorbent assay (ELISA) against many recombinant capsid proteins, including fibers, penton base, hexon and 100K (Shah *et al*., 2012; 2016; Schachner *et al*., 2014; Dar *et al*., 2015; Feichner *et al*., 2018a; 2018b). However, these antibodies are not necessarily protective as demonstrated for FAdV-4 fiber-1, hexon, penton base and 100K (Schachner *et al*., 2014; Shah *et al*., 2016; Wang *et al*., 2018b; Wang *et al*., 2019). Similar to the humoral response after infection, protection derived by vaccines based on a FAdV-D or -E template, and consequently for IBH, seems to rely mainly on the presence of nAbs (Junnu *et al*., 2015; Gupta *et al*., 2017; 2018; Popowich *et al*., 2018; Steer-Cope *et al*., 2019). However, the generation of nAbs against HHS following

vaccination is not consistent throughout the numerous studies that investigated such aspects. In fact, the detection of antibody response measured via ELISA does not always conform with the presence of neutralizing activity, despite still being associated with clinical protection: this phenomenon has been observed after immunization against FAdV-4 with live/attenuated and fiber subunit vaccines (Schonewille *et al*., 2010; Schachner *et al*., 2014). In contrast, other studies detected nAbs development after immunization against FAdV-4 with live/attenuated and inactivated formulations (Toro *et al*., 2002; Pan *et al*., 2021; Xie *et al*., 2021a; 2022; Zhang *et al*., 2021; 2022a; 2022b). Another study also observed neutralizing activity in the sera of birds vaccinated with fiber-2, although to a lesser amount compared with an inactivated formulation prepared from the same FAdV-4 strain (Chen *et al*., 2018). Generally, maternal antibodies against HHS and IBH protect the progeny of immunized breeders against the diseases (Toro *et al*., 2001; Mazaheri *et al*., 2003; Philippe *et al*., 2007), which marks a contrast compared to the immune response observed against AGE, for which the development of local immunity is crucial for protection (Okuda *et al*., 2001b; Grafl *et al*., 2013).

6.5.2. Cellular response

To this date, not many studies have focused on investigating the cellular immune response related to protection from FAdVs. Schonewille *et al.* (2008) demonstrated that vaccination with a live attenuated FAdV-4 strain was able to prevent lymphocytes depletion in the lymphoid organs of SPF chickens in context with HHS. Another study showed an increase of systemic helper T lymphocytes upon immunization with both FAdV-4 inactivated or a fiber-2 vaccine, although no differences in the level of cytotoxic T lymphocytes were observed (Chen *et al.*, 2018). A reduction of $CD4^+$:$CD8^+$ T cells ratio was demonstrated after booster injection of either FAdV-8b virus-like particles

(VLPs) or FAdV-8b fiber in commercial broiler breeders, suggesting the onset of a proliferation of $CD8^+$ T lymphocytes upon second contact with the antigen (Gupta *et al*., 2017). These results indicate that certain FAdV vaccines have an important effect on the cellular compartment of the chicken immune system, although their protection mechanism still needs to be fully unravelled.

7. Objectives of the thesis

The main goal of the present thesis was to develop a broad-spectrum immunization strategy to protect chickens against IBH and, subsequently, HHS using FAdV recombinant capsid proteins as immunizing antigens. Furthermore, this study aimed to investigate the role of the cell-mediated immune response to such vaccines in order to elucidate the mechanisms behind protection, so far poorly understood.

Overall, the following tasks were performed:

(a) assessment of serotype-specific protection provided by FAdV-8a recombinant fiber after experimental challenge with homotypic (8a) and heterotypic (8b) IBH-causing strains

(b) investigation of the systemic humoral and cellular immune response after successful vaccination with recombinant FAdV-8a fiber protein against homotypic challenge

(c) development of recombinant chimeric fiber proteins (crecFib) retaining epitopes from FAdV-8a and -8b as a novel subunit vaccines against IBH, and subsequent assessment of broad protection against both serotypes *in vivo*

(d) extension of the crecFib concept to simultaneously protect chickens against HHS and IBH through the development of a crecFib retaining epitopes from FAdV-4 and -11 (crecFib-4/11)

(e) in-depth investigation of humoral and cellular immune response elicited from crecFib-4/11 against FAdV-4 challenge not only on a systemic level, but also in target and lymphoid organs

(f) development of recombinant FAdV-E penton base subunit vaccines and assessment of their efficacy in providing protection against IBH

8. Publications

The present thesis includes four manuscripts. In the first manuscript, a recombinant FAdV-8a fiber protein (Fib-8a) was utilized to immunize SPF broiler chickens against IBH-causing strains from serotypes 8a or 8b (De Luca *et al*., 2020). The efficacy of homologous and heterologous vaccination was investigated, and the humoral and cellular immune responses were analysed within the homologous vaccine/challenge system.

The second manuscript describes the development of recombinant chimeric fiber proteins retaining epitopes from FAdV-8a and -8b, one of which successfully cross-protected chickens against both serotypes, representing the first instance in which a subunit vaccine provided successful immunization against different IBH-causing serotypes (Schachner *et al*., 2022).

The third manuscript outlines how this concept was applied to a new chimeric fiber protein retaining mixed identity from two different FAdV species (-C and -D), in order to extend the cross-protective spectrum to distinct FAdV-associated diseases, namely HHS and IBH (De Luca *et al*., 2022a).

Finally, in the fourth manuscript, the kinetics of the systemic and local cellular immune response against HHS after crecFib vaccination were investigated in depth (De Luca *et al.*, 2022b).

The first and second manuscripts are published in Veterinary Research and Microbiology Spectrum, respectively, while the third manuscript is published in Vaccine, and the fourth manuscript in Frontiers in Immunology.

8.1. Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH) provides type-specific protection guided by humoral immunity and regulation of B and T cell response

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH) provides type-specific protection guided by humoral immunity and regulation of B and T cell response

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Abstract

A recombinant fowl adenovirus (FAdV) fiber protein, derived from a FAdV-8a strain, was tested for its efficacy to protect chickens against inclusion body hepatitis (IBH). FAdV-E field isolates belonging to both a homotypic (FAdV-8a) and heterotypic (-8b) serotype were used as challenge. Mechanisms underlying fiber-induced protective immunity were investigated by fiber-based ELISA, virus neutralization assays and flow cytometry of peripheral blood mononuclear cells, monitoring the temporal developments of humoral and cellular responses after vaccination and challenge exposure. Birds were clinically protected from the homologous challenge and showed a significant reduction of viral load in investigated target organs, whereas fiber-based immunity failed to counteract the heterologous serotype infection. These findings were supported in vitro by the strictly type-specific neutralizing activity of fiber immune sera. In protected birds, fiber vaccination prevented a post-challenge drop of peripheral B cells in blood. Furthermore, fiber immunization stimulated $CD4^+$ T lymphocyte proliferation while moderating the $CD8\alpha^+$ T cell response and prevented challenge-induced changes in systemic monocytes/macrophages and $\gamma\delta^+$ T cell subpopulations. Both vaccinated and adjuvant-only injected birds experienced a priming of systemic B cells and $TCR\gamma\delta^+$ T lymphocytes, which masked possible pre-challenge effects due to the antigen. In conclusion, within FAdV-E, recombinant fiber represents a vaccine candidate to control the adverse effects of homotypic infection by eliciting an effective humoral immunity and regulating B and T cell response, whereas the failure of heterotypic protection suggests a primordial role of humoral immunity for this vaccine.

Keywords: Fowl adenovirus, fiber, inclusion body hepatitis, vaccine, humoral immunity, cellular immunity

Introduction

Fowl adenoviruses (FAdVs) are non-enveloped, dsDNA viruses belonging to the family *Adenoviridae*, genus *Aviadenovirus*. The current classification recognizes five species based on genomic criteria (*Fowl aviadenovirus A* to *Fowl aviadenovirus E* (FAdV-A–FAdV-E)), with 12 subordinate serotypes (FAdV-1 to -8a, and -8b to -11) defined by cross-neutralization [1, 2]. Particular FAdV types, belonging to different species, are associated with three disease complexes with relevance for the commercial poultry sector on a global scale [3]. Analogous to the extent of genetic separation between the responsible strains, adenoviral gizzard erosion (AGE) caused

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by FAdV-1 (species FAdV-A) represents a self-standing pathology, distinct from hepatitis-hydropericardium syndrome (HHS), caused by FAdV-4 (FAdV-C), and inclusion body hepatitis (IBH), caused by serotypes -2 and -11 (FAdV-D), and -8a and -8b (FAdV-E), which show relatively closer molecular relationship and similar features of pathogenesis and protection [3, 4].

To date, immunization strategies in the field are mainly limited to the use of inactivated autogenous vaccines, but the growing urgency for an efficacious and broad-coverage protection has led to the experimental development of FAdV subunit vaccines. Most of these efforts have utilized capsid components as target immunogens, based on the rationale that these are the main players in conferring antigenicity of adenoviruses [5]. In particular recombinant penton base and fiber, either alone or as a complex, were demonstrated to be efficacious vaccine candidates, providing the proof-of-concept with HHS as model system, with vaccination/challenge schemes based upon serotype -4 [6–8]. Subsequently, fiber-based vaccines were extended to IBH, addressing for the first time also vertical protection in the framework of a subunit vaccine [9]. However, differently from HHS with its mono-type etiology, the control of IBH by vaccination is complicated by a diverse spectrum of viral species and serotypes, ultimately requiring broad-protection strategies. The additional possibility of mixed infections in the field [10–12] indicates that chickens remain susceptible to heterologous infection despite pre-existing immunity against another FAdV serotype, and likely represents the molecular basis for the recently reported natural recombination of FAdVs, exchanging fibers between IBH-causing types [13]. Furthermore, enforced vaccination efforts against one serotype can cause a shift towards outbreaks with other serotypes [14–16]. On the other hand, experimental data on immunization against IBH collectively indicate a certain extent of heterotypic protection, albeit these studies remain ambiguous about coverage across the species boundary due to the use of bivalent FAdV-D/FAdV-E vaccines [17–19]. Cross protection amongst different IBH-causing serotypes was also reported using FAdV-C and FAdV-E strains as inactivated vaccines [20–22]. However, all these studies are based on whole virus as vaccine antigen, with protection likely resulting from a synergy of all antigenic components, an effect that does not apply for subunit vaccines.

Besides empirical demonstration of resistance to challenge of fiber-vaccinated birds, the immune mechanisms underlying protection are not well resolved. While both cellular and humoral immune responses are triggered by contact with live FAdV [23, 24], their participation in context with subunit antigens still needs to be clarified. Despite providing full protection, recombinant fiber derived from FAdV-4 elicited only moderate or no neutralizing antibodies in birds, raising questions about the immunological correlates of protection, especially outside the humoral repertoire, for such type of vaccines [7, 25]. Furthermore, type and number of fibers vary in a species-dependent manner, and this may prevent the extrapolation of results of recombinant fiber protection from the HHS to the IBH system.

The present study employed a recombinant fiber with genetic background of FAdV-8a to assess coverage against the complete, type-homologous (-8a) and -heterologous (-8b) spectrum of IBH. Moreover, this is the first study to extend the temporal profile of cellular and humoral immune responses to FAdV subunit vaccination beyond the time point of challenge, allowing the comparison between the pre-stimulated and the naïve (in our case, adjuvant-primed) response during infection.

Materials and methods

Virus and recombinant protein preparation

FAdV-E type reference strains TR59 (FAdV-8a) and 764 (-8b) (GenBank accession numbers KT862810 and KT862811) were used as template strains for cloning. Field isolates 11–16629 and 13–18153 (MK572859 and MK572862), identified by whole-genome sequencing and virus cross-neutralization as members of FAdV-8a and -8b, respectively [13], served as challenge strains. All strains were threefold plaque purified, propagated on primary chicken-embryo liver (CEL) cells, as described by Schat and Sellers [26], and viral titers were determined by endpoint titration [27]. Viral DNA for fiber gene cloning was extracted from cell culture supernatant with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The encoding regions for the FAdV-8a and FAdV-8b fiber (termed hereupon Fib-8a and Fib-8b) were cloned, expressed, purified and visualized as previously reported [7, 28].

Animal experiment

Specific pathogen-free (SPF) broiler chicks were hatched from embryonated eggs (Animal Health Service, Deventer, The Netherlands) at our facilities and randomly divided into six groups (n = 18/group) as summarized in Table 1, separately housed in isolator units (HM2500, Montair, The Netherlands).

At first day of life, chickens of groups I, II and III were vaccinated intramuscularly with 50 µg of recombinant Fib-8a protein, mixed 1:1 with GERBU Adjuvant P (GERBU Biotechnik GmbH, Heidelberg, Germany). Birds of groups IV and V (challenge controls) received a phosphate buffered saline (PBS)/adjuvant-mixture, and group VI (negative control) PBS instead. At 21 days of life (20 days post vaccination, dpv) birds were infected

Table 1 Design of the animal experiment

Group	Designation	Vaccination	Challenge strain (serotype)
I	Vaccination-only	Fib-8a	–
II	Vaccine/homologous challenge	Fib-8a	11–16629 (FAdV-8a)
III	Vaccine/heterologous challenge	Fib-8a	13–18153 (FAdV-8b)
IV	Challenge control FAdV-8a	Adjuvant only	11–16629 (FAdV-8a)
V	Challenge control FAdV-8b	Adjuvant only	13–18153 (FAdV-8b)
VI	Negative control	–[a]	–

[a] Not applicable.

intramuscularly with $10^{6.3}$ 50% tissue culture infective dose ($TCID_{50}$) of virulent FAdV-8a in groups II (vaccine/homologous challenge) and IV, and virulent FAdV-8b in groups III (vaccine/heterologous challenge) and V. Vaccination-only (group I) and negative control (group VI) were injected with PBS.

Five birds from each group were killed and necropsied at 3, 5 and 7 days post challenge (dpc), and the remaining birds on termination of the trial at 14 dpc. Birds that died due to infection were necropsied on the same day of their death. Endpoints for protection included clinical signs recorded during daily monitoring, *post mortem* findings, plasma analytes, and organ-body weight ratios for liver and spleen.

Coupled longitudinal monitoring of the humoral and cellular immune response in blood was restricted to the homologous protection setting, starting at 13 dpv with five vaccine recipients (from group I), five birds administered only adjuvant (group IV) and five non-vaccinated birds (group VI). At subsequent time points (20 dpv: prior to challenge, and 3, 5, 7, 14 dpc), serum for antibody investigation was collected from all the birds. Blood for investigation of cellular immunity was collected at the same time points in the homologous protection setting (groups I, II, IV, VI), keeping the sampled individuals consistent throughout the experiment (n = 5/group and all the remaining birds for the final measurement).

Clinical chemistry

During killing and bleeding the birds, blood was collected from the jugular vein into heparin tubes (VACUETTE®, Greiner Bio-One, Kremsmünster, Austria). Plasma concentrations of aspartate transaminase (AST) and lipase were determined as previously described [29].

Histopathology and immunohistochemistry (IHC)

Samples from liver, bursa of Fabricius and pancreas were fixed in 4% neutral buffered formalin and subsequently embedded in paraffin. In order to perform microscopic examination, 4–5 µm-thick tissue sections were cut with a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany) and mounted on glass slides before undergoing hematoxylin–eosin staining. Five birds from each group challenged with FAdV-8a (groups II and IV) and five birds from the negative control (group VI) were selected for histopathological analyses. All the selected birds were killed between 3 and 5 dpc, when the animals were most affected by the disease.

In order to identify virus-positive hepatocytes, liver sections were additionally mounted on coated glass slides (Superfrost ultra plus, Menzel Gläser, Braunschweig, Germany) to undergo IHC utilizing a polyclonal antibody against the FAdV-E/-7 reference strain YR36 (GenBank accession number KT862809) raised in rabbits, in a dilution of 1:5000. For this, the strain YR36 was propagated on primary CEL cells, pelleted through a CsCl cushion by ultracentrifugation, and the pellet dissolved in PBS. An equal amount of GERBU Adjuvant P (GERBU Biotechnik GmbH, Heidelberg, Germany) was added prior to repeated subcutaneous injections of rabbits. The paraffin was removed from the sections by sequential washing steps in ethanol 100%, 96%, 70%, and distilled water for 5 min each. The sections were then boiled for 10 min in citric acid-monohydrate buffer before being rinsed twice with PBS and left for 30 min in methanol + 1.5% H_2O_2, then 20 min in PBS. At that point the slides were covered in goat serum (Normal goat serum, Vector Laboratories, Burlingame, CA) and incubated in a humid chamber for 1 h; after removal of the serum, the sections were covered with the primary antibody solution and rested overnight in the humid chamber at +4 °C. The following day, the tissue was washed in PBS and incubated for 30 min with the secondary antibody (Goat Anti-Rabbit IgG Antibody (H + L), Vector Laboratories, Burlingame, CA) and then 1 h with the ABC-reagent (ABC kit, Vectastain®, Vector Laboratories, Burlingame, CA) in the humid chamber before being washed in PBS. As a last step, the DAB substrate (DAB Substrate Kit, Vector Laboratories, Burlingame, CA) was applied and the reaction was monitored under the microscope before being stopped after 2.5 min by washing the slides in distilled water. A hematoxylin counterstaining was then executed for each slide.

Quantitative polymerase chain reaction (qPCR) from tissues of target organs

Tissue samples from liver, spleen, pancreas and bursa of Fabricius were collected from birds challenged with FAdV-8a (groups II and IV) and from control birds (group VI) and stored at − 20 °C until processing. DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol and analyzed for quantification of viral DNA with a qPCR assay based on the 52K gene [30].

Fiber-based enzyme-linked immunosorbent assay (ELISA) and virus neutralization test (VNT)

All sera collected during the trial were tested on recombinant fiber ELISA (coated with either Fib-8a or Fib-8b proteins, representing both types used for challenge) following the protocol described by Feichtner et al. [31].

Sera from all the vaccinated birds (groups I, II, III) immediately before challenge (20 dpv), and from 5, 7 and 14 dpc were also tested for neutralizing activity against the two reference strains (TR59 and 764) which served as a template for fiber expression, and the challenge strains (11–16629 and 13–18153) according to a protocol described earlier [7].

Flow cytometry (FCM) analyses

Flow cytometry analyses on peripheral blood mononuclear cells (PBMCs) was performed on five birds per group within the homologous setting (group I, IV, VI at 13 dpv, and group I, II, IV, VI from 20 dpv onwards), keeping the sampled individuals consistent throughout the experiment.

Blood collection and preparation

For the separation of PBMCs, 2 ml of blood was collected from the wing vein of each bird in a heparin syringe. The blood was mixed with an equal volume of cold PBS, pH 7.4 (ThermoFisher Scientific, Vienna, Austria) with 2% fetal bovine serum (FBS) (ThermoFisher Scientific, Vienna, Austria). The prepared suspension was then slowly layered above a double volume of Histopaque®-1077 (Sigma-Aldrich, Vienna, Austria) for density gradient centrifugation. The cells from the interphase layer were collected and washed. Finally, the pellet was dissolved in 1 mL of the same solution.

FCM staining protocol

Mononuclear cells from the blood were examined for their viability using Nexcelom cellometer X2 fluorescent viability cell counter system (Nexcelom Bioscience, Manchester, UK). A concentration of 2×10^7 cells/mL of PBS + 2% FBS was adjusted before the cells were stained. Different combinations of monoclonal antibodies (mAbs) were used for immunophenotyping of $CD4^+$ T cells, $CD8\alpha^+$ T cells, B cells, monocytes/macrophages, $TCR\alpha\beta^+$ T cells and $TCR\delta\gamma^+$ T cells from the isolated cells. Gating strategy for PBMC is given as Additional file 1. A uniform gating hierarchy was used throughout all sampling days. Detailed information on antibody combinations and their fluorescence labelling by second-step reagents are given in Additional file 2. The final concentration of every antibody was determined by titration and the respective isotype controls were included.

For staining of mononuclear cells isolated from blood, 25 µl of the adjusted cell suspension was transferred into wells of 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) together with the respective primary antibodies for incubation for 20 min at 4 °C. Afterwards, cell pellets obtained by centrifugation at 4 °C, 450×*g* for 4 min were washed two times with cold PBS + 2% FBS. For biotinylated antibodies, the secondary reagent Brilliant Violet 421™ Streptavidin (BioLegend, San Diego, CA, USA) was applied. Following another incubation step for 20 min at 4 °C, further washing was performed. The cells were fixed with BD fixation buffer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Finally, the pellets were suspended in 200 µl cold PBS + 2% FBS kept at 4 °C until FCM analysis.

FCM analysis

FCM of stained cells was performed on a FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer equipped by FACSDiva Software version 6.1.3 (BD Biosciences). At least 40,000 lymphocytes per sample were recorded. Analysis of FCM raw data was performed by FlowJo_V10 software (BD Biosciences, San Jose, CA). Absolute quantification of the cells was performed according to Mitra et al. [32].

Statistical analyses

In order to verify the normal distribution assumptions, a preliminary analysis of the datasets was carried out using Shapiro–Wilk test associated with a visual inspection of histograms and normal Q–Q plots. The mean values from plasma analyses, liver- and spleen-body weight ratio, as well as cell populations in PBMC of vaccinated groups were compared with the negative control and their respective challenge control groups via unpaired Student's *t*-test. Pairwise comparisons for datasets not meeting the normality assumptions were carried out with Mann–Whitney *U* test. In each case, p values ≤ 0.05 were

Table 2 Summary of pathognomonic gross lesions recorded in necropsied birds at defined days post challenge (dpc)

	Liver dpc										Spleen dpc				Pancreas dpc				
	3			5			7			14	3	5	7		3	5	7		14
	Focal necroses	Hemorrhages	Marbled	Focal necroses	Hemorrhages	Marbled	Focal necroses	Hemorrhages	Marbled	Marbled	Marbled	Marbled	Marbled	Petechiae	Congestion	Hemorrhages	Congestion	Hemorrhages	Congestion
Vaccine/hom. chall.	–[a]	–	–	–	2/5	1/5	–	–	3/5	1/2	1/6	–	2/5	1/5	1/6	1/5	–	–	–
Chall. contr.-8a	2/5	2/5	2/5	–	–	4/5	–	–	3/5	1/3	–	–	2/5	–	–	–	–	–	–
Vaccine/het. chall.	–	–	3/5	1/5	1/5	1/5	–	–	5/5	-	–	–	1/5	–	–	–	–	1/5	1/3
Chall. contr.-8b	4/5	–	3/5	1/5	1/5	4/5	1/5	1/5	1/5	2/3	–	4/5	3/5	–	–	–	3/5	–	–
Neg. contr.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] No lesions recorded.

considered statistically significant. Statistical analyses were performed with the software package SPSS Version 26 (IBM SPSS Statistics; IBM Corp., Armonk, New York, USA).

Results

Clinical protection of recombinant Fib-8a against homologous and heterologous challenge

Following challenge, clinical signs were characterized by mild depression in one of the birds from the vaccine/heterologous challenge group, one bird of the FAdV-8a and three birds of the -8b challenge controls between 4–5 dpc, and one bird that died at 3 dpc in the vaccine/homologous challenge group. No clinical signs were recorded in the vaccination-only and negative control group throughout the whole experiment.

Frequent gross pathological lesions included severe swelling, marble-like appearance and hemorrhagic areas in most of the livers from the infection-only groups, with a tendency of being more prominent at 3 dpc and less severe at 7 and 14 dpc. Necrotic foci were present on the liver of two birds from the challenge control -8a at 3 dpc and six birds from challenge control -8b between 3–7 dpc. Similar lesions were observed in the vaccinated/challenged groups as well, although the general affection of liver was milder and necrotic lesions were observed in only one bird of the vaccine/heterologous challenge group at 5 dpc. Pathognomonic lesions recorded in liver, spleen and pancreas are summarized in Table 2. No specific lesions were recorded in birds of the vaccination-only and negative control group at any time point.

Mean liver-body weight ratios were not affected in the vaccine/homologous challenge group, whereas they were significantly increased in all FAdV-8b infected groups and -8a challenge control at 3 and 5 dpc compared to the negative control (Figure 1). At 7 dpc only values from the vaccine/heterologous challenge group were still significantly increased. Similarly, spleen-body weight ratio was found elevated up to 7 dpc in all infected groups except the vaccine/homologous challenge. Plasma AST significantly increased at 5 dpc for the vaccine/heterologous challenge birds and the infection-only groups, whereas the lipase was mostly increased at 7 dpc for birds challenged with FAdV-8b.

Viral load in target organs

The organ with the highest mean viral load was the liver at all time points post-challenge, except at 7 dpc, when the pancreas showed the highest viral load in the challenge control (Figure 2). However, in the vaccine/homologous challenge group the mean viral load in the liver was significantly reduced between 3–7 dpc compared to the challenge control, and a similar trend was recorded for spleen, pancreas and bursa of Fabricius, albeit not always statistically significant. At 7 dpc the viral DNA measured in the vaccinated birds was significantly lower ($p \leq 0.045$) in all analyzed organs. No viral DNA was detected at any time point in the organs from negative control birds.

Histopathology

Numerous areas with lymphocytic infiltration, and even necrosis in one case, were observed only in livers from challenge control FAdV-8a. Lymphocytic infiltration was also identified in the pancreas of only one out of five birds from the vaccine/homologous challenge group, in contrast with the challenge control where such lesions were noticed in four out of five birds, associated with necrotic areas in two cases. Necrosis was observed in the bursa of Fabricius of one challenge control bird, together with lymphoid depletion. Aggregation of viral material was observed via IHC in the nuclei of hepatocytes of all the five analyzed birds of the challenge control, whereas none of the tested vaccinated/challenged birds resulted positive. No lesions were recorded in the organs of the negative control. The microscopic lesions evaluated for each group are summarized in Table 3; histopathological changes in challenge control birds are exemplarily shown as Additional file 3.

Antibody development

Fiber-based ELISAs

At the earliest measurement after vaccination (13 dpv), based on five birds from the vaccination-only group, one bird exhibited an OD above the cut-off defined earlier by Feichtner et al. [28] on the homologous ELISA (mean OD from five tested birds: 0.55 ± 0.62). However, at 20 dpv (immediately prior challenge) the mean OD of all vaccinated birds (groups I, II and III) was 1.92 ± 1.17, with the majority of birds (80%) being above the cut-off (Figure 3A). Notably, the only vaccinated bird that died after homologous challenge had no measurable antibodies. As expected, no antibody development was noted prior challenge in adjuvant-only administered birds (mean OD from birds of groups IV and V: 0.05 ± 0.01). Between 20 dpv and subsequent time points, mean Fib-8a ODs of the vaccination-only and vaccine/heterologous challenge group remained relatively constant, while the vaccine/homologous challenge group experienced a further rise throughout the post-challenge period up to OD 3.35 ± 0.02 at 14 dpc. In the FAdV-8a challenge control, a mean OD above cut-off was first noted at 5 dpc, eventually reaching a similar magnitude as the vaccine/homologous challenge birds.

A small subset of Fib-8a vaccinated birds (10%) exhibited cross-reactivity with the Fib-8b reactant, although

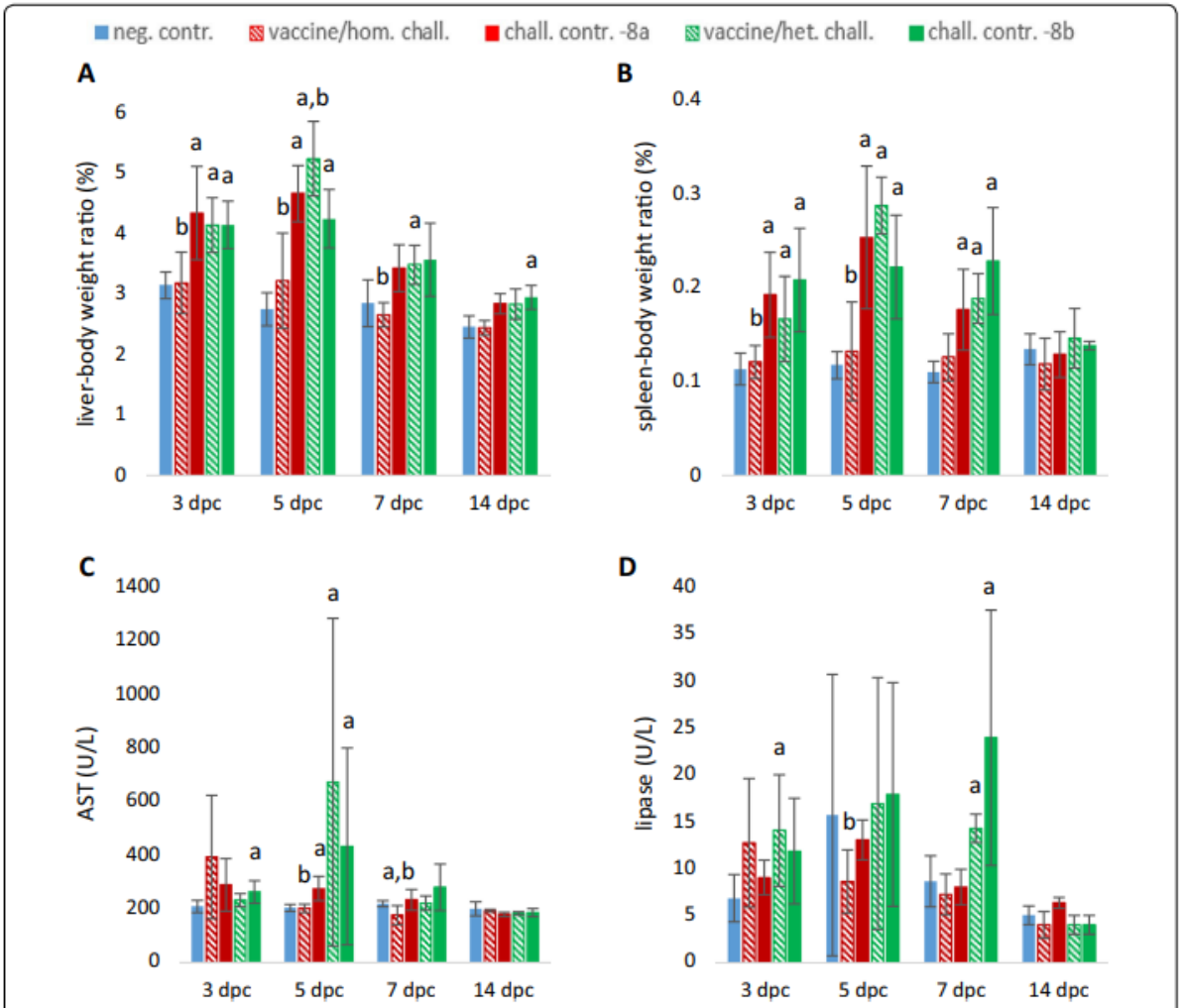

Figure 1 Organs-body weight ratio and plasma analytes. Mean and standard deviation of liver-body weight ratio (**A**), spleen-body weight ratio (**B**), AST in plasma (**C**) and lipase in plasma (**D**) measured for the six experimental groups at each time point. Significant differences against negative control are indicated with letter a, whereas significant differences against the respective challenge control are indicated with letter b.

with generally lower levels than the homologous reaction. Mean Fib-8b ODs of the FAdV-8b challenge control exceeded those of the vaccine/heterologous challenge group at 14 dpc (Figure 3B).

Negative control sera from all time points remained well below the earlier defined cut-offs.

VNT

At 20 dpv, 73.5% of all vaccinated birds exhibited neutralizing antibodies (nAbs) against FAdV-8a/TR59 (mean titer 4.4 $\log_2 \pm 3.1$) (Figure 4). A subset of these birds (38.8%) had nAbs against FAdV-8a/11–16629 (1.7 $\log_2 \pm 2.3$), while only one vaccinated bird exhibited cross-neutralization with the lowest detectable titer level against FAdV-8b/764. In the vaccination-only group, mean nAb titers against TR59 continued to increase up to 7 $\log_2 \pm 1.7$ until the end of the experiment, while mean nAbs against 11–16629 reached a maximum of 4.1 $\log_2 \pm 2.3$ one week earlier and decreased afterwards. Upon challenge, 8a-specific nAbs of the vaccinated/FAdV-8a challenged group continuously increased, whereas titers of the vaccinated/FAdV-8b challenged group peaked at 7 dpc before decreasing, with generally lower levels compared to the homologous challenge. In the FAdV-8a challenge control, 8a-specific neutralizing activity was first recorded

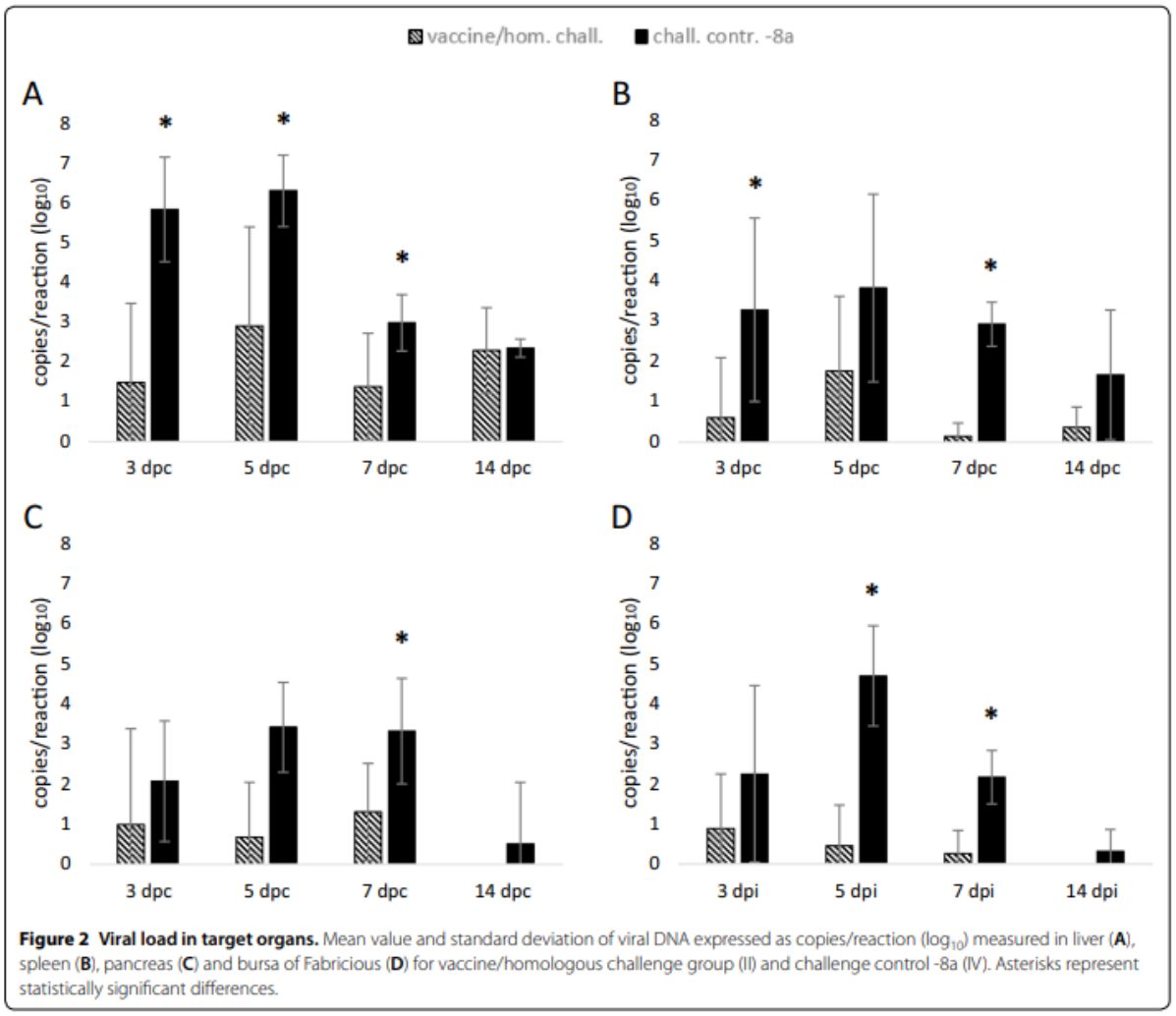

Figure 2 Viral load in target organs. Mean value and standard deviation of viral DNA expressed as copies/reaction (log_{10}) measured in liver (**A**), spleen (**B**), pancreas (**C**) and bursa of Fabricious (**D**) for vaccine/homologous challenge group (II) and challenge control -8a (IV). Asterisks represent statistically significant differences.

Table 3 Summary of histopathological lesions observed in 5 birds/group euthanized between 3 and 5 dpc

	Liver			Pancreas		Bursa of Fabricius	
	Lymphoid infiltration	Necrosis	Virus-positive hepatocytes (IHC)	Lymphoid infiltration	Necrosis	Lymphoid depletion	Necrosis
Vaccin./hom. chall.	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Chall. contr. -8a	5/5	1/5	5/5	4/5	2/5	1/5	1/5
Neg. contr.	0/5	0/5	x[a]	0/5	0/5	0/5	0/5

[a] Not performed.

at 5 dpc and increased continuously until the end of the experiment, reaching values at the far end of the measured range (14 $log_2 \pm 0$ against TR59 and 13 $log_2 \pm 1$ against 11–16629).

Neutralizing activity against FAdV-8b was found in all vaccinated/infected groups independent of type of challenge. However, the vaccinated/FAdV-8a challenged group showed higher final titers than the vaccinated/FAdV-8b challenged birds (7 $log_2 \pm 0$ against 764 and

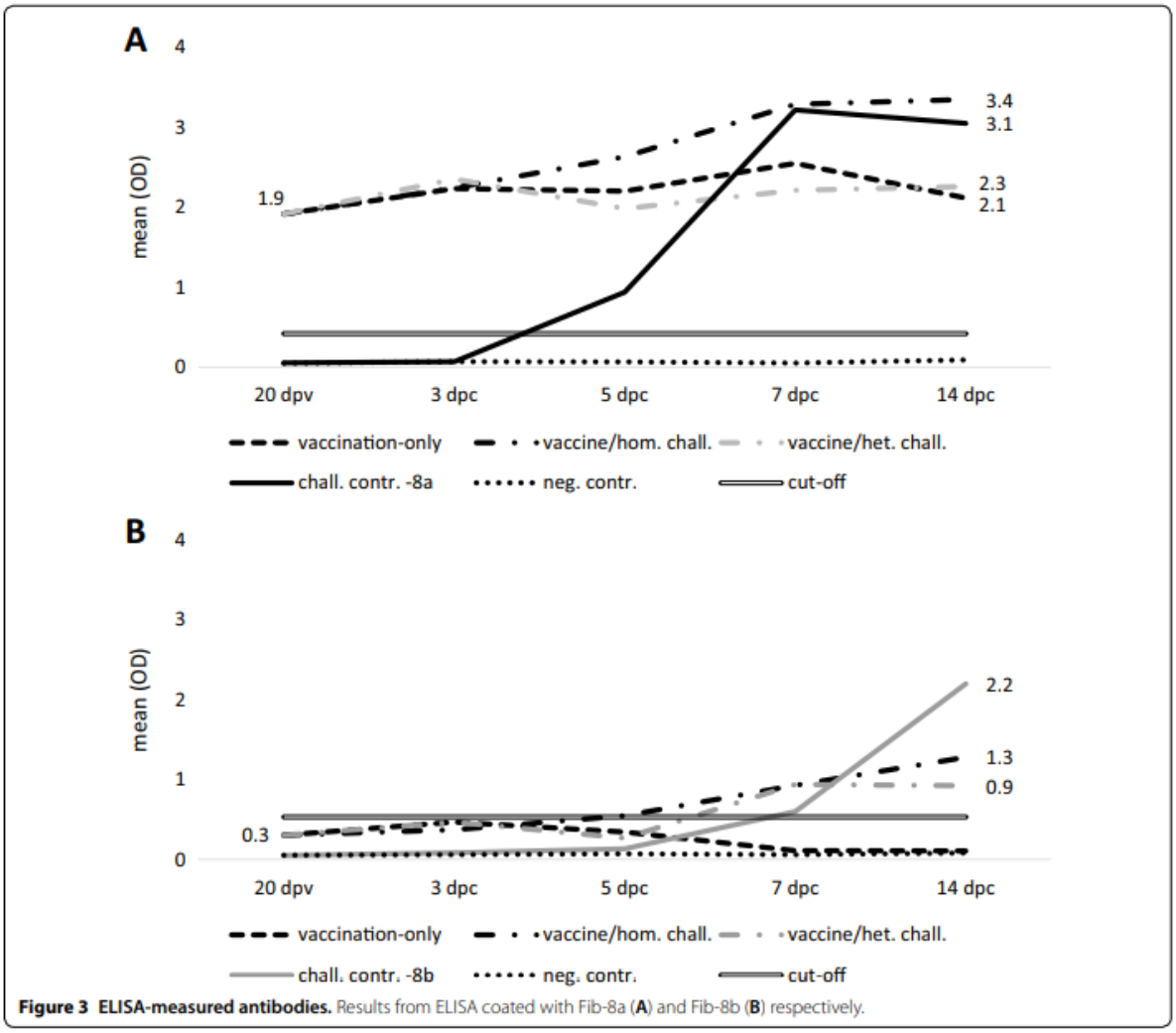

Figure 3 ELISA-measured antibodies. Results from ELISA coated with Fib-8a (**A**) and Fib-8b (**B**) respectively.

7 $\log_2 \pm 1.4$ against 13–18153 vs. 3 $\log_2 \pm 2.6$ and 1.7 $\log_2 \pm 2.9$, respectively). In comparison, neutralizing antibodies were present in only one bird (8 $\log_2$ against 764 and 13–18153) of the FAdV-8b challenge control at 14 dpc.

PBMC flow cytometry

B cells

Means of the B cell population in blood were significantly higher at 20 dpv in all investigated groups (I, II, IV—homologous system) compared to negative control (basal level), with the values of vaccinated birds returning comparable to basal level from the subsequent time point (Figure 5A). After infection, a sharp and statistically significant decline was registered in the challenge control at 3 dpc, followed by a rapid increase, significant at 7 dpc. No significant changes were recorded at 13 dpv and 14 dpc. On an individual level, the highest intra-group deviation occurred at 13 dpv in vaccination-only birds, at 7 dpc for vaccinated/challenged birds, and at 5 dpc for challenge control (Additional file 4).

Monocytes/macrophages

Monocyte/macrophage populations in blood remained comparable between vaccinated groups and negative control throughout the whole experiment (Figure 5B). In contrast, the challenge control showed a decrease in the cell population following infection (from 3 to 7 dpc) and returned to basal level only at 14 dpc. Intra-group

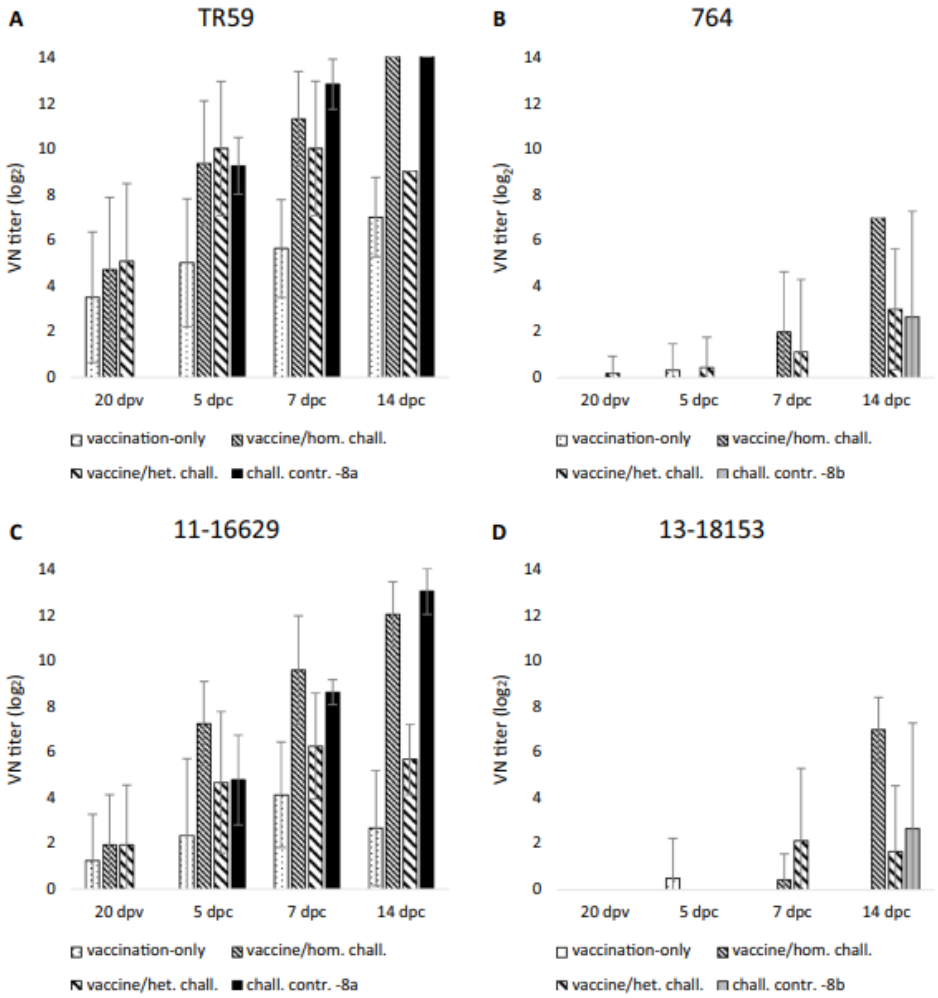

Figure 4 Development of neutralizing antibodies. Mean level and standard deviation of nAbs for each experimental group expressed in VN titers (log_2) tested against different FAdV reference strains [TR59 (**A**), 764 (**B**)] and challenge viruses [11–16629 (**C**), 13–18153 (**D**)].

deviation was not prominent for challenge control birds, whereas in the vaccinated groups individual values showed a greater extent of variation (Additional file 5).

TCRαβ⁺ T cells

TCRαβ$^+$ T cells in PBMC significantly increased in the vaccination-only group at 23 and 25 dpv (corresponding to 3 and 5 dpc) before returning to basal levels (Figure 5C). The population was also consistently increased in the vaccinated/challenged group in the week following challenge. A similar tendency was recorded for challenge control birds, with a significant increase starting immediately before infection and peaking at 5 dpc, surpassing the vaccinated/challenged group, before returning to basal level at 14 dpc. No significant changes were recorded at 13 dpv and 14 dpc. Challenged birds tended to be subjected to higher individual variations (Additional file 6).

TCRγδ⁺ T cells

Blood TCRγδ$^+$ T cells significantly increased in vaccination-only birds at 23 and 25 dpv before returning to basal levels (Figure 5D). Immediately before challenge, the cell population increased in both subsequently infected groups. The vaccinated/challenged group did not show any other significant change, whereas levels of challenge control remained significantly elevated up to 7 dpc, peaking at 5 dpc before returning comparable to the negative control at 14 dpc. No significant changes

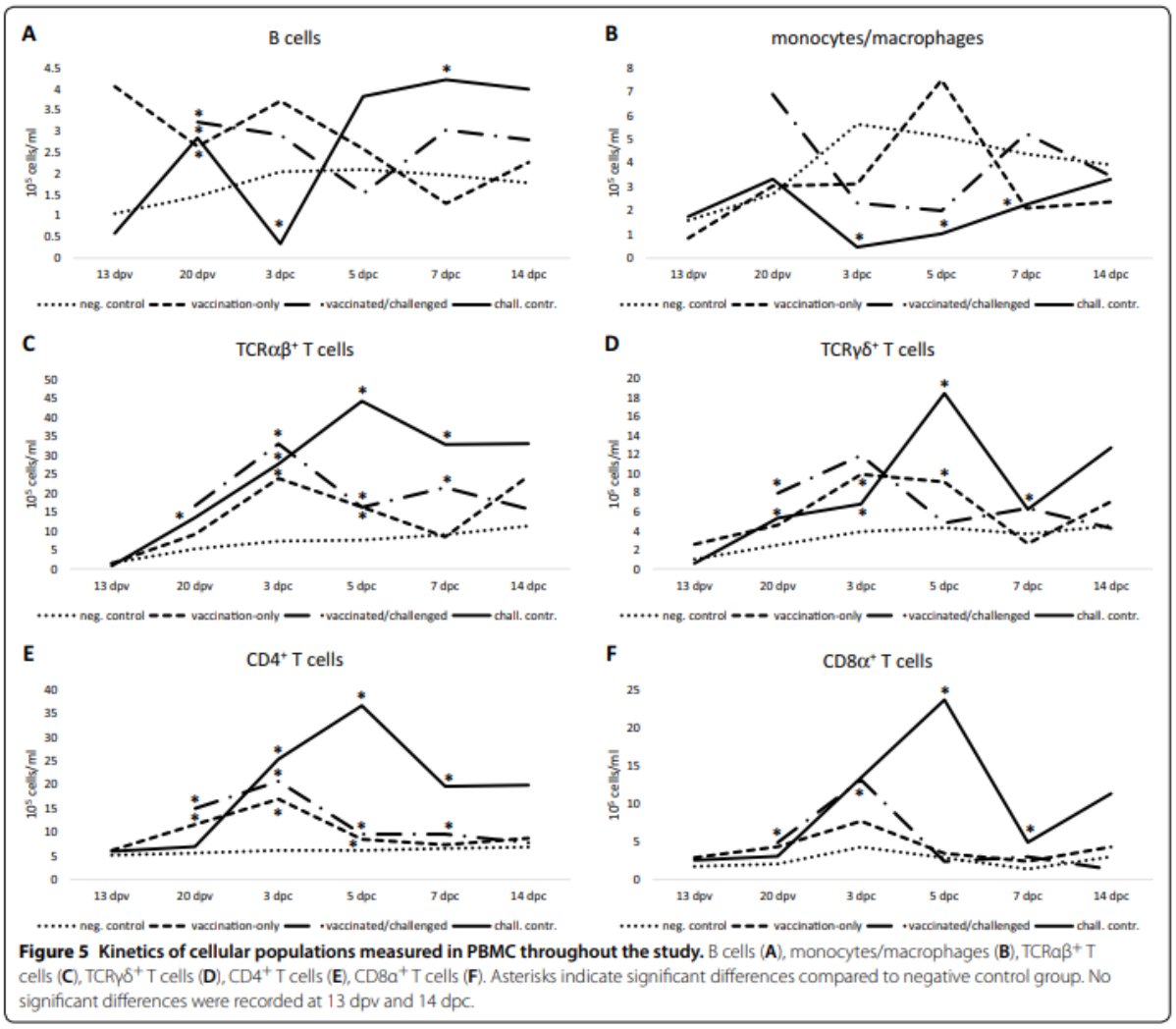

Figure 5 Kinetics of cellular populations measured in PBMC throughout the study. B cells (**A**), monocytes/macrophages (**B**), TCRαβ$^+$ T cells (**C**), TCRγδ$^+$ T cells (**D**), CD4$^+$ T cells (**E**), CD8α$^+$ T cells (**F**). Asterisks indicate significant differences compared to negative control group. No significant differences were recorded at 13 dpv and 14 dpc.

were recorded at 13 dpv and 14 dpc. The highest intra-group variations were recorded at 5 dpc in the challenge control group (Additional file 7).

CD4$^+$ T cells

CD4$^+$ T cells started to be significantly increased in PBMC at 20 dpv in both vaccinated groups before returning to basal levels at 27 dpv (corresponding to 7 dpc) and 14 dpc respectively (Figure 5E). Mean values of CD4$^+$ T cells in challenge control significantly increased from 3 to 7 dpc and returned to basal level at 14 dpc. No significant changes were recorded at 13 dpv and 14 dpc. Intra-group deviation was also more prominent in the challenge control within the week after infection (Additional file 8).

CD8α$^+$ T cells

CD8α$^+$ T cells of vaccination-only and negative control birds remained comparable at each investigated time point (Figure 5F). Birds of the vaccinated/challenged group showed a significant increase in their CD8α$^+$ T cell population at 20 dpv and 3 dpc before returning to basal levels; challenge control birds showed a similar trend, with a sharp significant rise at 5 dpc, whereas values at 7 dpc were lower but still significantly increased compared to the negative control. No significant changes were recorded at 13 dpv and 14 dpc. Once again, challenge control birds tended to be subjected to higher individual variations (Additional file 9).

Discussion

Inclusion body hepatitis (IBH) as a primary disease caused by certain FAdVs has become a growing concern to poultry industry in the past two decades, with an increasing documentation of outbreaks worldwide [3]. At the same time, intensified sequencing efforts, with mounting genomic data for FAdVs, have contributed to a more refined understanding of the diversity of strains involved in IBH. Based on their molecular composition, the spectrum of IBH strains encompasses types -2/-11 (FAdV-D), which constitute a narrow antigenic category due to being closely related [13], and the genetically much more divergent types -8a and -8b (FAdV-E). Given the greater antigenic variability of causative strains, the application of subunit vaccines, which are reasonably efficacious against HHS in experimental settings, should undergo a re-evaluation with regard to IBH. This is particularly relevant in case of the fiber, which was reported as the most type specific of all antigenic domains in FAdVs [13]. Furthermore, recombinant strains with exchanges in antigenic domains mark a possible gap in the established typing practices, which can lead to serious distortions when addressing cross-protection [13]. In light of this recent acknowledgment of recombinant FAdVs, all strains applied in this study were fully characterized with special focus on the relationship between fiber genes of vaccine and challenge strains.

Based on the serotype duality (FAdV-8a/-8b) of IBH, the present study shows that fiber-mediated response efficiently interferes with the homologous serotype infection even if it is a more distantly related strain of the same serotype, as shown by the preserved target organ-body weight ratios and levels of plasma analytes. At the same time, recombinant fiber fails to provide heterotypic coverage. Overall, vaccination could not completely prevent gross lesions in the target organs. Major lesions on the liver of vaccine/homologous challenge birds only appeared from 5 dpc, marking a delay in the onset of gross hepatic damage compared to the respective challenge control group. In contrast, vaccine/heterologous challenge birds presented hepatic lesions at each time point of the first week post infection.

Underlining the importance of antibodies and their type specificity in combating FAdV infection, the observed clinical protection in the homologous system (or, vice versa, its absence in the heterologous system) correlated well with in vitro findings. Although neutralizing titers of up to 10 $\log_2$ were found in vaccinated birds, they generally failed to cross-neutralize FAdV-8b. Despite potent homologous neutralization (up to 14 $\log_2$) from 5 dpc onwards, which possibly led to the significant decrease of viral load in target organs of vaccinated birds infected with homologous challenge, cross-reactivity was not evident before 7–14 dpc, but then also occurred in some sera with intermediate homologous titers. This indicates that immune sera containing only fiber antibody fractions, even if potently neutralizing, remain type-specific and thus less favorable to induce cross-protection, although this should be confirmed for other antigenic settings.

Efficacious fiber-based vaccines have been frequently associated with seroconversion in ELISA, but not necessarily neutralizing activity, as shown for the HHS/FAdV-4 system using fiber-2 as vaccine antigen [7, 25, 33]. Contrarily, induction and vertical transfer of nAbs, conforming with progeny protection, was reported for a prime-boost regimen with FAdV-8b fiber [9], and fiber-based neutralization is achieved independent of a second contact with the antigen, as our results show. However, variations in number and types of FAdV fibers suggest that controversial findings are due to differences in individual fibers´ immune functions, depending on the usage of a system with two types (FAdV-4) or one type of fiber (FAdV-8a/8b).

Data on PBMC cellular immune subpopulations stimulated by FAdV fiber subunits are so far limited to reports on a proliferation in $CD4^+$, besides unchanged $CD8\alpha^+$, T lymphocytes following vaccination with FAdV-4 fiber-2 [25], and an increase in $CD8\alpha^+$ T cells after booster immunization with FAdV-8b fiber [9]. However, these findings only refer to vaccinated but non-infected birds, and do not account for the role of the adjuvant, which can have a self-standing effect on the chicken immune system [34–36]. The importance of the adjuvant is illustrated by the present study, which shows that, except for a pre-challenge increase in $CD4^+$ T cells obviously related to fiber-dependent priming, fiber-vaccinated birds were distinctive from adjuvant-only administered birds only after challenge. In our setting, immune stimulation of the adjuvant was evident by an increase in B cells and $TCR\gamma\delta^+$ T cells compared to the negative control. However, upon challenge, the non-specific nature of this stimulation was exposed, with challenge control birds experiencing an acute and significant drop in B cells and monocytes/macrophages compared to the negative control. This could be explained by the immunosuppressive effect of FAdVs [24, 37–39] and/or recruitment of immune cells to the target organs. The decrease of peripheral B cells was followed by a rise well above the level of the vaccinated/challenged group, in which B cells remained unchanged vs. the vaccination-only group and comparable to the negative control, while maintaining a continued production of antibodies. Final peaks of the antibodies were comparable between protected birds and their challenge control, however, the actual levels are likely masked by reaching saturation of the ELISA. This makes the post-challenge

antibody development not a conclusive marker for protection in the applied setting. However, the steep rise of systemic B cell levels in the challenge control birds, immediately upon recovery within the week following challenge, highlights the importance of humoral effectors for limiting the infection in response to replicating virus.

Similar to B cells, a significant post-challenge drop in blood monocytes/macrophages, lasting up to 7 dpc, was prevented by the vaccine, with both vaccinated groups remaining comparable to the negative control. TCRαβ$^+$ and TCRγδ$^+$ T cells were vigorously stimulated early after viral challenge, and to a lesser extent by the vaccination. This was observed mainly in the vaccination-only group at 23 and 25 dpv (corresponding to 3 and 5 dpc), indicating that certain vaccine-induced effects may have overlapped closely with the time point of challenge. However, in the absence of an adjuvant-only control beyond 20 dpv, it cannot be confirmed that stimulation of TCRαβ$^+$ and TCRγδ$^+$ T cells was exclusively antigen-specific and their role in protection remains more speculative. Similarly, a CD4$^+$ T cell proliferation was detected in response to both challenge and vaccination, with the difference that T helper cells were already distinctive for vaccinated groups at 20 dpv, and could thus serve as an indicator for subsequent protection.

CD8α$^+$ T cell proliferation due to vaccination was noted, but only in one of the groups. In another study this effect was obtained only after booster [9], suggesting the necessity of robust sequential fiber administration for priming of CD8α$^+$ T cells to activate cytotoxic T lymphocytes as a defense mechanism. Non-vaccinated birds relied on a vigorous cytotoxic defense, at least at a systemic level, reflected by the abrupt, steep rise of CD8α$^+$ T cells in the challenge control group, whereas lower levels were reached in vaccinated/challenged birds before returning to basal level.

In conclusion, our data suggest that resistance to infection conferred by fiber critically depends on a humoral response by type-specific virus neutralization, which can also be linked to systemic B cell and CD4$^+$ T cell priming by vaccination. On the individual birds' level, this hypothesis is also supported by the death of a bird that had no vaccine-induced antibodies prior to challenge. The faster recruitment of humoral effectors in vaccinated birds, with more rapid clearance of virus, may contribute to limiting cytotoxic responses and immune-mediated tissue damage during progressive infection. The reliance on humoral immunity with a specific antibody fraction, however, explains why fiber fails to protect across the serotype boundary, imposing certain obstacles to use fiber as vaccine candidate for broad protection against IBH.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-020-00869-8.

Additional file 1. Gating strategy for peripheral blood mononuclear cells in multicolor flow cytometry analysis applying three different panels of antibody combination. The cells were gated according to their light scatter properties. Potential leukocytes were gated with FSC/SSC and afterwards for CD45$^+$ cells (double lined). In the first panel, CD45$^+$ cells were further analyzed for CD45$^+$CD4$^+$CD8α$^-$ T cells (orange gate) and CD45$^+$CD4$^-$CD8α$^+$ T cells (blue gate). In the second panel, B cells and monocytes/macrophages were identified by CD45$^+$Bu1$^+$KuI01$^-$ (purple gate) and CD45$^+$Bu1$^-$KuI01$^+$ phenotype (green gate) respectively. The last panel analyzed CD45$^+$TCRαβ$^+$TCRδγ$^-$ T cells (brown gate) and CD45$^+$TCRαβ$^-$TCRδγ$^+$ T cells (pink gate). The gating strategy is shown as a representative example for isolated PBMCs from a bird at 14 dpc and was performed accordingly for all analyzed samples.

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

Additional file 3. Histopathological lesions in different organs from a challenge control bird infected with FAdV-8a at 5 dpc. Necrosis in liver (A), lymphocytic infiltration and degeneration of glandular acini in pancreas (B), lymphocytic depletion and necrotic area in bursa of Fabricius (C), immunohistochemistry showing aggregation of viral material in the nuclei of hepatocytes (D); bar in lower right corner indicates magnification.

Additional file 4. Individual distribution of B cells in PBMC for each experimental group. Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.

Additional file 5. Individual distribution of monocytes/macrophages in PBMC for each experimental group. Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.

Additional file 6. Individual distribution of TCRαβ$^+$ T cells in PBMC for each experimental group. Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.

Additional file 7. Individual distribution of TCR γδ$^+$ T cells in PBMC for each experimental group. Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.

Additional file 8. Individual distribution of CD4$^+$ T cells in PBMC for each experimental group. Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.

Additional file 9. Individual distribution of CD8α$^+$ T cells in PBMC for each experimental group. Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.

Abbreviations

AGE: adenoviral gizzard erosion; AST: aspartate transaminase; CD: cluster of differentiation; CEL: chicken embryo liver; dpc: days post challenge; dpv: days post vaccination; ELISA: Eenzyme-linked immunosorbent assay; FAdV: Fowl adenovirus; FBS: fetal bovine serum; FCM: flow cytometry; HHS: hepatitis-hydropericardium syndrome; IBH: inclusion body hepatitis; IHC: immunohistochemistry; mAbs: monoclonal antibodies; nAbs: neutralizing antibodies; OD: optical density; PBMCs: peripheral blood mononuclear cells; PBS: phosphate

buffered saline; qPCR: quantitative polymerase chain reaction; SPF: specific pathogen-free; $TCID_{50}$: 50% tissue culture infective dose; TCR: T cell receptor; VNT: virus neutralization test.

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Authors' contributions
MH, AS, CDL, DL and TM conceived and designed the work. CDL and AS performed the animal trial. CDL, AS, SH and TM performed the laboratory analysis. CDL, AS, TM and MH interpreted the data. CDL, AS and TM drafted the manuscript. MH and DL revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate
All the procedures on experimental animals were discussed and approved by the institutional ethics committee and licensed by the Austrian government (animal licenses GZ: 68.205/0100-BrGT/2005 and GZ: 68.205/0035-V/3b/2019).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

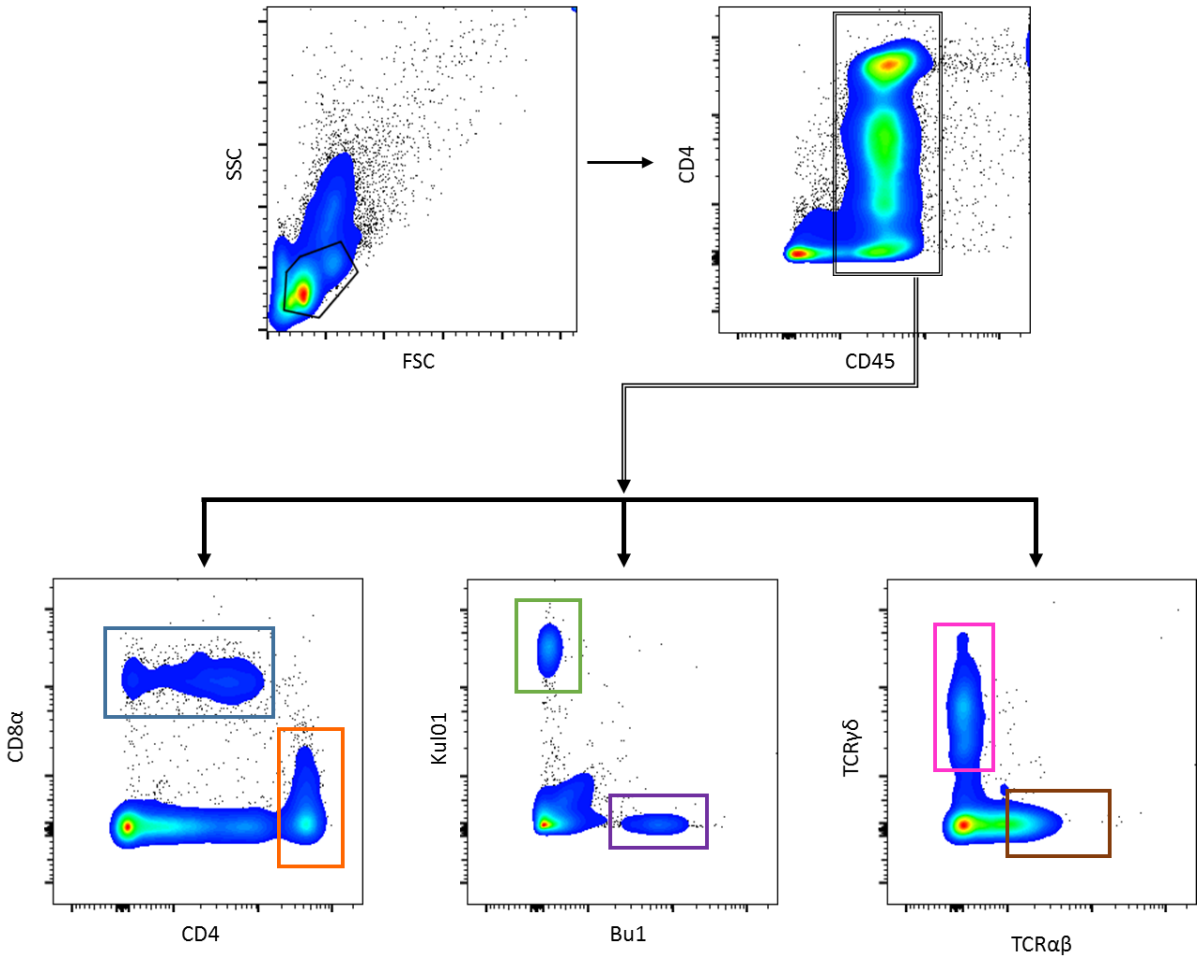
1. Harrach B, Benkö M, Both GW, Brown M, Davison AJ, Echavarría M, Hess M, Jones MS, Kajon A, Lehmkuhl HD, Mautner V, Mittal SK, Wadell G (2012) Family Adenoviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses, 9th edn. Elsevier Academic Press, San Diego
2. Hess M (2000) Detection and differentiation of avian adenoviruses: a review. Avian Pathol 29:195–206
3. Schachner A, Matos M, Grafl B, Hess M (2018) Fowl adenovirus-induced diseases and strategies for their control—a review on the current global situation. Avian Pathol 47:111–126
4. Hess M (2020) Aviadenovirus infections. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL (eds) Diseases of poultry, 14th edn. Wiley-Blackwell, Hoboken
5. Norrby E (1969) The relationship between the soluble antigens and the virion of adenovirus type 3. IV. Immunological complexity of soluble components. Virology 37:565–576
6. Shah MS, Ashraf A, Rahman M, Khan MI, Qureshi JA (2012) A subunit vaccine against hydropericardium syndrome using adenovirus penton capsid protein. Vaccine 30:7153–7156
7. Schachner A, Marek A, Jaskulska B, Bilic I, Hess M (2014) Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis-hydropericardium syndrome (HHS). Vaccine 32:1086–1092
8. Wang X, Tang Q, Qiu L, Yang Z (2019) Penton-dodecahedron of fowl adenovirus serotype 4 as a vaccine candidate for the control of related diseases. Vaccine 37:839–847
9. Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya R, Ojkic D, Tikoo SK, Willson P, Gomis S (2017) Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. Vaccine 35:2716–2722
10. Gomis S, Goodhope R, Ojkic D, Willson P (2006) Inclusion body hepatitis as a primary disease in broilers in Saskatchewan, Canada. Avian Dis 50:550–555
11. Mittal D, Jindal N, Tiwari AK, Khokhar RS (2014) Characterization of fowl adenoviruses associated with hydropericardium syndrome and inclusion body hepatitis in broiler chickens. Virusdisease 25:114–119
12. Schachner A, Marek A, Grafl B, Hess M (2016) Detailed molecular analyses of the hexon loop-1 and fibers of fowl aviadenoviruses reveal new insights into the antigenic relationship and confirm that specific genotypes are involved in field outbreaks of inclusion body hepatitis. Vet Microbiol 186:13–20
13. Schachner A, Gonzalez G, Endler L, Ito K, Hess M (2019) Fowl adenovirus (FAdV) recombination with intertypic crossovers in genomes of FAdV-D and FAdV-E, displaying hybrid serological phenotypes. Viruses 11:1094
14. Steer PA, O'Rourke D, Ghorashi SA, Noormohammadi AH (2011) Application of high-resolution melting curve analysis for typing of fowl adenovirus in field cases of inclusion body hepatitis. Aust Vet J 89:184–192
15. Venne D (2013) Field data of the changing clinical picture over time of inclusion body hepatitis in Canada with an emphasis on diagnosis, prevention and trials on supportive treatments. In: Proceedings of the 24th Annual Australian Poultry Science Symposium, Sydney, New South Wales, Australia, February 2013. The Poultry Research Foundation and the World's Poultry Science Association, pp 222–229
16. Wang J, Wang S, Zou K, Zhang Y, Xu S, Yin Y (2018) Variant serotypes of fowl adenovirus isolated from commercial poultry between 2007 and 2017 in some regions of China. Avian Dis 62:171–176
17. Gupta A, Popowich S, Ojkic D, Kurukulasuriya S, Chow-Lockerbie B, Gunawardana T, Goonewardene K, Karunarathna R, Ayalew LE, Ahmed KA, Tikoo SK, Willson P, Gomis S (2018) Inactivated and live bivalent fowl adenovirus (FAdV8b + FAdV11) breeder vaccines provide broad-spectrum protection in chicks against inclusion body hepatitis (IBH). Vaccine 36:744–750
18. Alvarado IR, Villegas P, El-Attrache J, Jensen E, Rosales G, Perozo F, Purvis LB (2007) Genetic characterization, pathogenicity, and protection studies with an avian adenovirus isolate associated with inclusion body hepatitis. Avian Dis 51:27–32
19. Popowich S, Gupta A, Chow-Lockerbie B, Ayalew L, Ambrose N, Ojkic D, Gunawardana T, Kurukulasuriya S, Willson P, Tikoo SK, Gomis S (2018) Broad spectrum protection of broiler chickens against inclusion body hepatitis by immunizing their broiler breeder parents with a bivalent live fowl adenovirus vaccine. Res Vet Sci 118:262–269
20. Kim M, Lim T, Lee D, Youn H, Yuk S, Kim B, Choi S, Jung C, Han J, Song C (2014) An inactivated oil-emulsion fowl adenovirus serotype 4 vaccine provides broad cross-protection against various serotypes of fowl adenovirus. Vaccine 32:3564–3568
21. Pallister JA, Erny KM, Fahey KJ (1993) Serological relationships within the group E fowl adenoviruses. Intervirology 36:84–90
22. Steer-Cope PA, Sandy JR, O'Rourke D, Scott PC, Browning GF, Noormohammadi AH (2019) Vaccination with FAdV-8a induces protection against inclusion body hepatitis caused by homologous and heterologous strains. Avian Pathol 48:396–405
23. Calnek BW, Shek WR, Menendez NA, Stiube P (1982) Serological cross-reactivity of avian adenovirus serotypes in an enzyme-linked immunosorbent assay. Avian Dis 26:897–906
24. Schonewille E, Singh A, Göbel TW, Gerner W, Saalmüller A, Hess M (2008) Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid organs in specific pathogen-free chickens following experimental infection. Vet Immunol Immunopathol 121:130–139

25. Chen L, Yin L, Zhou Q, Li Q, Luo Y, Xu Z, Zhang Y, Xue C, Cao Y (2018) Immunogenicity and protective efficacy of recombinant fiber-2 protein in protecting SPF chickens against fowl adenovirus 4. Vaccine 36:1203–1208
26. Schat K, Sellers HS (2008) Cell culture methods. In: Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR (eds) A laboratory manual for the isolation and identification of avian pathogens, 5th edn. OmniPress Inc, Madison
27. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Hyg 27:493–497
28. Feichtner F, Schachner A, Berger E, Hess M (2018a) Fiber-based fluorescent microsphere immunoassay (FMIA) as a novel multiplex serodiagnostic tool for simultaneous detection and differentiation of all clinically relevant fowl adenovirus (FAdV) serotypes. J Immunol Methods 458:33–43
29. Matos M, Grafl B, Liebhart D, Schwendenwein I, Hess M (2016) Selected clinical chemistry analytes correlate with the pathogenesis of inclusion body hepatitis experimentally induced by fowl aviadenoviruses. Avian Pathol 45:520–529
30. Günes A, Marek A, Grafl B, Berger E, Hess M (2012) Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). J Virol Methods 183:147–153
31. Feichtner F, Schachner A, Berger E, Hess M (2018b) Development of sensitive indirect enzyme-linked immunosorbent assays for specific detection of antibodies against fowl adenovirus serotypes 1 and 4 in chickens. Avian Pathol 47:73–82
32. Mitra T, Gerner W, Kidane FA, Wernsdorf P, Hess M, Saalmüller A, Liebhart D (2017) Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens. Vaccine 35:4184–4196
33. Ruan S, Zhao J, He Z, Yang H, Zhang G (2018) Analysis of pathogenicity and immune efficacy of fowl adenovirus serotype 4 isolates. Poult Sci 97:2647–2653
34. Fodey TL, Delahaut P, Charlier C, Elliott CT (2008) Comparison of three adjuvants used to produce polyclonal antibodies to veterinary drugs. Vet Immunol Immunopathol 122:25–34
35. Gupta SK, Bajwa P, Deb R, Chellappa MM, Dey S (2014) Flagellin a toll-like receptor 5 agonist as an adjuvant in chicken vaccines. Clin Vaccine Immunol 21:261–270
36. Cui D, Zhang J, Zuo Y, Huo S, Zhang Y, Wang L, Li X, Zhong F (2018) Recombinant chicken interleukin-7 as a potent adjuvant increases the immunogenicity and protection of inactivated infectious bursal disease vaccine. Vet Res 49:10
37. Naeem K, Niazi T, Malik SA, Cheema AH (1995) Immunosuppressive potential and pathogenicity of an avian adenovirus isolate involved in hydropericardium syndrome in broilers. Avian Dis 39:723–728
38. Shivachandra SB, Sah RL, Singh SD, Kataria JM, Manimaran K (2003) Immunosuppression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4 (FAV-4) associated with hydropericardium syndrome. Vet Res Commun 27:39–51
39. Niu YJ, Sun W, Zhang GH, Qu YJ, Wang PF, Sun HL, Xiao YH, Liu SD (2016) Hydropericardium syndrome outbreak caused by fowl adenovirus serotype 4 in China in 2015. J Gen Virol 97:2684–2690

Publisher's Note

Additional file 1. Gating strategy for peripheral blood mononuclear cells in multicolor flow cytometry analysis applying three different panels of antibody combination.

The cells were gated according to their light scatter properties. Potential leukocytes were gated with FSC/SSC and afterwards for $CD45^+$ cells (double lined). In the first panel, $CD45^+$ cells were further analyzed for $CD45^+CD4^+CD8\alpha^-$ T cells (orange gate) and $CD45^+CD4^-CD8\alpha^+$ T cells (blue gate). In the second panel, B cells and monocytes/macrophages were identified by $CD45^+Bu1^+Kul01^-$ (purple gate) and $CD45^+Bu1^-Kul01^+$ phenotype (green gate) respectively. The last panel analyzed $CD45^+TCR\alpha\beta^+TCR\delta\gamma^-$ T cells (brown gate) and $CD45^+TCR\alpha\beta^-TCR\delta\gamma^+$ T cells (pink gate). The gating strategy is shown as a representative example for isolated PBMCs from a bird at 14 dpc and was performed accordingly for all analyzed samples.



Additional file 2. Antibody panels.

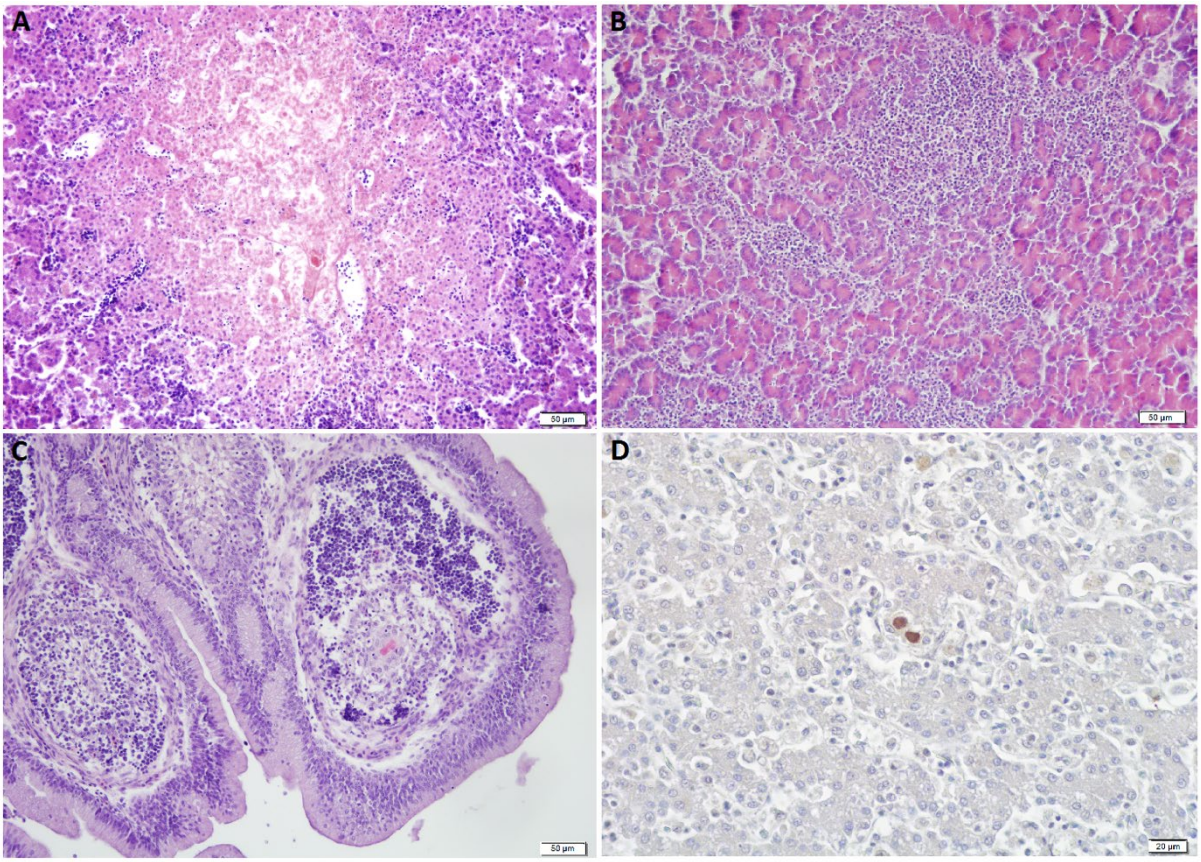
List of antibodies and antibody combinations used in this study.

species specificity	antigen	clone	isotype	fluorochrome	labeling strategy	source of primary mAb
panel 1						
chicken	CD45	LT40	mouse IgM	APC	directly conjugated	Southern-Biotech
chicken	CD4	CT4	mouse IgG1	BV421	biotin-streptavidin[a]	Southern-Biotech
chicken	CD8α	3-298	mouse IgG2b	R-PE	directly conjugated	Southern-Biotech
panel 2						
chicken	CD45	LT40	mouse IgM	APC	directly conjugated	Southern-Biotech
chicken	Bu-1	AV20	mouse IgG1	BV421	biotin-streptavidin[a]	Southern-Biotech
chicken	monocytes/ macrophags	Kul-01	mouse IgG1	R-PE	directly conjugated	Southern-Biotech
panel 3						
chicken	CD45	LT40	mouse IgM	APC	directly conjugated	Southern-Biotech
chicken	TCR-γδ	TCR1	mouse IgG1	BV421	biotin-streptavidin[a]	Southern-Biotech
chicken	TCR-αβ/ VB1	TCR2	mouse IgG1	FITC	directly conjugated	Southern-Biotech

[a]Brilliant Violet 421™ Streptavidin, BioLegend

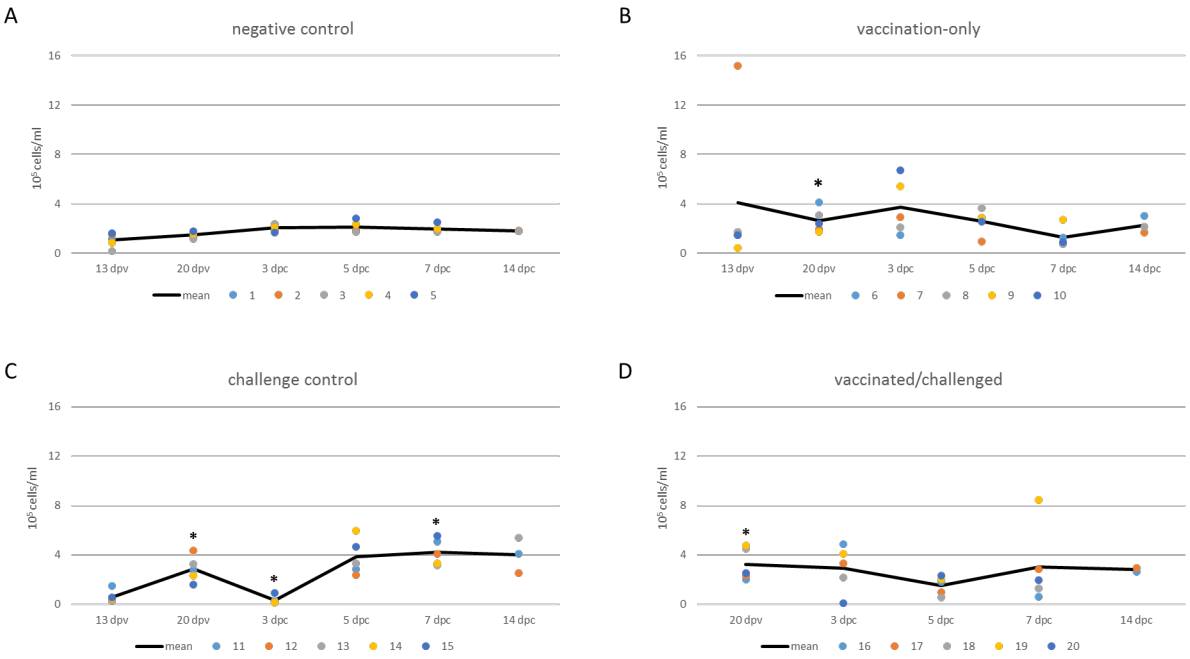
Additional file 3. Histopathological lesions in different organs from a challenge control bird infected with FAdV-8a at 5 dpc.

Necrosis in liver (A), lymphocytic infiltration and degeneration of glandular acini in pancreas (B), lymphocytic depletion and necrotic area in bursa of Fabricius (C), immunohistochemistry showing aggregation of viral material in the nuclei of hepatocytes (D); bar in lower right corner indicates magnification.



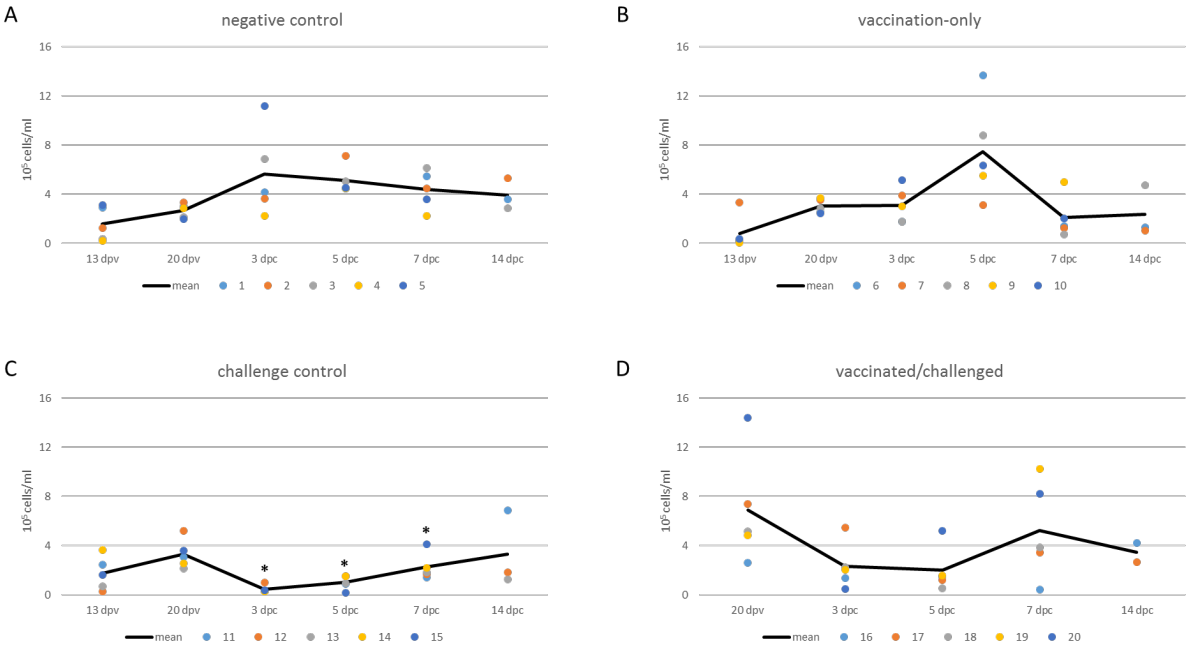
Additional file 4. Individual distribution of B cells in PBMC for each experimental group.

Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.



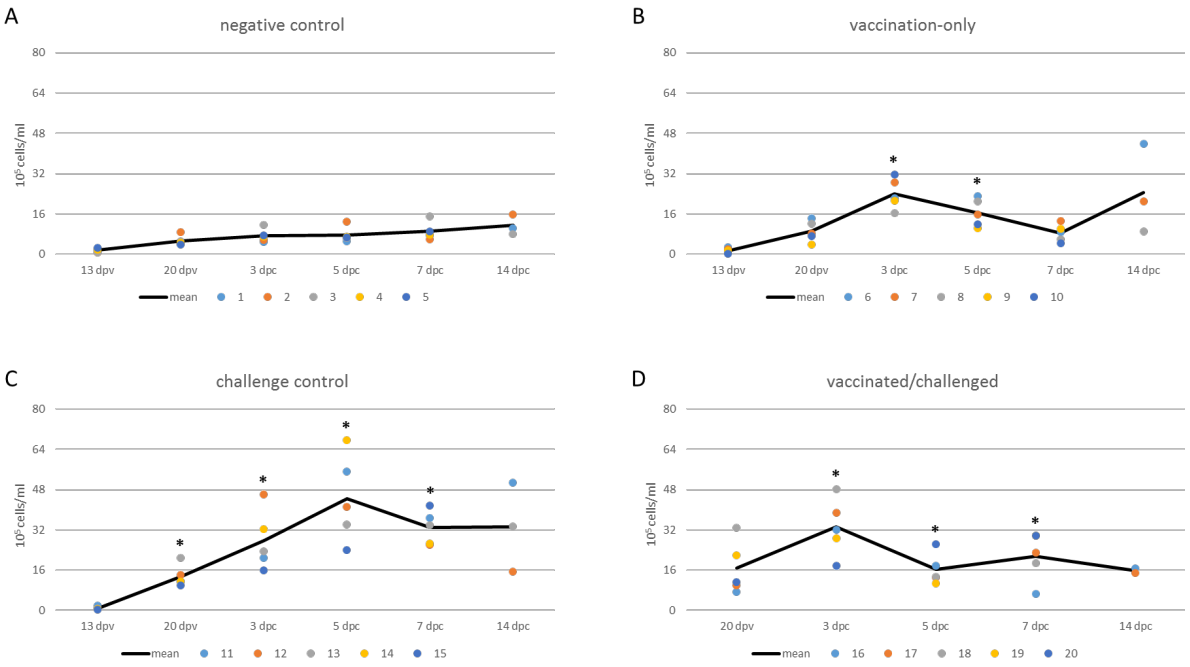
Additional file 5. Individual distribution of monocytes/macrophages in PBMC for each experimental group.

Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.



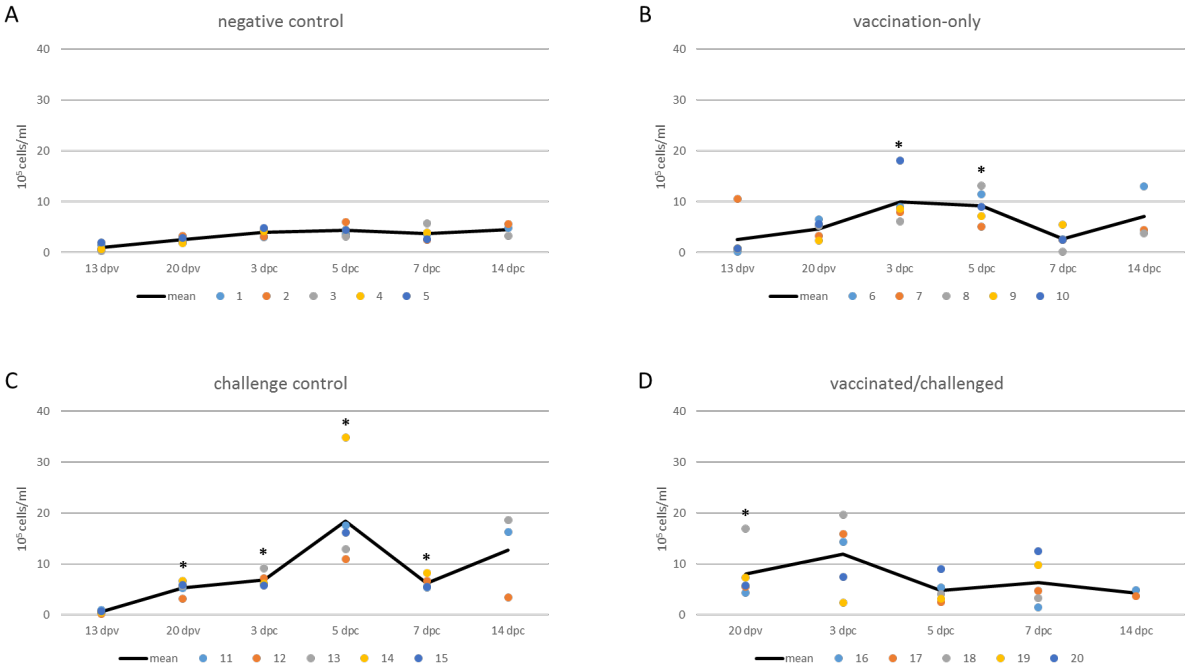
Additional file 6. Individual distribution of TCRαβ⁺ T cells in PBMC for each experimental group.

Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.



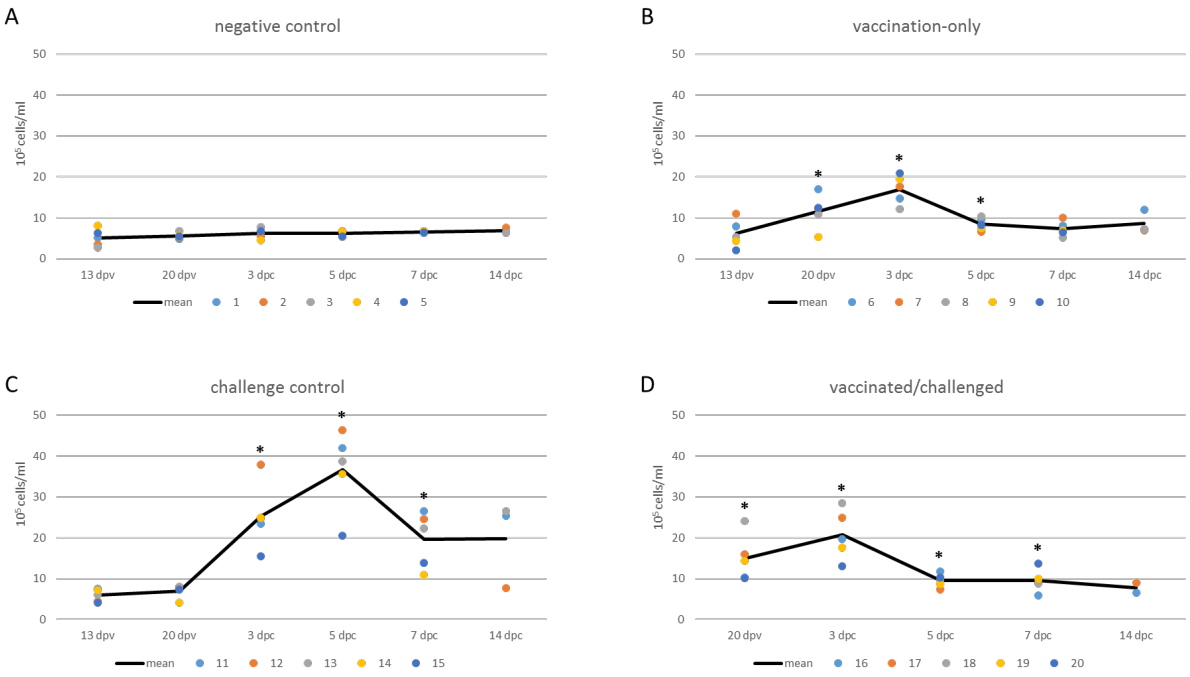
Additional file 7. Individual distribution of TCR γδ⁺ T cells in PBMC for each experimental group.

Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.



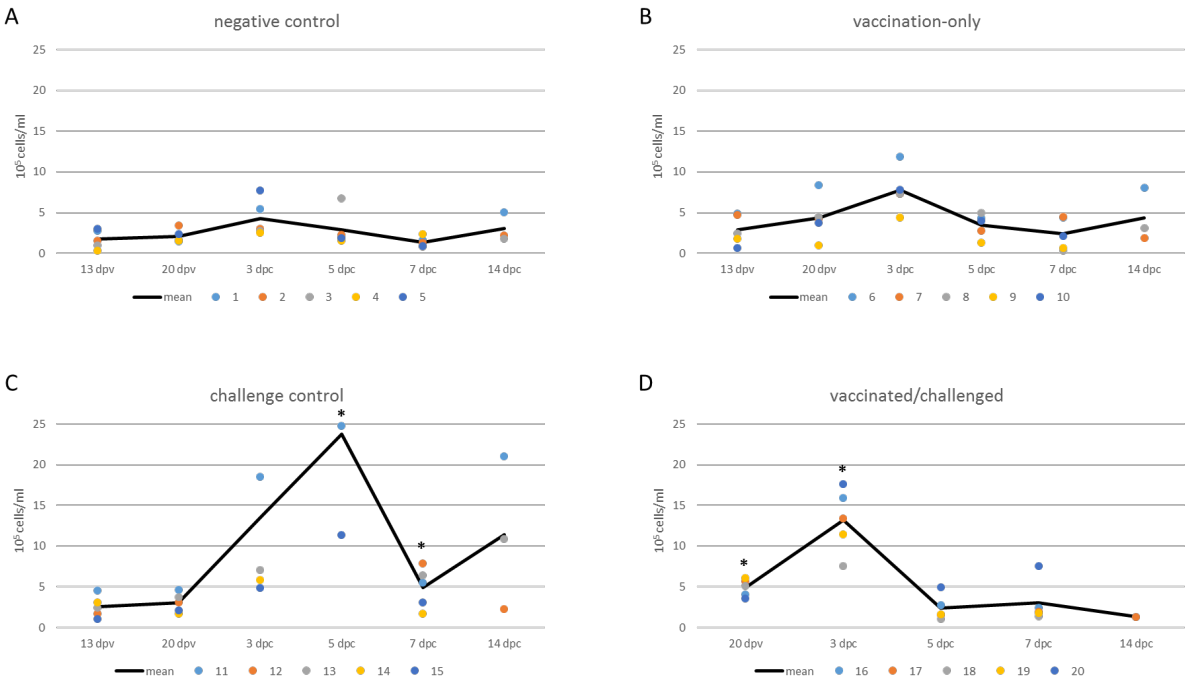
Additional file 8. Individual distribution of CD4⁺ T cells in PBMC for each experimental group.

Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.



Additional file 9. Individual distribution of CD8α⁺ T cells in PBMC for each experimental group.

Negative control (A), vaccination-only (B), challenge control (C) and vaccinated/challenged group (D). The asterisk indicates statistical significance ($p \leq 0.05$) compared to the negative control.



8.2. Recombinantly expressed chimeric fibers demonstrate discrete type-specific neutralizing epitopes in the *Fowl aviadenovirus E* (FAdV-E) fiber, promoting the optimization of FAdV fiber subunit vaccines towards cross-protection *in vivo*

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RESEARCH ARTICLE

Recombinantly Expressed Chimeric Fibers Demonstrate Discrete Type-Specific Neutralizing Epitopes in the *Fowl Aviadenovirus E* (FAdV-E) Fiber, Promoting the Optimization of FAdV Fiber Subunit Vaccines towards Cross-Protection *in vivo*

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ABSTRACT Vaccines against inclusion body hepatitis in chickens are complicated by the involvement of antigenically diverse fowl adenovirus types. Though immunization with fiber protein confers robust protection, type specificity of fiber antibodies is an obstacle for the desired broad coverage. In this study, we utilized information on multiple linear epitopes predicted in the *Fowl Aviadenovirus E* (FAdV-E) fiber head (knob) to develop chimeric fibers with an exchange between two serotypes' sequences, each containing proposed epitopes. Two consecutive segments pertaining to amino acid positions 1 to 441 and 442 to 525/523 in the fibers of FAdV-8a and -8b, types of *Fowl Aviadenovirus E* that cause inclusion body hepatitis, were swapped reciprocally to result in novel chimeras, crecFib-8a/8b and crecFib-8b/8a. crecFib was indistinguishable from monospecific recombinant fibers in its eactivity with different FAdV antisera in Western blotting. However, contrary to the results for monospecific fibers, crecFib induced cross-neutralizing antibodies against both serotypes in chickens. This demonstrates three nonidentical epitopes in the FAdV-E fiber, the conserved epitope detected in Western blotting and at least two epitopes participating in neutralization, being type specific and located opposite residue position 441-442. Furthermore, we supply conformational evidence for a site in the fiber knob with accessibility critical for neutralization. With such an extended neutralization spectrum compared to those of individual fibers, crecFib was anticipated to fulfill and even extend the mechanistic basis of fiber-mediated protection toward bivalent coverage. Accordingly, crecFib, administered as a single-antigen component, protected chickens simultaneously against challenge with FAdV-8a or -8b, demonstrated by up-to-complete resistance to clinical disease, prevention of target organ-related changes, and significant reduction of viral load.

IMPORTANCE The control of inclusion body hepatitis, a disease of economic importance for chicken production worldwide, is complicated by an etiology involving multiple divergent fowl adenovirus types. The fiber protein is principally efficacious in inducing neutralizing and protective antibodies in vaccinated chickens; however, it faces limitations due to its intrinsic type specificity for neutralization. In this study, based on an *in silico*-guided prediction of multiple epitopes in the fowl adenovirus fiber head's loops, we designed chimeric proteins, swapping N- and C-distal fiber portions, each containing putative epitopes, between divergent types FAdV-8a and -8b. In *in vitro* and *in vivo* studies, the chimeric fiber displayed extended properties compared to those of individual monotype-specific fibers, allowing the number, distribution, functionality, and conformational bearings of epitopes of the fowl adenovirus fiber to be characterized in more detail. Importantly, the chimeric fiber induced cross-neutralizing antibodies and protective responses in chickens against infections by both serotypes, promoting the advancement of broadly protective subunit vaccination strategies against FAdV.

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Knowledge about adenoviral antigenicity is predominantly derived from human adenoviruses, from the pioneer work on the major determinants by Norrby (1) to a range of studies on epitope identification. More recently, such studies have also advanced beyond the immunodominant antigen, hexon, and started to involve novel *in silico* approaches (2–4). Such information is widely unavailable for adenoviruses outside the genus *Mastadenovirus*, despite holding clinical applications, not only for certain veterinary areas but also for potential development of new vectorization platforms. In the field of avian adenoviruses, several early works on typing by investigation of antigenic relationships contributed to a principal understanding of the relevant domains (5). However, studies to identify and characterize individual epitopes of bird adenoviruses are still exceptionally rare.

The group-reactive antigen (designated α, residing in the hexon), noted shortly after its discovery as principally discriminatory between mammalian and avian adenoviruses, is reflected in the historical taxonomic division of avian adenoviruses into groups I, II, and III, subsequently recognized as members of different genera (6, 7). Especially because of the diversity of *Fowl Aviadenovirus* (FAdVs), the classical adenoviruses of chickens belonging to the genus *Aviadenovirus*, a need for further differentiation shifted focus to the main type-specific (ε) determinant harbored by the hexon. This antigen with its neutralizing properties serves for serological distinction of 12 types (FAdV-1 to -8a and -8b to -11) within the recognized species *Fowl Aviadenovirus A* to *Fowl Aviadenovirus E* (FAdV-A to FAdV-E).

However, with the fiber as an additional carrier of different reactivities (subgroup and type specific, referred to as δ and λ determinants), this has recently exposed a possible pitfall of stand-alone hexon typing. Based on the discovery of natural FAdV recombinants with exchanges between hexon and fiber of different types, fiber was shown as probably the second-most-potent contributor to type-specific neutralization besides hexon (8).

The type-specific component of fiber has already proven amenable to use in refining serological detection of FAdVs, advancing from whole-virus-based enzyme-linked immunosorbent assays (ELISAs) with broad-spectrum detection to type differentiation with isolated fiber proteins as coating antigens (9–11). Furthermore, fiber has been repeatedly confirmed as a subunit vaccination antigen with high protective efficacy against different FAdV-induced pathologies in chickens. However, a dilemma arises for certain fiber subunit vaccines, due to the fact that neutralization is the mechanistic basis of fiber-induced protection, while fiber antibodies are intrinsically type specific (12). This severely limits fiber as an antigen for tackling multitype disease complexes, importantly in the case of inclusion body hepatitis (IBH), caused by three major antigenically discriminate clusters (FAdV-2/11 of species FAdV-D and FAdV-8a and FAdV-8b of species FAdV-E) (13). Moreover, neutralizing antibodies (NAbs) to fiber were absent in some studies and in others supported as a correlate of protection (12, 14–16), but there are indications for a more important role in FAdV species with a singular fiber (rather than those with dual fibers), underlining the relevance of neutralization for IBH. And yet, the only *in vitro* functional data on fiber epitopes are based on an exceptional model with two fiber genes, FAdV-4 (of species FAdV-C) (17), and contrary to findings *in vivo*, those data support that all serotypes' fibers possess a neutralizing epitope.

In this study, we used *in silico*-generated information on multiple epitopic sites in the fiber head domain for engineering a sequence exchange between the two discrete serotypes FAdV-8a and -8b, anticipated to result in new full-length fiber proteins with mixed-epitope compositions. The antibody subsets induced by such chimeric fibers *in vivo* resolved the coexistence of multiple epitopes, along with their discriminate specificities and functionality. In accordance with this, a challenge experiment proved the newly developed chimeric fiber efficacious for simultaneous control of the major serotypes causing IBH, which was previously unattainable with native fibers.

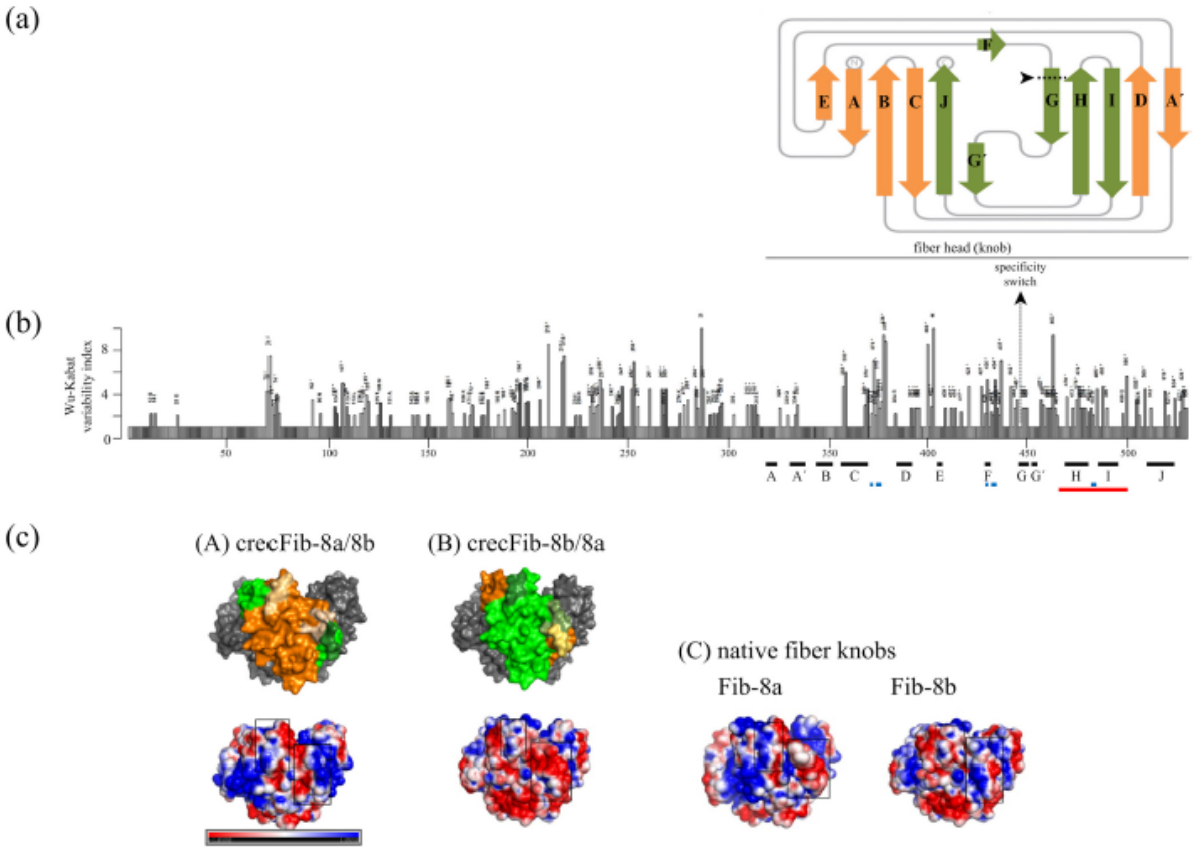

FIG 1 (a) The crecFib knob domain, represented schematically as a 2-dimensional ribbon model (adapted from reference 32). Arrows represent β-strands (designated A to J, in linear order from the N to the C terminus of the knob sequence) connected by lines representing the intervening loops. Structures encoded by sequences of discriminate serotypes are shown in different colors opposite the chimeric exchange site (the "specificity switch," indicated by the dashed line at the left end of the G strand). (b) Coordinates of β-strands of the fiber knob (black bars), candidate epitopes (blue bars, epitopes predicted by DiscoTope 2.0; red bar, a previously reported epitope [17]), and the specificity switch, annotated in a Wu-Kabat plot of the FAdV-E fiber sequence. The degree of variability, indicated by the bar size for each residue position, has been calculated as the Wu-Kabat index from available fibers representing all types of the species (n = 29). Amino acid positions of the multiple sequence alignment are annotated along the horizontal axis. Candidate epitopes conform well with less tightly conserved residues, while the specificity switch is located at an intraspecies consensus site. (c) Comparisons of the two crecFib knobs (8a/8b and 8b/8a) by similarity modeling of the amino acid sequences (A and B) with their predicted surface electrostatics in the panels underneath, with the molecule shown in side view. In the top panels, only one chain of the fiber trimer is colored, with orange tones for the sequence portion derived from FAdV-8a and green tones for the sequence portion from FAdV-8b; muted tones indicate the three epitopes predicted in this study. The same epitopes are highlighted with black squares in the electrostatic surface charge images with color transitions from red (negative charge) to blue (positive charge) and are compared to the corresponding sites in the cognate native knobs of FAdV-8a and -8b (C).

RESULTS

***In silico* design and recombinant expression of crecFib constructs.** *In silico* epitope analysis of the strain TR59 and strain 746 fiber knobs suggested sites that, based on homology modeling, were assigned to the CD loop (amino acids G.SSD and N.PTG), the β-strand F/FG loop (amino acids V.DANP and I.DASS), and the HI loop of the knob (amino acids QSQ and RSQ) (Fig. 1a). According to 3-dimensional models created on the basis of the chimeric knobs' sequences, all predicted epitopes were localized externally, fulfilling the criterion of surface accessibility, as well as apically on the molecule (Fig. 1c), corresponding to the side in HAdVs that participates in virus-host interaction (18). Furthermore, the two sites in the β-strand F/FG loop and HI loop, though noncontiguous in the primary sequence, were conformationally in contact, revealing a coherent surface patch formed by residues encoded N and C distally from the chimeric junction.

Both chimeric proteins, designated crecFib-8a/8b and crecFib-8b/8a, were successfully recovered from the soluble fraction of infected Sf9 cells, as confirmed by bands with the

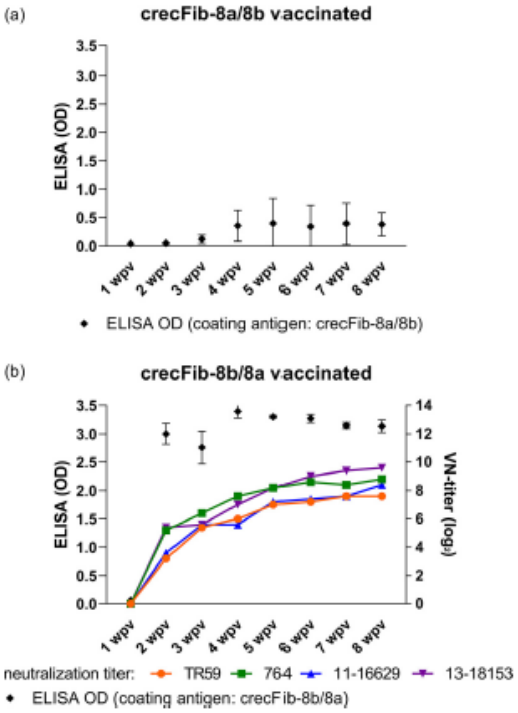

FIG 2 Antibody development following immunization with crecFib constructs (n = 5 birds for each construct). The header of each panel indicates the construct used for immunization, which also corresponds to the coating antigen of the ELISA for determination of weekly ODs from 1 to 8 weeks post vaccination (wpv). Mean neutralization titers, if detectable, are plotted with the respective colors for the viruses against which the sera were tested (including the chimeric template and the challenge strains of this study from FAdV-8a and -8b). Error bars show standard deviations.

appropriate monomer size in Western blots (identical to the control proteins used for the Western blots shown in Fig. 3). The yields of purified chimeric fibers were approximately 13.4 mg (crecFib-8a/8b) and 14.8 mg (crecFib-8b/8a) per liter of Sf9 cell culture.

Immunogenicity and *in vitro* reactivity spectrum of crecFib. (i) crecFib antibody induction detected by ELISA. Based on the homologous-antigen ELISA, sera from birds immunized with crecFib-8a/8b showed a flat increase in optical density (OD) magnitude, with only sporadic occurrence of ODs of >1 in a single individual at three successive time points from 5 to 7 weeks post vaccination (wpv). Sera from all other birds did not exceed an OD of 0.5 at any time point during an 8-week monitoring period (Fig. 2a). In contrast, sera from birds immunized with the reverse-order crecFib-8b/8a developed sharp rises in ODs in the homologous-antigen ELISA as early as 2 wpv, when sera from 5/5 birds already presented with ODs close to 3 (mean OD ± standard deviation, 3.00 ± 0.18), independent of the antigen dose (50 versus 100 μg). The crecFib-8b/8a ODs remained at a constant level in all 5 individuals until the end of the monitoring period at 8 wpv, with mean weekly ODs in the range of 2.76 ± 0.29 to 3.39 ± 0.12 (Fig. 2b).

(ii) Neutralizing activity of crecFib antisera. No neutralizing activity was detected in any of the birds immunized with crecFib-8a/8b, including the individual with an indicative ELISA OD (Fig. 2a).

Among crecFib-8b/8a antisera, neutralization was first present at 2 wpv, with all birds showing low to moderate titers (4 to 7 $\log_2$) against at least one of the constitutive types (Fig. 2b). In fact, 4/5 individuals had already developed bilateral NAbs against both types at this time point. The neutralizing titers of the crecFib-8b/8a antisera continuously increased during the monitoring period; from 5 wpv onwards, all birds had titers of ≥6 $\log_2$ against the complete set of tested strains, with one bird reaching the maximum of the

measured range (14 $\log_2$) against FAdV-8b. On an individual-bird level, the neutralizing responses showed an overall balanced distribution, with similar titers against both types, regardless of whether the corresponding virus was a template (reference) or field strain.

Immunofluorescent staining of a virus neutralization (VN) setting, which directly compared crecFib-8b/8a antiserum side by side with antifiber antisera against Fib-8a or Fib-8b on the same microtiter plate, demonstrated that only the chimeric serum could efficiently inhibit the infection of both FAdV-8a (strain TR59) and FAdV-8b (strain 764) *in vitro* (Fig. S1 in the supplemental material). While the monospecific Fib-8a and Fib-8b antisera exerted neutralizing activity against their cognate serotypes at titers similar to those of the crecFib antiserum, neither of them could inhibit the opposite serotype.

(iii) Recognition of fibers in Western blotting. All recombinant fibers investigated, independent of their genetic background and their monospecific or chimeric composition, were detected by immune sera representing the complete spectrum of FAdV types, defined by fiber specificity (Fig. 3).

Mutual recognition was even possible between reaction partners with differential fiber expression (fiber-1 and fiber-2 of FAdV-A and FAdV-C versus the singular fiber of the remaining FAdV species) and between chimeric counterparts with the reverse template order (e.g., crecFib-8b/8a antiserum was able to recognize crecFib-8a/8b) (Fig. 3b to d).

Of note, no differences were noted between antisera raised against live (native) FAdV and inactivated, adjuvant-formulated virus preparations, as well as subunit-directed antisera, in their ability to recognize the fiber monomer in immunoblots (data not shown as identical signals were obtained for the same protein when incubated with any type of antiserum).

Protection study 1: protective efficacy of crecFib-8a/8b and crecFib-8b/8a. An overview of the *in vivo* experimental designs is provided in Fig. 4. A group designated $VV^{8a/8b}C^{8b}$, according to the treatments carried out, was vaccinated twice with crecFib-8a/8b at 3 and 21 days of life, followed by FAdV-8b challenge at 36 days of life (15 days post booster [dpb]). A second group, designated $VV^{8b/8a}C^{8b}$, was vaccinated at the same time points with the reverse-order crecFib-8b/8a and then also challenged with FAdV-8b. The challenge control, mock vaccinated with a phosphate-buffered saline (PBS)/adjuvant mixture prior to FAdV-8b challenge, was designated C^{8b}, and the negative control, administered only sterile PBS at each occasion, was designated N.

The onset of vaccine-induced antibody development was not detected by ELISA until after the booster (7 dpb, 28 days of life), and only at a low level, indicating the beginning of a rise, in birds of the $VV^{8a/8b}$ group (mean OD, 0.51 $\pm$ 0.65), while peak detectable levels were already reached in the $VV^{8b/8a}$ group (3.14 $\pm$ 0.73) (Fig. 5a). In comparison, sera from birds of the N group and the C^{8b} group (prior to challenge) never exceeded an OD of 0.07 $\pm$ 0.01. Immediately before challenge (14 dpb), sera from birds of the $VV^{8a/8b}$ group reached moderate ODs (1.45 $\pm$ 0.95), but none of the sera exhibited neutralizing activity (VN titers of $\leq$8 $\log_2$). In sera from the $VV^{8b/8a}$ group, the OD levels remained rather constant (2.60 $\pm$ 0.85), and with the exception of a single bird, all sera exhibited neutralizing activity, ranging from low titers against at least one of the tested serotypes (in 3 birds) to titers at the highest end of the measured range (14 $\log_2$) against both serotypes (FAdV-8a and -8b reference and challenge viruses) in the remaining birds. As a general trend, higher titers (and earlier onset, based on those sera with only unilateral neutralization) were noted against FAdV-8a; in addition, NAbs against FAdV-8b never diverged by more than 3 titer levels between reference and challenge strains, while NAbs against FAdV-8a diverged by up to 5 titer levels, with more pronounced reactivity against the challenge than the reference strain (Fig. 5b).

Following challenge, mild depression (inappetence, huddling, and ruffled plumage) was recorded in two birds of the C^{8b} group at 4 to 5 dpc. During necropsy at this time point, the same individuals had significantly increased liver/body weight (BW) ratios. An increase of liver/BW ratios was also recorded in birds of the the $VV^{8a/8b}C^{8b}$ and $VV^{8b/8a}C^{8b}$ groups, but the occurrence was delayed, at 7 dpc (Fig. 6i). The C^{8b} group additionally experienced a significant increase of the spleen/BW ratio (7 dpc), which was absent in the $VV^{8b/8a}C^{8b}$ group, whereas it occurred at a moderate but not significant level in the $VV^{8a/8b}C^{8b}$ group.

The highest plasma aspartate transaminase (AST) values were recorded in birds in the C^{8b} group at 3 and 5 dpc, being significant at 5 dpc, whereas the plasma AST values in

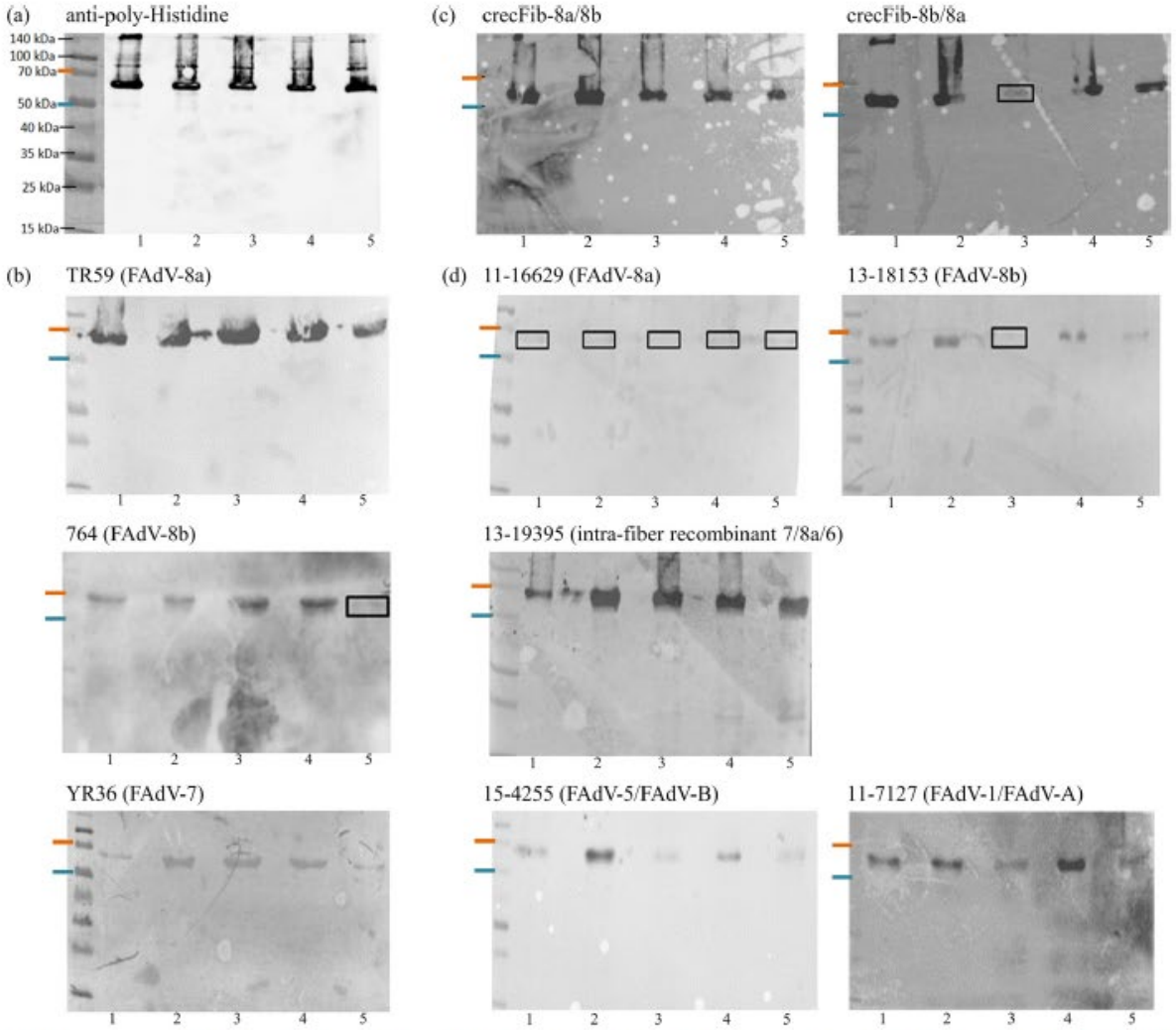

FIG 3 Western blot analysis to assess the reactivities of crecFib constructs side by side with monospecific control fibers from types FAdV-8a, FAdV-8b, and FAdV-7 (all from species FAdV-E). The order of antigens is the same on all membranes as labeled for the first membrane: 1, Fib-8a; 2, Fib-8b; 3, crecFib-8a/8b; 4, crecFib-8b/8a; 5, Fib-7. However, each membrane was incubated with a different antiserum, specified in the header of each membrane, with the corresponding serotype in parentheses. Molecular weight sizes are indicated by the standard in the first lane in panel a; in all subsequent blots, only the relevant 50 kDa-to-70 kDa range is marked. Signals that are present but difficult to distinguish from the background are boxed. (a) Confirmation of monomeric fibers via the polyhistidine tag. (b) Detection of crecFib by antisera raised against the constitutive strains from FAdV-8a and -8b and FAdV-7 as the outlier type. (c) Detection of crecFib by antisera from birds vaccinated with the same or the reverse-order construct. CrecFib antisera also recognized all monospecific control fibers. (d) Detection of crecFib and control fibers by antisera against field isolates, including the two challenge strains of this study, a naturally recombinant strain, and exemplary strains from species other than FAdV-E with either one (FAdV-B) or two (FAdV-A) fiber genes.

birds in the $W^{8a/8b}C^{8b}$ and $W^{8b/8a}C^{8b}$ groups remained comparable to the values in birds in the N group (Fig. 6ii). At 7 dpc, plasma AST values were not distinguishable between the groups anymore.

A reduction of hepatic viral loads versus the loads in birds in the C^{8b} group was noted in birds in both the $W^{8a/8b}C^{8b}$ and $W^{8b/8a}C^{8b}$ groups at all time points investigated, with the lowest values throughout and significance at 5 dpc in the $W^{8b/8a}C^{8b}$ group (Fig. 6iii). In the pancreas, viral DNA was completely absent in birds in the $W^{8b/8a}C^{8b}$ group at 3 dpc and significantly reduced at 5 and 7 dpc. In birds in the $W^{8a/8b}C^{8b}$ group, the pancreatic viral load was significantly reduced only at 5 dpc.

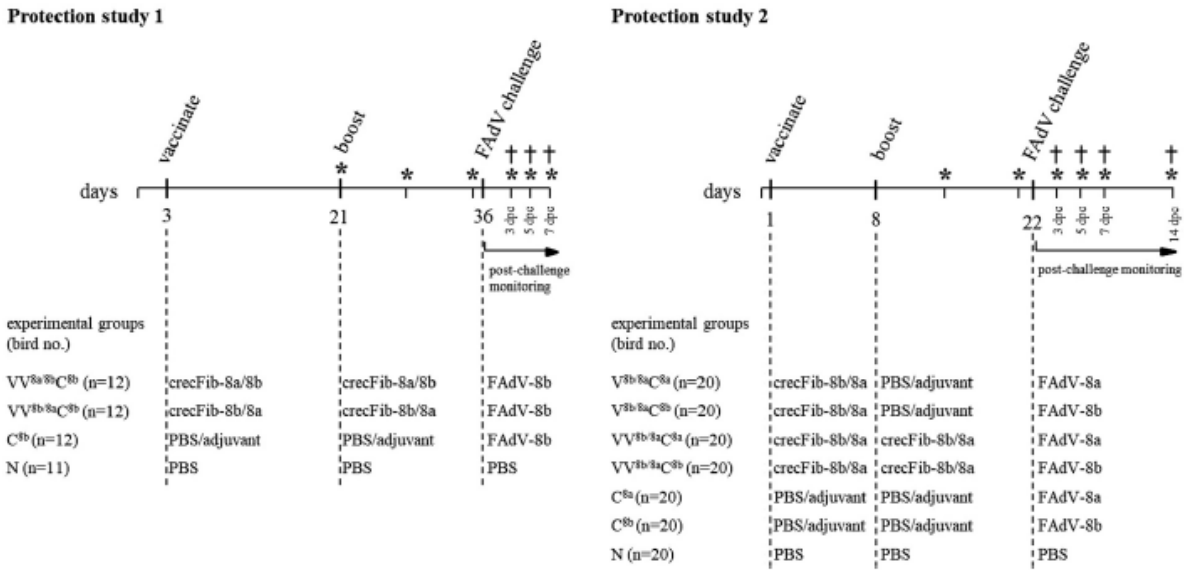

FIG 4 Experimental design of protection studies 1 and 2. The procedures (vaccination, booster, and challenge) carried out on birds are indicated on the timelines (as days of life and days post challenge [dpc]). Individual treatment schemes of each group are specified below. Asterisks indicate blood sampling, and cross symbols indicate sequential killings, with necropsy and organ sampling.

Protection study 2: broad protective efficacy of crecFib-8b/8a applying different vaccination regimens. A prime-boost vaccination regimen at 1 day old and 7 days postvaccination (dpv) was administered to two groups, one of which was challenged at 22 days of age with FAdV-8a (designated $VV^{8b/8a}C^{8a}$) and the other with FAdV-8b ($VV^{8b/8a}C^{8b}$). Additionally, the results of the experiment were compared to those of a single-vaccination

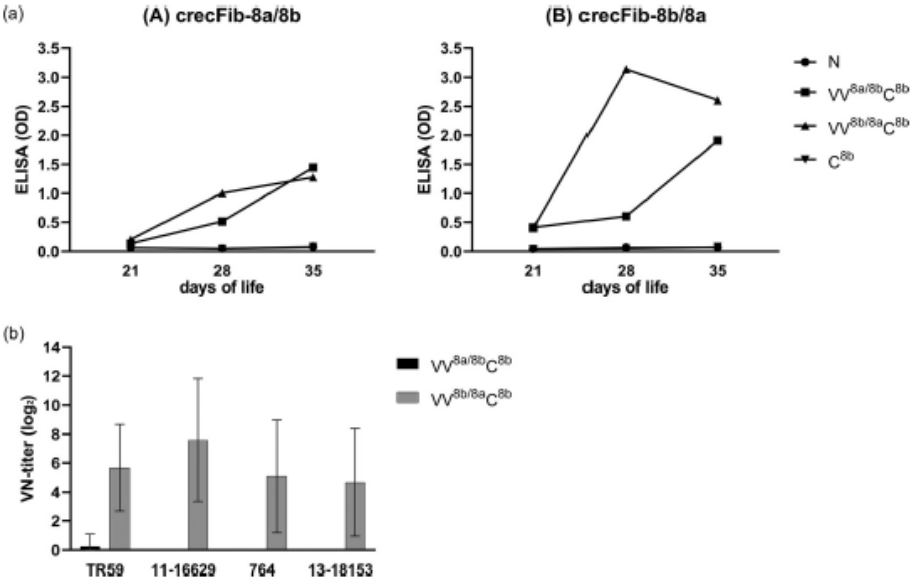

FIG 5 Prior challenge antibody development in protection study 1 (prime-boost vaccination with crecFib-8a/8b or crecFib-8b/8a). (a) Mean OD values for each treatment (groups receiving the same treatment were pooled), measured by crecFib-8a/8b ELISA (A) and crecFib-8b/8a ELISA (B) at 21 (18 dpv), 28 (7 dpb), and 35 (14 dpb) days of life. (b) Neutralizing antibody titers at 35 days of life (14 dpb, immediately prior to challenge) against FAdV-8a (strains TR59 and 11-16629) and FAdV-8b (strains 764 and 13-18153). Error bars show standard deviations.

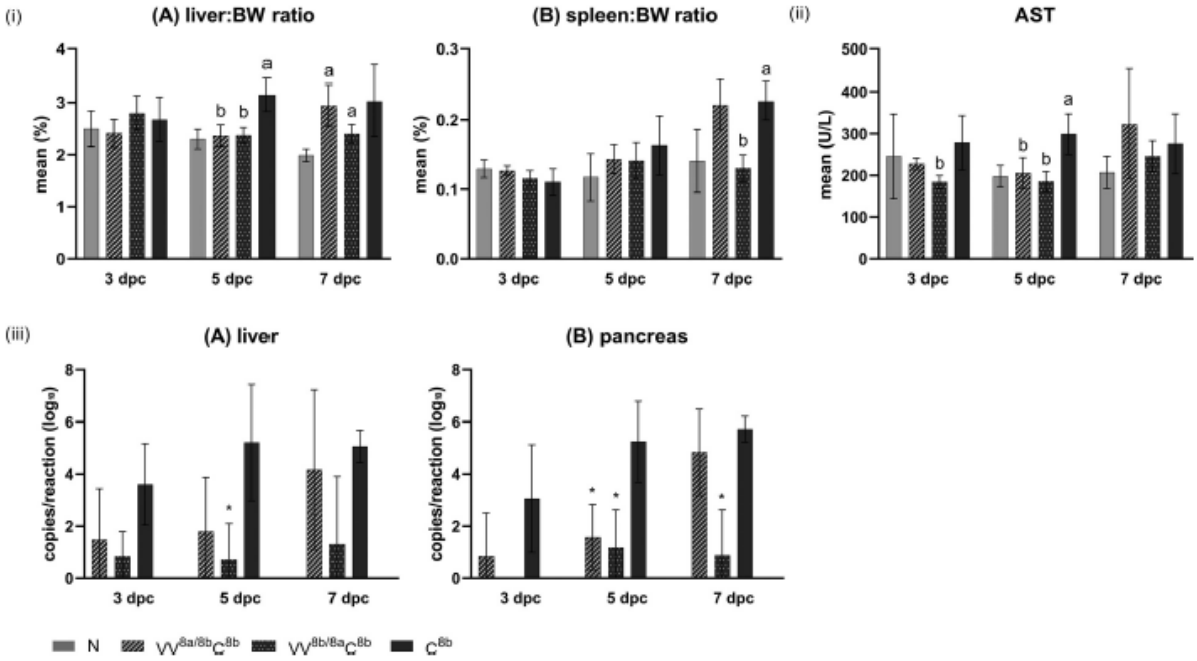

FIG 6 Endpoints of protection, evaluated after FAdV-8b challenge in protection study 1 (at 3, 5, and 7 dpc). (i) Organ-body weight (BW) ratios for liver (A) and spleen (B). Lowercase letters above bars indicate significant differences (a, versus negative control; b, versus challenge control). (ii) Plasma AST (a, significantly different versus negative control; b, significantly different versus challenge control). (iii) Viral loads in liver (A) and pancreas (B). Significant differences versus challenge control are indicated with asterisks ($P \leq 0.05$). Error bars show standard deviations.

regimen administered at 1 day old (groups $V^{8b/8a}C^{8a}$ and $V^{8b/8a}C^{8b}$). Mock-vaccinated challenge control groups were included for each challenge type (C^{8a} and C^{8b}), as well as a negative-control (N) group receiving PBS at time points analogous to the prime-boost regimen.

At 21 days of life, immediately prior to challenge, birds from groups receiving each vaccination regimen had developed antibody levels detectable by ELISA. However, sera from birds in the prime-boost group ($VV^{8b/8a}$) had higher ODs, with only 1/40 individuals having an OD of <3 (mean OD, 3.3 ± 0.1), while sera from birds receiving the single-vaccine regimen ($V^{8b/8a}$) had overall lower, more unevenly distributed titers (2.65 ± 0.79) (Fig. 7). Post challenge and irrespective of the challenge virus type, the ODs between sera from the $V^{8b/8a}$ and $VV^{8b/8a}$ groups were not distinguishable anymore, as they reached (and then remained at) the highest measurable endpoint. In contrast, sera from nonvaccinated challenge control birds showed a steady incline of titers to 2.15 ± 1.02 until 14 dpc (C^{8a}) or an abrupt rise to 2.02 ± 0.95 at 7 dpc, thereupon remaining stable (C^{8b}).

In 36/40 $VV^{8b/8a}$ birds, prechallenge Abs had neutralizing activity against both FAdV-8a and -8b, while the remaining four birds showed only unilateral neutralizing activity against one of the serotypes. In comparison, single-vaccinated $V^{8b/8a}$ birds showed a more infrequent presence of prechallenge NAbs, with 11/40 birds having unilateral NAbs and 7 birds having a complete absence of NAbs. Furthermore, the titer levels were lower in $V^{8b/8a}$ birds than in $VV^{8b/8a}$ birds, although the regimens were similar in eliciting stronger neutralization against FAdV-8a (3.28 ± 3.22 $\log_2$ in $V^{8b/8a}$ birds versus 5.58 ± 2.00 $\log_2$ in $VV^{8b/8a}$ birds against FAdV-8a and 2.88 ± 2.44 $\log_2$ in $V^{8b/8a}$ birds versus 4.30 ± 3.13 $\log_2$ in $VV^{8b/8a}$ birds against FAdV-8b).

The FAdV-8a challenge resulted in a relatively abrupt increase of NAbs in naive birds (C^{8a}), reaching higher levels than in any of the vaccinated groups at 7 dpi (Fig. 7a). In $VV^{8b/8a}C^{8a}$ birds, overall higher titers against FAdV-8b than FAdV-8a were found, while the reverse trend was seen in $V^{8b/8a}C^{8a}$ birds; by 14 dpc, birds of all three groups reached comparable mean titers against FAdV-8a. FAdV-8b challenge induced mean titers in naive birds (C^{8b}) that were similar to those induced by FAdV-8a challenge at 7 dpc (10.70 ± 1.34 $\log_2$) but did not significantly exceed the mean titers of vaccinated birds ($V^{8b/8a}C^{8b}$ and $VV^{8b/8a}C^{8b}$)

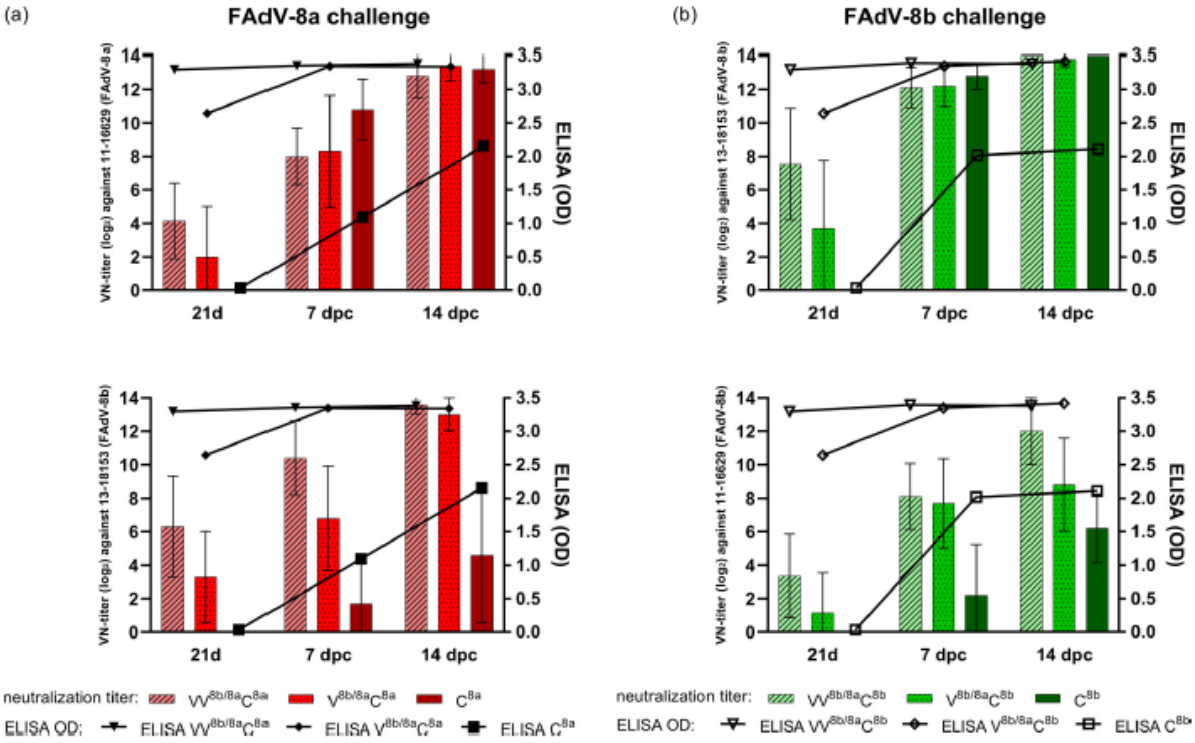

FIG 7 Antibody development (ELISA and VN test) in protection study 2 (comparing the $V^{8b/8a}$ and $VV^{8b/8a}$ regimens against challenge with either FAdV-8a or -8b) immediately prior to (21 days), and after (7 and 14 dpc) challenge. Only groups that received the same challenge were compared among each other, with a summary of data for FAdV-8a-challenged birds (strain 11-16629) in panel a and of FAdV-8b challenged birds (strain 13-18153) in panel b. Top, neutralization against the virus type used for challenge; bottom, neutralization against the respective heterologous virus type. Error bars show standard deviations.

(Fig. 7b). Although $V^{8b/8a}C^{8b}$ and $VV^{8b/8a}C^{8b}$ birds continued to develop NAbs against FAdV-8a, the mean titers against FAdV-8b were still always higher.

Of note, individual challenged birds of the C^{8a} and C^{8b} groups also showed a certain cross-neutralization of the heterologous viral type, though those were exclusively birds with peak homologous titers (12 to >14 $\log_2$).

As a benchmark for clinical effects following challenge, mild depression was recorded in one (C^{8a}) and four (C^{8b}) birds between 2 and 5 dpc, and an additional dead bird at 4 dpc in the C^{8b} group. Among all birds of the vaccinated groups, only one case of transient depression occurred at 2 to 3 dpc, less surprisingly in an individual of the $V^{8b/8a}C^{8b}$ groups that lacked prechallenge NAbs.

The mean liver/BW and spleen/BW ratios were most affected at 3 and 5 dpc, with significant increases in birds in both challenge control groups (Fig. 8i). Despite levelling off, this effect still persisted for the spleen at 7 dpc in the C^{8a} group and for the liver at 14 dpc in both the C^{8a} and C^{8b} groups. In contrast, the liver/BW and spleen/BW ratios in the vaccinated/challenged groups remained comparable to those in the negative control (N) group throughout the whole experiment, except for the liver/BW ratio in the $V^{8b/8a}C^{8b}$ group at one time point (14 dpc). Bursa/BW ratios were most affected at 5 dpc, with a significant reduction in all groups versus the ratio in the N group, except for the $V^{8b/8a}C^{8b}$ group, in which the same trend was shifted to 14 dpc.

In addition, birds in the C^{8a} and C^{8b} groups had the highest plasma AST values of all groups from 3 to 7 dpc; significant differences were found compered to the plasma AST values in two of the vaccinated groups ($VV^{8b/8a}C^{8b}$ and $V^{8b/8a}C^{8a}$) (Fig. 8ii). Furthermore, vaccinated groups exhibited consistently lower viral loads in target organs compared to their challenge controls, while vaccination even prevented detectable infection at several time points (Fig. 8iii). With few exceptions, liver, pancreas, and spleen samples of birds in the C^{8a} and C^{8b}

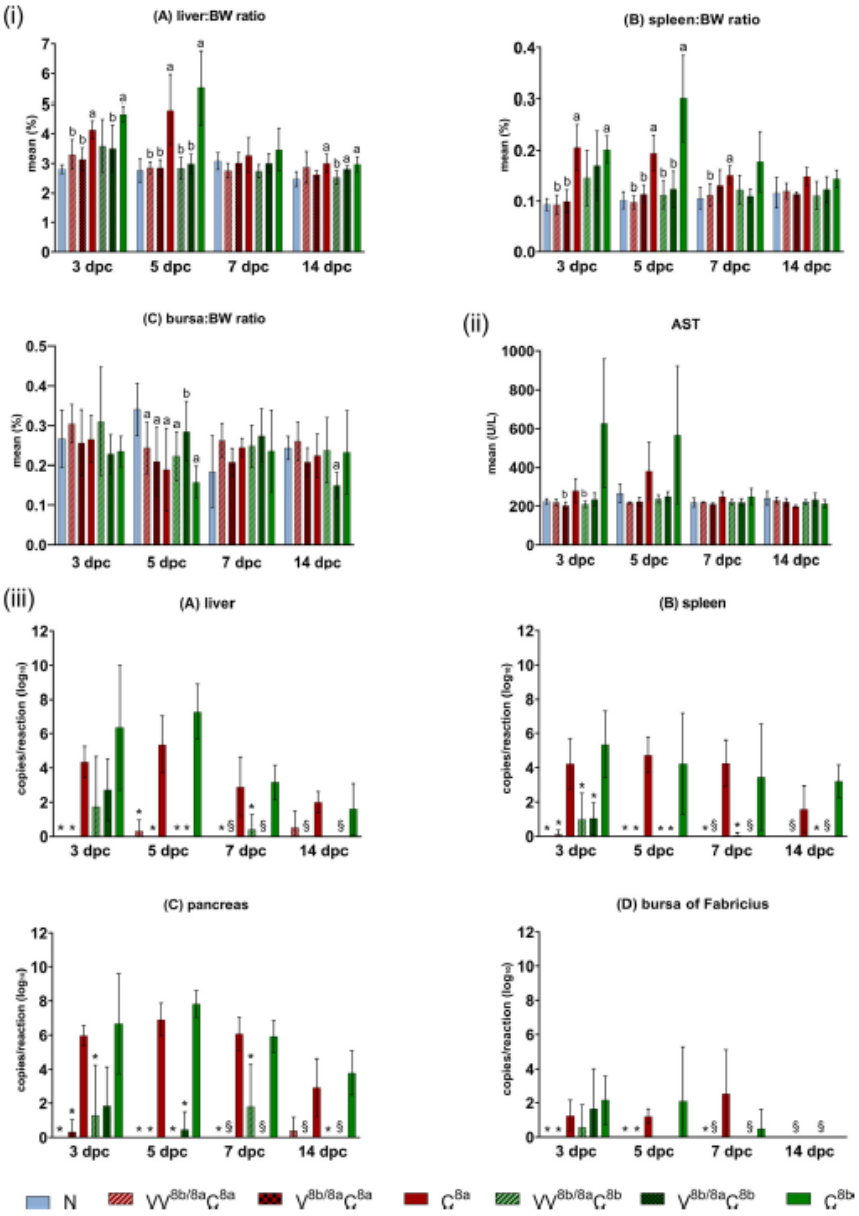

FIG 8 Endpoints of protection in protection study 2 (evaluated at 3, 5, 7, and 14 dpc). The groups are designated with V or VV according to single or prime-boost regimen with crecFib-8b/8a, followed by C with the respective challenge virus indicated (either FAdV-8a or FAdV-8b); N designates the negative control. (i) Organ-body weight (BW) ratios for liver (A), spleen (B), and bursa of Fabricius (C). Lowercase letters above bars indicate significant differences (a, versus negative control; b, versus the corresponding challenge control). (ii) Plasma AST levels (a, significantly different versus negative control; b, significantly different versus challenge control). (iii) Viral loads in liver (A), pancreas (B), spleen (C), and bursa of Fabricius (D). Significant differences versus the corresponding challenge control are indicated with asterisks ($P \leq 0.05$). Data exclude the single-vaccination regimen at 7 and 14 dpc due to low sample size (marked with §). Error bars show standard deviations.

groups were positive at all time points (only one liver at 7 dpc and the pancreas and spleen from another bird at 14 dpc in the C^{8a} group were negative, as well as two livers at 14 dpc from birds in the C^{8b} group); in contrast, viral DNA was detected only in one bird's liver at 5 dpc and another bird's liver and pancreas at 14 dpc in the $VV^{8b/8a}C^{8a}$ group, with viral loads

significantly reduced from 3 to 7 dpc. Similar results were achieved in the $V^{8b/8a}C^{8a}$ group between 3 and 5 dpc, with only one bird's pancreas and spleen testing positive at the earliest time point (3 dpc). In the $V^{8b/8a}C^{8b}$ group, the mean viral loads were significantly reduced at 5 to 7 dpc in the liver and at all time points in the pancreas and spleen; in fact, all of the target organs remained even completely negative at 5 and 14 dpc. In the $V^{8b/8a}C^{8b}$ group, significant reductions of viral loads occurred only in the spleen at 3 to 5 dpc and in the liver and pancreas at 5 dpc. The bursa of Fabricius was the organ with the lowest mean viral load, although positive results were still found in all but 2 samples in the C^{8a} group and in 4, 3, and 1 sample(s) in the C^{8b} group from 3 to 7 dpc. Of all vaccinees, only three individuals, having no prechallenge NAbs and challenged with FAdV-8b, were positive at 3 dpc. At 14 dpc, viral DNA was generally not detectable in the bursa of Fabricius anymore.

DISCUSSION

Most of the knowledge about epitopes in *Fowl Aviadenovirus* (FAdV) capsid proteins is still deduced from the heavily investigated human adenoviruses (HAdVs), lacking, however, a directly comparable antigenic constellation. Particularly the fiber, despite its increasing popularity as a candidate subunit vaccine against FAdVs, is less resolved in regard to its antigenic properties. Complicating the situation, adenoviral fibers show individual variations within their common morphological framework. This includes the number and type of fibers present on the capsid, but also variations in the surface accessibility of residue sites, with consequences for receptor tropism and immunity (3, 19).

In the FAdV-8b fiber (species FAdV-E), two B cell epitopes were previously identified (20). Located in the fiber shaft, those epitopes were, however, nonneutralizing, which conforms with similar reports from HAdVs (21). In contrast, we recently found evidence for at least 2 discrete neutralizing epitopes in the FAdV-E fiber, mapping inside or near the knob, based on hybrid neutralization of a wild-type strain with a fiber gene recombined between two serotypes (8). As the only further information about neutralizing fiber epitopes derives from FAdV-4 (17), a member of the species FAdV-C, which peculiarly encodes two fiber proteins in two separate genes, differences between the studied models were expected. Notably, however, the FAdV-E fiber sequences analyzed in the present study had a predicted epitope in the same location as that of the FAdV-4 fiber-2 reported for type-specific *in vitro* neutralization. This, alongside two other *in silico*-identified candidate epitopes in the FAdV-E knob, prompted us to design a crosswise sequence exchange between fibers of FAdV-8a and -8b, the two FAdV-E affiliate serotypes causing inclusion body hepatitis (IBH) (22).

The resulting protein, termed crecFib, was amenable to confer simultaneous protection against each viral type included in the chimeric construct, as demonstrated by up to complete prevention of clinical disease and significant limitation (or, in some instances, delay) of effects of virus in target organs, as well as organic and metabolic damage.

Sera from chimera-vaccinated birds neutralized both virus types, in contrast to sera against monospecific control fibers, which strictly neutralized virus of the same serotype. This reinforces the results of our previous study in which the FAdV-8a fiber failed to protect chickens against FAdV-8b challenge, showing that fiber is a monovalent vaccine antigen due to the requirement of functionally neutralizing Abs for protection (12). Though the reliance on NAbs seems to pertain more to those types causing IBH (i.e., types with one fiber gene), considering controversial findings with fiber vaccines against hepatitis-hydropericardium syndrome (HHS) (FAdV-4, the type with two fiber genes), a greater practical interest arises for IBH due to (i) its multitype etiology and (ii) the resistance of the involved types to cross-neutralization by fiber Abs.

Of the two recombinant chimeras tested in this study, only one combination, crecFib-8b/8a, was adequately immunogenic, as shown by robust ELISA antibody titers, the presence of neutralizing Abs, and up to complete protection following challenge.

With three-dimensional models of the crecFib knobs made available, we speculate on a possible role of structural differences between the two constructs, specifically in a region that evoked our interest because it merges residues from opposite sides of the chimeric

switch, each accounting for separate epitopes in the linear sequences. Besides discriminate topologies, the corresponding region showed overall inverted surface electrostatics with positively charged residues in the weakly immunogenic crecFib-8a/8b, as opposed to a predominantly negative potential in the superior crecFib-8b/8a. Charge conversion can substantially influence the immunogenicity of antigens, sometimes resulting from only a single mutation (23, 24). It is therefore possible that the changed residue constellations at the point of contact between sequences from different template strains in chimeras altered their surface charge, with consequences for presentation to the immune system.

The conformational nature of this region alongside local changes of structure and electrostatics in crecFib proteins with different immunogenicities provides intriguing hints toward a major neutralizing epitope. The importance of conformational epitope(s) for functional neutralization by the FAdV fiber is also supported by an earlier work, in which non-hexon-specific MAbs against FAdV-1 (species FAdV-A) full virus with neutralizing activity (identifying them with some certainty as fiber Abs) could not detect denatured viral proteins (25). This also resembles the situation in HAdVs, where fiber-directed NAbs preferentially recognize conformational epitopes and trimeric fiber (26, 27).

Aside from the structural effects discussed, our data on the immunogenicities of chimeras with reciprocally oriented sequences are similar to those of another study, conducted with HAdV fibers, where the outcome of *in vitro* reactivities of chimeric knobs varied due to different locations of the dominant neutralization epitope depending on the serotype (3). Although this hypothesis remains to be investigated for FAdVs, our results would indicate the location of the neutralizing epitope to be anywhere to the right in the FAdV-8a fiber, as opposed to being to the left in the FAdV-8b fiber, relative to the exchange site G441-R442, rendering crecFib-8b/8a the only, but very powerful, antigen attainable by such means.

Despite its inability to elicit a humoral response, the reverse-order crecFib-8a/8b still showed good reactivity with diverse antifiber antisera in Western blotting. As Western blotting, unlike fiber ELISA and VN test, was also the only method that allowed cross-detection of fibers, this pinpoints an epitope that is broadly shared but accessible only in the denatured protein. We propose that this epitope, which is distinct from the epitope(s) inducing NAbs and does not contribute to protection, resides N distally in the tail, the most strongly conserved of all fiber domains, similarly to the broadly reactive epitope identified in HAdV fiber monomers (28, 29).

In conclusion, we resolved distinct antigenic interfaces, together with their putative locations in the FAdV fiber, having different structural and functional properties that can be exploited for the design of chimeric proteins. *In vivo*, chimeric fibers were confirmed to extend the protective spectrum of conventional fiber subunits. Allowing diverse neutralizing epitopes to be condensed into a single-antigen component for broad coverage, chimeric fibers are a suitable vaccination strategy to address the advancing emergence of FAdV types worldwide.

MATERIALS AND METHODS

***In silico* design for recombinantly expressed chimeric fibers.** The FAdV fiber open reading frame was divided into an amino (N)- and a carboxy (C)-distal segment, amplified separately from different template strains and fused seamlessly via Gibson Assembly cloning to reconstitute a novel, full-length fiber, herein referred to as "crecFib" (Fig. 1a and b). As a result, crecFib constructs contain a crossover between heterologous sequences of serotypes FAdV-8a and -8b at the junction of N- and C-distal segments. The junction (termed a "specificity switch") was engineered at consensus residue positions of amino acids 441-442 in the pairwise sequence alignment of the FAdV-8a and FAdV-8b fibers, mapping inside the proposed fiber head (knob) domain. Accommodating *in silico*-predicted epitopes to both sides, this strategy was anticipated to combine the antigenic specificity of both constitutive serotypes into a single antigen. By engineering the specificity switch inside the knob, we also sought to maintain sequence integrity at the presumed shaft-knob boundary, which represents an important element for trimerization of the fiber (30).

Candidate epitopes were (i) inferred from a previously reported epitope in fiber-2 of FAdV-4 (species FAdV-C) (17) and (ii) *in silico* prediction with DiscoTope 2.0 software (31), whereby the closest related fiber knob for which a molecular model is currently available, fiber-2 of FAdV-1 (species FAdV-A) reference strain CELO (32), served for homology modeling. Positional homologies between members of different types were determined by multiple sequence alignments created with MegAlign software (DNAStar, Madison, WI, USA). Homology modeling was also used for assigning structural domains (tail, shaft, and knob) of the FAdV fiber based on existing information (33).

Cloning and expression of crecFib constructs. Based on the FAdV reference strains selected as expression templates, the above-defined N- and C-distal fiber segments were amplified using primer

pairs featuring overhangs with the flanking sequence of the EheI-/StuI-digested pFastBac expression vector (Invitrogen, Vienna, Austria) and between the two segments themselves. Detailed information on the cloning strategy is provided in Table S1. Ligation into the linearized pFastBac vector was performed with the Gibson Assembly master mix (NEB, Ipswich, MA) according to the manufacturer's instructions. Two constructs were generated for each chimeric combination, with reciprocal specificity order, designated crecFib-8a/8b and crecFib-8b/8a accordingly.

Correct insertion of the segments into the vector was confirmed by Sanger sequencing across the multiple-cloning site (LGC Genomics, Berlin, Germany).

Recombinant proteins were expressed in *Spodoptera frugiperda* Sf9 cells and purified via polyhistidine tags on affinity chromatography columns as described previously (14), and their concentrations determined by Bradford assay (Thermo Fisher Scientific, Vienna, Austria).

Assessment of the immunogenicity and *in vitro* reactivity spectrum of crecFib. Specific-pathogen-free (SPF) chickens (Valo BioMedia GmbH, Osterholz-Scharmbeck, Germany), hatched and housed at our facilities, were immunized intramuscularly (i.m.) with crecFib-8a/8b ($n = 5$, 3-day-old) or crecFib-8b/8a ($n = 5$, 14-day-old). Three birds of each group received 50 μg and two birds 100 μg of recombinant protein mixed 1:1 with GERBU adjuvant P (GERBU Biotechnik GmbH, Heidelberg, Germany). Postimmunization sera were collected at weekly intervals for parallel monitoring by enzyme-linked immunosorbent assay (ELISA) and virus neutralization test.

The procedures on experimental birds were discussed and approved by the institutional ethics committee and licensed by the Austrian government according to the approval requirements of the Animal Experiments Act 2012 (34) (license number GZ 68.205/0006-V/3b/2019).

Additional immune sera for comparative purposes were sourced from sample collections of previously published studies or recruited under animal trial license numbers GZ 68.205/0217-WF/V/3b/2016 and GZ 68.205/0006-V/3b/2019 (summarized in Table S2).

Briefly, these sera were derived from SPF chickens injected with whole virus or immunized i.m. with monospecific FAdV fibers (i.e., recombinantly expressed fibers based on the sequence of a singular serotype). Whole virus for antiserum production was 3-fold plaque purified and characterized at least by partial analysis of the hexon and fiber genes but in most cases by full-genome sequencing and cross-neutralization test, as described previously (35). Whenever available, our test setting included sera against inactivated, adjuvanted FAdV (prepared with 1% formaldehyde, administered in a 1:1 mixture with GERBU adjuvant) and live virus, in order to assess a possibly differential recognition of denatured antigen in the immunoblot.

crecFib-based ELISA. Sera from birds immunized with each type of crecFib were tested on ELISA plates coated with the corresponding crecFib, following the protocol described by Feichtner et al. (10).

Virus (cross-)neutralization (VN) test. Neutralizing antibodies (NAbs) in sera were determined in a microtiter assay on primary chicken embryo liver (CEL) cells. Serial 2-fold serum dilutions (1:8 to 1:16,384) were incubated with 100 50% tissue culture infective doses ($TCID_{50}$) of virus. The crecFib antisera were tested against the FAdV-8a and -8b reference (expression template) strains and two field isolates from each serotype which served as challenge strains in the protection study. For comparative purposes, antifiber antisera with monotype specificity (Fib-8a and Fib-8b serum) were included in certain settings. Additionally, each microtiter plate included positive (cells plus virus) and negative (cells only) control wells. After 5 days at 37°C in 5% CO_2, the wells were investigated for cytopathic effect (CPE), with the titer defined by the highest serum dilution inhibiting CPE.

Immunofluorescence staining. Following a virus cross-neutralization test against FAdV-8a and -8b reference strains, as described above, the cells were fixed by adding ice-cold methanol for 5 min. Following serial washing steps and blocking with 3% bovine serum albumin (BSA) for 1 h, in-house-generated polyclonal rabbit anti-FAdV antiserum (diluted 1:500 in PBS) was added to each well overnight. After removal of the rabbit antiserum and washing, the plates were incubated with 1:200-diluted Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Life Technologies, Carlsbad, CA, USA) in the dark for 1 h. After another wash, cells were stained with 1:1,000 DAPI (4′6-diamidino-2-phenylindole solution; Roche Diagnostics GmbH, Vienna, Austria) for 5 min, subjected to a final wash, and covered with PBS.

Internalization of viral particles was examined and documented with a Zeiss Axiovert 200 M fluorescence microscope (Zeiss, Jena, Germany) coupled to a Flexacam C1 camera and Leica Application Suite X (LAS X) (Leica Microsystems GmbH, Wetzlar, Germany).

Western blotting. The crecFib constructs were screened side by side with the most closely related, in-house-expressed monospecific fibers (Fib-8a of strain TR59, Fib-8b of strain 764, and Fib-7 of strain YR36, the reference type representatives of FAdV-E) using polyclonal immune sera. In order to minimize variations in the ratios of reactants, the concentration of recombinant protein loaded per lane was adjusted to 7.5 μg, and detection sera with similar ELISA titers ($2.5 \leq OD \leq 3.0$) against recombinant fiber of the homologous type and, if applicable, neutralization titers in the range of 11 to 12 $\log_2$ were used. Briefly, recombinant purified proteins were separated by 12% SDS–PAGE and transferred onto BioTrace polyvinylidene difluoride (PVDF) transfer membrane (Pall, Vienna, Austria) with the Trans-Blot Turbo transfer system (Bio-Rad, Vienna, Austria). After blocking with 3% (wt/vol) skim milk, membranes were incubated separately with polyclonal sera, preabsorbed with 1% Sf9 cell powder, and diluted 1:2,000. As a control for the presence and size of monomeric fibers, one membrane was incubated with antipolyhistidine antibody (Sigma-Aldrich, Vienna, Austria). Following incubation with secondary rabbit anti-chicken IgG-horseradish peroxidase (HRP) (Sigma-Aldrich, Vienna, Austria), or goat anti-mouse IgG(H+L)-HRP (Bio-Rad, Vienna, Austria) for controls, and intermediate washes, blots were developed with Clarity Western ECL substrate (Bio-Rad, Vienna, Austria). Visualization was performed with the ChemiDoc Imager (Bio-Rad, Vienna, Austria).

Protection studies with crecFib constructs. Two vaccination-challenge trials were performed to sequentially address whether (i) crecFib constructs confer *in vivo* protection and (ii) crecFib-induced

protection is amenable for broad coverage of the inclusion body hepatitis complex. An overview of both experimental designs is summarized in Fig. 4. The corresponding procedures on birds were discussed and approved by the institutional ethics committee and licensed by the Austrian government (license numbers GZ 68.205/0156-V/3b/2019 and GZ 68.205/0215-V/3b/2019).

(i) Protection study 1: protective efficacy of crecFib-8a/8b and crecFib-8b/8a. In the first study, two groups of SPF broiler chickens ($n = 12$) were prime-boost vaccinated with either crecFib-8a/8b or the reverse-order crecFib-8b/8a, followed by challenge with FAdV-8b in each case. SPF broilers were obtained from Animal Health Service (Deventer, The Netherlands) and housed in separate isolator units (HM2500; Montair, The Netherlands). Vaccination consisted of 50 μg of the respective crecFib formulated in a 40% (wt/vol) antigen-oil-based adjuvant phase, administered i.m., while challenge was carried out i.m. with 106.2 $TCID_{50}$ FAdV-8b (strain 13-18153). Further groups served as a challenge control group, injected with a PBS/adjuvant mixture instead of vaccination, and a negative-control group, administered only sterile PBS according to the same scheme. Blood was collected weekly from booster until challenge and at 3, 5, and 7 days post challenge (dpc). Four birds per group were killed and submitted to necropsy at 3 and 5 dpc, analogous to the remaining birds at 7 dpc. Endpoints of protection included clinical signs, assessed daily in the time period post challenge, organ-body weight (BW) ratios for liver and spleen, the aspartate transaminase (AST) content in plasma as previously described (36), and viral load quantification in liver and pancreas by a quantitative PCR (qPCR) protocol adapted from Günes et al. (37).

(ii) Protection study 2: broad protective efficacy of crecFib-8b/8a applying different vaccination regimens. In this setting, we proceeded with only one of the chimeras, crecFib-8b/8a, this time testing its protective efficacy against challenge with both viral types of interest (FAdV-8a or FAdV-8b). Additionally, a prime-boost vaccination regimen was compared to a single-shot regimen, using groups of 20 SPF broilers. Each vaccination contained 50 μg crecFib-8b/8a formulated in a 40% (wt/vol) antigen-oil-based adjuvant phase, administered i.m. Challenge was carried out i.m. with 106.2 $TCID_{50}$ of FAdV-8a (strain 11-16629) or FAdV-8b (strain 13-18153), while negative-control birds again received PBS instead.

Blood was collected weekly from the second week of life until challenge and then at each of the following sampling time points: 3, 5, 7, and 14 dpc. Up to five birds/group were killed and necropsied at 3, 5, 7, and 14 dpc alongside individuals that died due to the infection.

Endpoints of protection included organ-BW ratios for liver, spleen, and bursa of Fabricius, the AST content in plasma, and viral loads in liver, pancreas, spleen, and bursa of Fabricius.

Statistical analysis of the data sets was carried out using the Shapiro-Wilk test together with a visual inspection of histograms and normal Q-Q plots in order to verify the normal distribution assumption. The mean values for organ-BW ratios, plasma AST levels, and viral loads in target organs of vaccinated groups were compared with the values for the negative-control group and the corresponding challenge control group via unpaired Student's t test. Data sets that did not meet the normality assumptions were analyzed through pairwise comparisons with Mann-Whitney U test. In each case, P values of ≤ 0.05 were considered statistically significant. Statistical analyses were performed with SPSS version 26 (IBM SPSS Statistics; IBM Corp., Armonk, NY, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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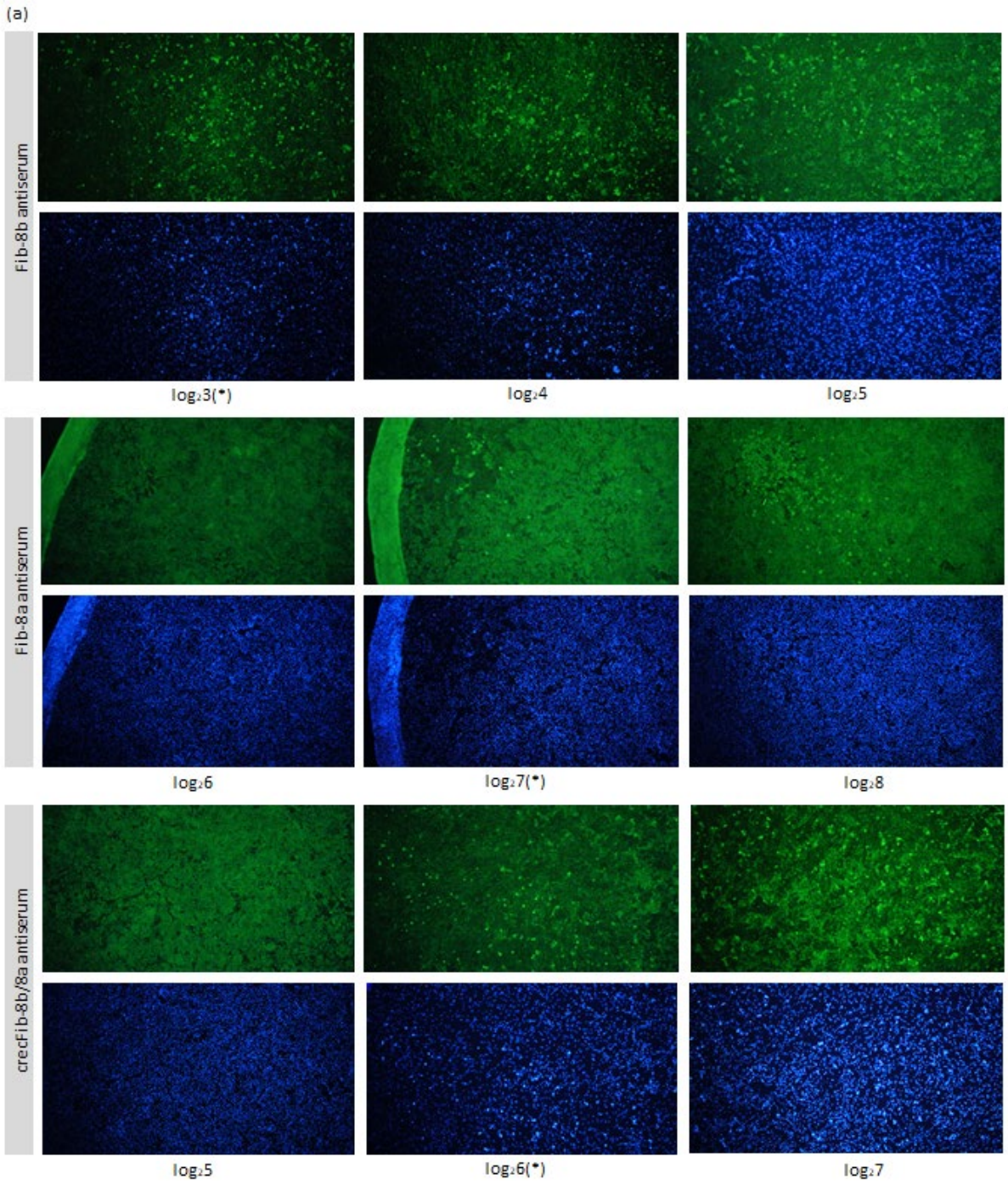
REFERENCES

1. Norrby E. 1969. The structural and functional diversity of adenovirus capsid components. J Gen Virol 5:221–236. https://doi.org/10.1099/0022-1317-5-2-221.
2. Liebermann H, Lotz K, Seidel W. 2002. Mapping of epitopes on the fiber knobs of human adenovirus serotypes 8 and 15. Intervirology 45:59–66. https://doi.org/10.1159/000050089.
3. Lang S, Wang L, Wang Z, Zhu R, Yan J, Wang B, Wu J, Zhang H, Wu H, Zhou Y, Kong W, Yu B, Yu X. 2016. Localization of neutralization epitopes on adenovirus fiber knob from species C. J Gen Virol 97:955–962. https://doi.org/10.1099/jgv.0.000410.
4. Hossain R, Yasmin T, Hosen MI, Nabi AHMN. 2018. *In silico* identification of potential epitopes present in human adenovirus proteins for vaccine design and of putative drugs for treatment against viral infection. J Immunol Methods 455:55–70. https://doi.org/10.1016/j.jim.2018.01.005.
5. McFerran JB, Adair BM. 1977. Avian adenoviruses - a review. Avian Pathol 6:189–217. https://doi.org/10.1080/03079457708418228.
6. McFerran JB, Adair B, Connor TJ. 1975. Adenoviral antigens (CELO, QBV, GAL). Am J Vet Res 36:527–529.
7. Hess M. 2000. Detection and differentiation of avian adenoviruses: a review. Avian Pathol 29:195–206. https://doi.org/10.1080/03079450050045440.
8. Schachner A, Gonzalez G, Endler L, Ito K, Hess M. 2019. Fowl adenovirus (FAdV) recombination with intertypic crossovers in genomes of FAdV-D and FAdV-E, displaying hybrid serological phenotypes. Viruses 11:1094. https://doi.org/10.3390/v11121094.
9. Calnek BW, Shek WR, Menendez NA, Stiube P. 1982. Serological cross-reactivity of avian adenovirus serotypes in an enzyme-linked immunosorbent assay. Avian Dis 26:897–906. https://doi.org/10.2307/1589878.
10. Feichtner F, Schachner A, Berger E, Hess M. 2018. Development of sensitive indirect enzyme-linked immunosorbent assays for specific detection of antibodies against fowl adenovirus serotypes 1 and 4 in chickens. Avian Pathol 47:73–82. https://doi.org/10.1080/03079457.2017.1372561.

11. Feichtner F, Schachner A, Berger E, Hess M. 2018. Fiber-based fluorescent microsphere immunoassay (FMIA) as a novel multiplex serodiagnostic tool for simultaneous detection and differentiation of all clinically relevant fowl adenovirus (FAdV) serotypes. J Immunol Methods 458:33–43. https://doi.org/10.1016/j.jim.2018.03.002.
12. De Luca C, Schachner A, Mitra T, Heidl S, Liebhart D, Hess M. 2020. Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH) provides type-specific protection guided by humoral immunity and regulation of B and T cell response. Vet Res 51:143. https://doi.org/10.1186/s13567-020-00869-8.
13. Schachner A, Matos M, Grafl B, Hess M. 2018. Fowl adenovirus-induced diseases and strategies for their control—a review on the current global situation. Avian Pathol 47:111–126. https://doi.org/10.1080/03079457.2017.1385724.
14. Schachner A, Marek A, Jaskulska B, Bilic I, Hess M. 2014. Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis–hydropericardium syndrome (HHS). Vaccine 32:1086–1092. https://doi.org/10.1016/j.vaccine.2013.12.056.
15. Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya S, Goonewardene K, Gunawardana T, Karunarathna R, Ojkic D, Tikoo SK, Willson P, Gomis S. 2017. Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. Vaccine 35:2716–2722. https://doi.org/10.1016/j.vaccine.2017.03.075.
16. Chen L, Yin L, Zhou Q, Li Q, Luo Y, Xu Z, Zhang Y, Xue C, Cao Y. 2018. Immunogenicity and protective efficacy of recombinant fiber-2 protein in protecting SPF chickens against fowl adenovirus 4. Vaccine 36:1203–1208. https://doi.org/10.1016/j.vaccine.2018.01.028.
17. Wang P, Zhang J, Wang W, Li T, Liang G, Shao H, Gao W, Qin A, Ye J. 2018. A novel monoclonal antibody efficiently blocks the infection of serotype 4 fowl adenovirus by targeting fiber-2. Vet Res 49:29. https://doi.org/10.1186/s13567-018-0525-y.
18. Arnberg N, Kidd AH, Edlund K, Nilsson J, Pring-Åkerblom P, Wadell G. 2002. Adenovirus type 37 binds to cell surface sialic acid through a charge-dependent interaction. Virology 302:33–43. https://doi.org/10.1006/viro.2002.1503.
19. Nicklin SA, Wu E, Nemerow GR, Baker AH. 2005. The influence of adenovirus fiber structure and function on vector development for gene therapy. Mol Ther 12:384–393. https://doi.org/10.1016/j.ymthe.2005.05.008.
20. Lu H, Shao H, Chen H, Zhang J, Wang W, Li T, Xie Q, Qin A, Ye J. 2019. Identification of novel B cell epitopes in the fiber protein of serotype 8 fowl adenovirus. AMB Express 9:172. https://doi.org/10.1186/s13568-019-0895-1.
21. Liu T, Fan Y, Li X, Gu S, Zhou Z, Xu D, Qiu S, Li C, Zhou R, Tian X. 2018. Identification of adenovirus neutralizing antigens using capsid chimeric viruses. Virus Res 256:100–106. https://doi.org/10.1016/j.virusres.2018.08.007.
22. Schachner A, Grafl B, Hess M. 2021. Spotlight on fowl adenovirus (FAdV) in chickens and beyond—an unresolved host-pathogen interplay. Avian Pathol 50:2–5. https://doi.org/10.1080/03079457.2020.1810629.
23. Ohkuri T, Nagatomo S, Oda K, So T, Imoto T, Ueda T. 2010. A protein's conformational stability is an immunologically dominant factor: evidence that free-energy barriers for protein unfolding limit the immunogenicity of foreign proteins. J Immunol 185:4199–4205. https://doi.org/10.4049/jimmunol.0902249.
24. Yoshida K, Kuroda D, Kiyoshi M, Nakakido M, Nagatoishi S, Soga S, Shirai H, Tsumoto K. 2019. Exploring designability of electrostatic complementarity at an antigen-antibody interface directed by mutagenesis, biophysical analysis, and molecular dynamics simulations. Sci Rep 9:4482. https://doi.org/10.1038/s41598-019-40461-5.
25. Taharaguchi S, Ito H, Ohta H, Takase K. 2006. Characterization of monoclonal antibodies against fowl adenovirus serotype 1 (FAV1) isolated from gizzard erosion. Avian Dis 50:331–335. https://doi.org/10.1637/7460-102605R.1.
26. Fender P, Kidd AH, Brebant R, Oberg M, Drouet E, Chroboczek J. 1995. Antigenic sites on the receptor-binding domain of human adenovirus type 2 fiber. Virology 214:110–117. https://doi.org/10.1006/viro.1995.9949.
27. Yu B, Dong J, Wang C, Zhan Y, Zhang H, Wu J, Kong W, Yu X. 2013. Characteristics of neutralizing antibodies to adenovirus capsid proteins in human and animal sera. Virology 437:118–123. https://doi.org/10.1016/j.virol.2012.12.014.
28. Tarassishin L, Szawlowski P, Kidd AH, Russell WC. 2000. An epitope on the adenovirus fibre tail is common to all human subgroups. Arch Virol 145:805–811. https://doi.org/10.1007/s007050050673.
29. Zubieta C, Schoehn G, Chroboczek J, Cusack S. 2005. The structure of the human adenovirus 2 penton. Mol Cell 17:121–135. https://doi.org/10.1016/j.molcel.2004.11.041.
30. Hong JS, Engler JA. 1996. Domains required for assembly of adenovirus type 2 fiber trimers. J Virol 70:7071–7078. https://doi.org/10.1128/JVI.70.10.7071-7078.1996.
31. Vindahl Kringelum J, Lundegaard C, Lund O, Nielsen M. 2012. Reliable B cell epitope predictions: impacts of method development and improved benchmarking. PLoS Comput Biol 8:e1002829. https://doi.org/10.1371/journal.pcbi.1002829.
32. El Bakkouri M, Seiradake E, Cusack S, Ruigrok RWH, Schoehn G. 2008. Structure of the C-terminal head domain of the fowl adenovirus type 1 short fibre. Virology 378:169–176. https://doi.org/10.1016/j.virol.2008.05.011.
33. Hess M, Cuzange A, Ruigrok RWH, Chroboczek J, Jacrot B. 1995. The avian adenovirus penton: two fibres and one base. J Mol Biol 252:379–385. https://doi.org/10.1006/jmbi.1995.0504.
34. Bundesgesetz über Versuche an lebenden Tieren. 2012. Federal Act on Experiments on Live Animals (Tierversuchsgesetz 2012—TVG 2012). § 26 Approval of projects. Federal Law Gazette I No. 114/2012.
35. Schachner A, Marek A, Grafl B, Hess M. 2016. Detailed molecular analyses of the hexon loop-1 and fibers of fowl aviadenoviruses reveal new insights into the antigenic relationship and confirm that specific genotypes are involved in field outbreaks of inclusion body hepatitis. Vet Microbiol 186:13–20. https://doi.org/10.1016/j.vetmic.2016.02.008.
36. Matos M, Grafl B, Liebhart D, Schwendenwein I, Hess M. 2016. Selected clinical chemistry analytes correlate with the pathogenesis of inclusion body hepatitis (IBH) experimentally induced by fowl aviadenoviruses. Avian Pathol 45:520–529. https://doi.org/10.1080/03079457.2016.1168513.
37. Günes A, Marek A, Grafl B, Berger E, Hess M. 2012. Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). J Virol Methods 183:147–153. https://doi.org/10.1016/j.jviromet.2012.04.005.

Supplemental material



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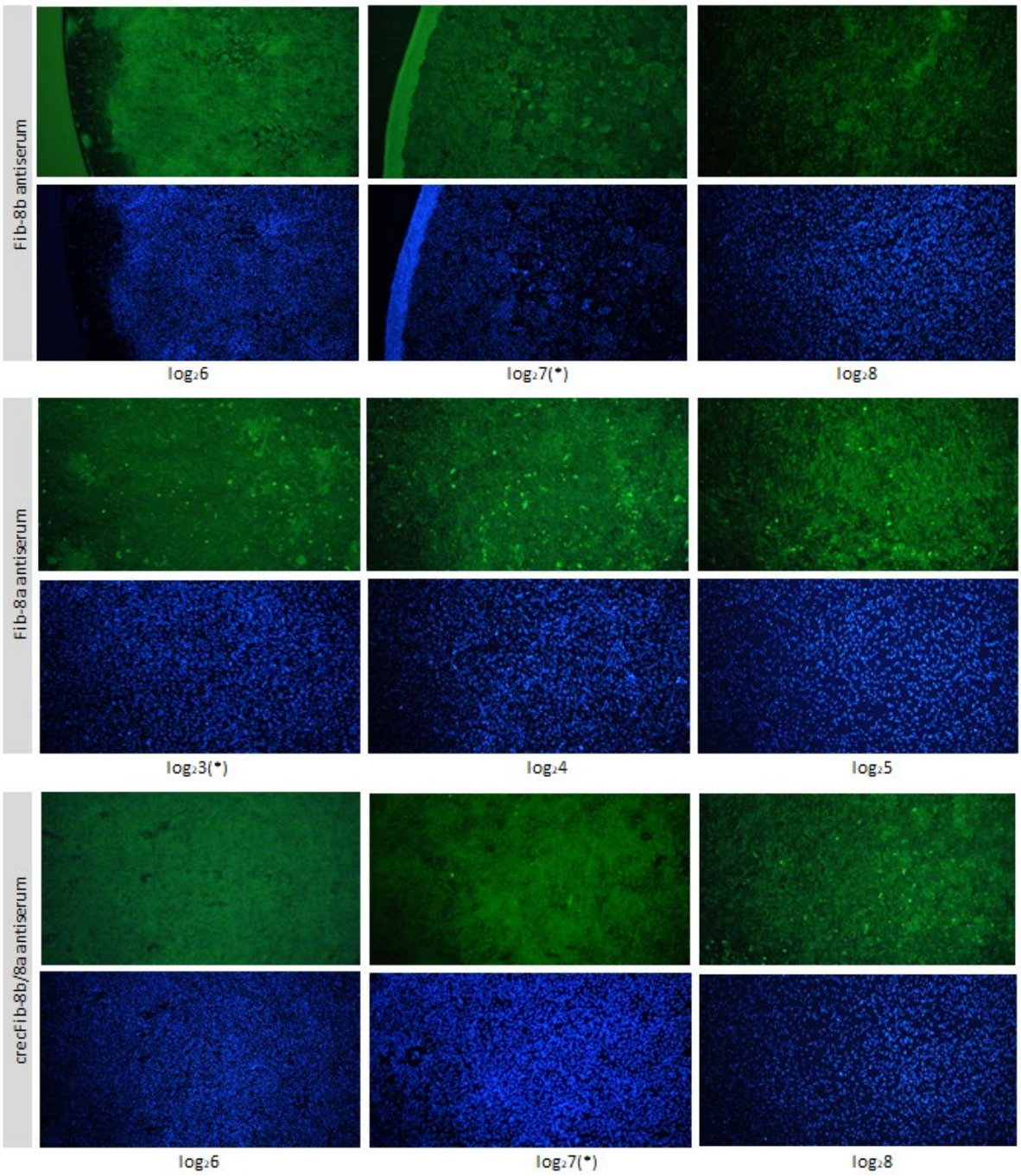
Fib-8b antiserum
log2 6
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Fib-8a antiserum
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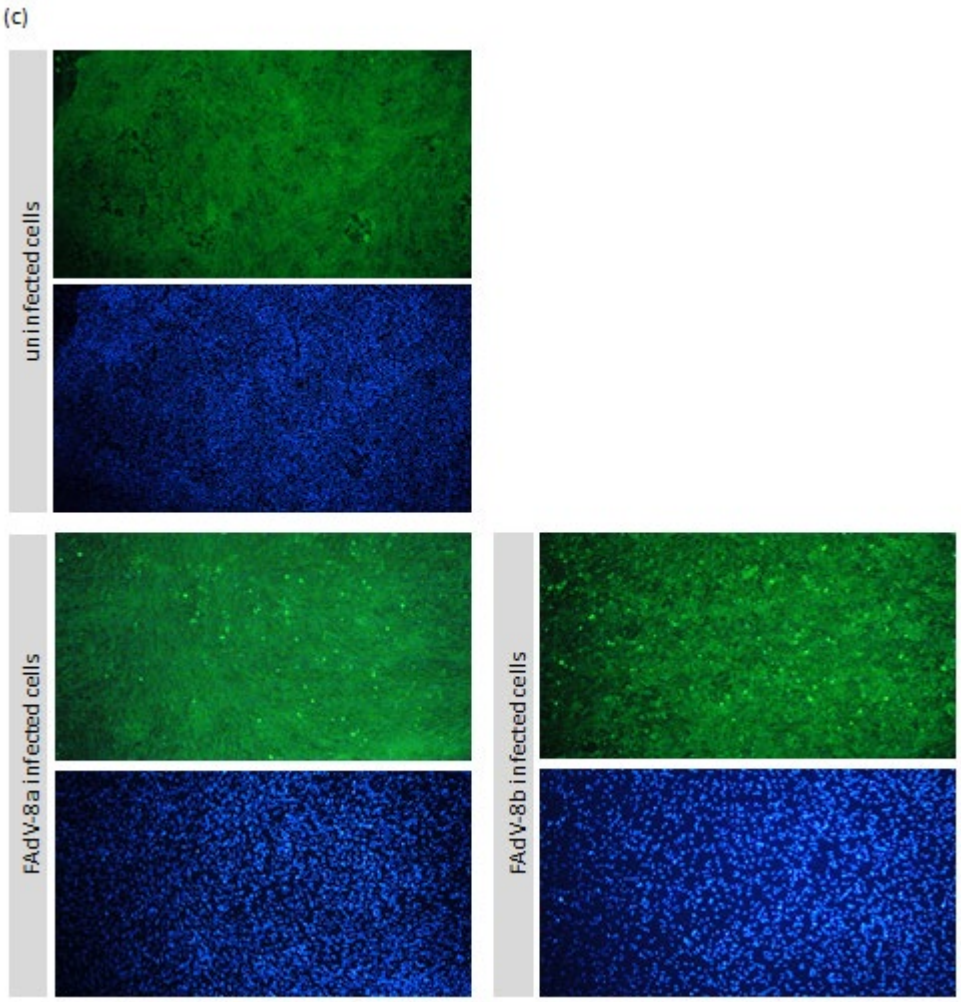
crecFib-8b/8a antiserum
log2 6
log2 7(*)
log2 8



Supplemental figure S1. Immunofluorescence staining of viral particles´ internalization into CEL cells, and inhibition by different categories of fiber antisera (objective 5x). The same three antisera (indicated at the left margins), tested side-by-side against (a) FAdV-8a reference strain TR59 and (b) FAdV-8b reference strain 764. A series of three successive serum dilutions is exemplarily shown, with titers indicated below each image. The lowest titer level at which stained viral particles (and CPE) were detected is marked by an asterisks. Identical sections of the wells are shown on top of each other for Alexa Fluor 488 and cell nuclei staining (DAPI). Panel (c) shows control wells with uninfected, FAdV-8a/TR59-infected and FAdV-8b/764-infected cells. Bar, 100 µm.

Supplemental table S1. Cloning information on the crecFib proteins of this study. Overhangs at the 5´-termini of primer sequences with the flanking vector sequence (FP1 and RP2 primers) or with the counterpart template sequence (RP1 and FP2 primers) are represented by underlined nucleotides.

Designation of chimeric construct	Fragment (position in template sequence)	Template strain (GenBank accession number)	Primer sequences	Expression vector (restriction sites used for cloning)
crecFib-8a/8b	I (nt1-1323)	TR59, FAdV-8a reference strain (KT862810)	FP1-TR59[a]: 5´-AAA CCT GTA TTT TCA GGG CAT GGC GAC CTC GAC TC-3´ RP1-TR59: 5´-GAT AGC TTT CCA GTC GCC CGG TGT TTG GTT GGA AA-3´	pFAST BAC HTb (*EheI/StuI*)
	II (nt1324-1569)	764, FAdV-8b reference strain (KT862811)	FP2-764: 5´- ACC GGG CGA CTG GAA AGC TAT CTC CCC GTC CTT AC-3´ RP2-764: 5´- GTG AGC TCG TCG ACG TAG GTT AAG GAG CGT TGG CG-3´	
crecFib-8b/8a	I (nt1-1323)	764, FAdV-8b reference strain (KT862811)	FP1-764[a]: 5´-AAA CCT GTA TTT TCA GGG CAT GGC GAC CTC GAC TC-3´ RP1-764: 5´-GGT ATG TGT CCA CTC GAC CAC TGG TGG GTT CAA AA-3´	pFAST BAC HTb (*EheI/StuI*)
	II (nt1324-1575)	TR59, FAdV-8a reference strain (KT862810)	FP2-TR59: 5´-AGT GGT CGA GTG GAC ACA TAC CTG CCG GTT CTC AC-3´ RP2-TR59: 5´- GTG AGC TCG TCG ACG TAG GTT ATG ACA CGT CCG CA-3´	

[a] Primers with identical sequence.

Supplemental table S2. Summary of immune sera used in this study, according to their specificity.

Specificity based on cross-neutralization		Category of antigen used for immunization			Source[a]
		Live FAdV	Inactivated FAdV	Recombinant protein	
FAdV-A	FAdV-1	11-7127 (MK572848)			Grafl *et al.* (2014)
FAdV-B	FAdV-5		340 (KC493646) 15-4225		Feichtner *et al*. (2018) this study
FAdV-C	FAdV-4	KR5 (HE608152) AG234 (MK572849)		KR5 Fib-1 (HE608152) KR5 Fib-2 (HE608152)	Feichtner *et al*. (2018) Schachner *et al.* (2014) Schachner *et al.* (2014) Schachner *et al.* (2014)
	FAdV-10	C-2B (MK572851)			Feichtner *et al*. (2018)
FAdV-D	FAdV-2	685 (KT862805) 08-12809 (LN907547) 11-16628 (LN907545)			Feichtner *et al.* (2018) GZ 68.205/0217-WF/V/3b/2016 GZ 68.205/0217-WF/V/3b/2016
	FAdV-3	SR49 (KT862807)			Feichtner *et al*. (2018)
	FAdV-9	A-2A (AF083975)			Feichtner *et al.* (2018)
	FAdV-11	380 (KT862812) 13-18966 (LN907556)	13-14796		Feichtner *et al.* (2018) GZ 68.205/0217-WF/V/3b/2016 this study
FAdV-E	FAdV-6	CR119 (KT862808)	CR119 (KT862808)		Feichtner *et al*. (2018)
	FAdV-7	YR36 (KT862809)	YR36 (KT862809)		Feichtner *et al*. (2018)
	FAdV-8a	TR59 (KT862810) 11-16629 (MK572865)	TR59 (KT862810)		Feichtner *et al.* (2018)
	FAdV-8b	764 (KT862811) 13-18153 (MK572862)	764 (KT862811)		Feichtner *et al*. (2018)
hybrid fiber specificity	7/8a/6		13/19395 (MK572863)		Schachner *et al*. (2019)
	8a/8b			crecFib-8a/8b crecFib-8b/8a	this study this study

[a] Serum generated in the framework of published studies, or under the indicated animal trial license number.

8.3. Vaccination with a fowl adenovirus chimeric fiber protein (crecFib-4/11) simultaneously protects chickens against hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH)

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Vaccination with a fowl adenovirus chimeric fiber protein (crecFib-4/11) simultaneously protects chickens against hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH)

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ABSTRACT

In the past decades, fowl adenovirus (FAdV)-related diseases became an increasing concern for the poultry industry worldwide. Various immunization strategies against FAdVs have been experimentally investigated, with a particular focus on subunit vaccines against hepatitis-hydropericardium syndrome (HHS), caused by FAdV serotype 4, and inclusion body hepatitis (IBH), caused by serotypes 2, 8a, 8b and 11. In this study, we extended our innovative concept of recombinant chimeric fiber proteins to design a novel chimera combining epitopes from two distinct serotypes, FAdV-4 and -11, and we investigated its efficacy to simultaneously protect chickens against HHS and IBH. Specific pathogen-free chickens were vaccinated with the novel recombinant chimeric fiber and subsequently challenged with either a HHS- or IBH-causing strain. Vaccinated/challenged birds exhibited a reduction of clinical signs, limited hepatomegaly and lower levels of AST compared to the respective challenge controls. Furthermore, the vaccine prevented atrophy of HHS-affected lymphoid organs, such as thymus and bursa of Fabricius, and viral load in the target organs was significantly reduced. Clinical protection was associated with high levels of pre-challenge antibodies measured on ELISA plates coated with the vaccination antigen. Interestingly, the development of neutralizing antibodies was limited against FAdV-11 and absent against FAdV-4, indicating that protection granted by such an antigen may be linked to different immunization pathways. In conclusion, we proved that the concept of chimeric fiber vaccines can be extended across viral species boundaries and represents the first single-component FAdV subunit vaccine providing comprehensive protection against different FAdV-associated diseases.

1. Introduction

Fowl adenoviruses (FAdVs), from the family *Adenoviridae*, genus *Aviadenovirus*, are non-enveloped dsDNA viruses classified into five specie (*Fowl aviadenovirus A* to *Fowl aviadenovirus E* (FAdV-A to FAdV-E)) based on genomic features, and 12 subordinate serotypes (FAdV-1 to -8a, -8b to -11) defined by cross-neutralization [1,2]. Though all three fowl adenoviral disease complexes are caused by different FAdV species, hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH) share characteristic pathogenic and immune mechanisms, being overall very distinct from those of adenoviral gizzard erosion (AGE). One of the underlying reasons appears to be a closer molecular relationship between the causative types of HHS (FAdV-4, species FAdV-C) and IBH (FAdV-2/-11, FAdV-D; FAdV-8a and -8b, FAdV-E) as compared to the genetically separated FAdV-1 (species FAdV-A) responsible for AGE [3,4]. Despite worldwide distribution and severe economic impact of FAdV-associated diseases, commercially available immunization strategies rely on a few registered vaccines mainly to prevent HHS with the extension towards autogenous

Abbreviations: AGE, adenoviral gizzard erosion; AST, aspartate transaminase; BW, body weight; CEL, chicken embryo liver; dpb, days post booster; dpc, days post challenge; dpv, days post vaccination; ELISA, enzyme-linked immunosorbent assay; FAdV, fowl adenovirus; HHS, hepatitis-hydropericardium syndrome; IBH, inclusion body hepatitis; nAbs, neutralizing antibodies; non-nAbs, non-neutralizing antibodies; OD, optical density; ORF, open reading frame; PBS, phosphate buffered saline; qPCR, quantitative polymerase chain reaction; SPF, specific pathogen-free; $TCID_{50}$, 50% tissue culture infective dose; VNT, virus neutralization test.

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vaccines in recent years. In addition, this has been tackled by experimental development of alternative vaccination antigens, including certain live and inactivated, as well as subunit vaccines, with a particular focus on the latter given their relative ease and efficiency of production, as recently summarized by Schachner *et al.* [3]. In the majority of studies, structural proteins of the FAdV capsid were used for subunit vaccine formulation, based on their immunogenic potential and role in conferring antigenicity to the virus [5]. In particular, recombinant penton base and fiber, obtained from FAdV-4, proved successful for protecting chickens against the cognate disease, HHS [6,7,8,9].

Despite this, vaccines with an extended protection spectrum are still pending and highly required due to simultaneous occurrence and mixed infections with diverse FAdV strains in the field [10,11,12,13,14,15,16,17]. However, the variety of etiological FAdV types, concordant with genetically distinct fibers, somewhat antagonizes the idea of a broadly efficacious subunit vaccine.

Dependent on the diversification of fibers within the same species, cross-protection is even problematic for targeting a single disease complex, as confirmed for the fibers of types -8a and -8b, which are each efficient antigens against the homotypic IBH challenge while providing no vice-versa protection [18,19]. In addition, natural recombination of fibers, mainly between those two FAdV types, has been identified as another potential hurdle for vaccination strategies [20].

In order to address this issue and provide coverage against the entire spectrum of IBH strains within species FAdV-E, a recombinant chimeric fiber (crecFib) protein containing epitopes from both FAdV-8a and -8b was recently designed, and proved to be efficient in protecting chickens from both serotypes [21]. It is not known, however, if this principle can be extended and therefore applied to comprehensively protect chickens against different FAdV-caused diseases, especially given the phylogenetic distance between the amino acidic structures of fibers belonging to distinct FAdV species [20], as well as the dissimilarities in the immune mechanisms associated with FAdV subunit vaccines. In particular, protection from IBH seems to be linked to the production of neutralizing antibodies, whereas experimental studies point towards other mechanisms involved to protect birds against HHS [7,22]. Independent of this, the chimeric fiber concept potentially holds the key to unlock a comprehensive broad protection against the different FAdV diseases, although merging epitopes from different FAdV species has never been attempted so far. Therefore, this study represents the first description of a chimeric protein retaining the individual epitopic identities (-C/4 and -D/11), and its subsequent *in vivo* testing to protect chickens from both HHS and IBH simultaneously.

2. Materials and methods

2.1. Design and expression of chimeric fiber

A chimeric fiber protein retaining epitopes from FAdV-4 and FAdV-11 was designed in this study following the strategy and methods described by Schachner *et al.* [21]. Briefly, the open reading frames (ORFs) of fibers from FAdV-4 (fib-2) and FAdV-11 were divided into an amino (N)- and a carboxy (C)-distal segment, then amplified and fused seamlessly via Gibson assembly cloning. The specificity switch was engineered at an intertype consensus motif corresponding to positions aa491-492 in the pairwise sequence alignments of FAdV-4 fiber-2 and FAdV-11 fiber. The resulting construct was named crecFib-4/11. Detailed information on the cloning strategy is provided in Supplementary table 1. The recombinant chimeric fiber protein was expressed in *Spodoptera frugiperda* Sf9 cells after transfection with recombinant baculovirus DNA obtained from transformed *Escherichia coli* DH10Bac (Invitrogen, Vienna, Austria). The construct was then purified via polyhistidine tag on affinity chromatography columns (His GraviTrap, GE Healthcare, Freiburg, Germany) as described previously [7]. Protein concentration was determined via Bradford assay (Thermo Scientific, Vienna, Austria).

2.2. Virus preparation

Field isolates AG234 and 08–18926 (GenBank accession no. MK572849 and MK572871) were analyzed through Next-generation sequencing and virus neutralization test (VNT) and thus identified as members of types FAdV-4 and -11, respectively [20], to be used as challenge strains during the protection studies. The strains were 3-fold plaque purified and propagated on primary chicken-embryo liver (CEL) cells [23]; viral titers were determined by endpoint titration [24].

2.3. Animal experiment

One hundred and twenty specific pathogen-free (SPF) chicks were hatched, individually tagged and divided into six groups (n = 20) under the following designation: vaccinated-only (v/crecFib-4/11), vaccinated and challenged with FAdV-4 (v/c/FAdV-4), vaccinated and challenged with FAdV-11 (v/c/FAdV-11), FAdV-4 challenge control (c/FAdV-4), FAdV-11 challenge control (c/FAdV-11), and negative control (summarized in Table 1). Each group was housed separately in isolator units (HM2500, Montair, The Netherlands). For immunization, a prime-booster vaccination scheme with intramuscular administrations at 1 and 7 days of age was adopted, according to the model proposed by Schachner *et al.* [21]. A dose of 0.5 ml of the vaccine containing 50 μg of crecFib-4/11 homogenized in an oil-based adjuvant was injected intramuscularly in the *Musculus tibialis lateralis* of 1-day-old birds. Birds of the challenge control groups were only given phosphate buffered saline (PBS) mixed with adjuvant, and the negative control group was administered with sterile PBS only. The booster injection was administered in the same way at 7 days of age (6 days post vaccination, dpv) in the three vaccinated groups, and birds of the challenge and negative controls received an injection of adjuvant mixed with PBS and PBS only, respectively, as described above. Blood was collected in weekly intervals from 14 days of age up to challenge, then at each of the following sampling time points: 3, 5, 7 and 14 days post challenge (dpc). The birds were challenged intramuscularly at 22 days of age (15 days post booster, dpb) using 200 μl of 10^7 50% tissue culture infectious dose ($TCID_{50}$)/ml of FAdV-4 strain AG234 (groups v/c/FAdV-4 and c/FAdV-4) or FAdV-11 field strain 08–18926 (groups v/c/FAdV-11 and c/FAdV-11). In the time period after challenge, birds were monitored daily for clinical signs related to HHS and IBH, such as depression, ruffled feathers, huddling behavior, and reluctance to move and take in feed [3]. Up to five birds per group were sacri-

Table 1
Design of animal experiment

group	designation	vaccination	challenge strain
v/crecFib-4/11	vaccine-only	crecFib-4/11	–[a]
v/c/FAdV-4	vaccine vs. FAdV-4	crecFib-4/11	AG234 (FAdV-4)
v/c/FAdV-11	vaccine vs. FAdV-11	crecFib-4/11	08–18926 (FAdV-11)
c/FAdV-4	FAdV-4 challenge control	adjuvant only	AG234 (FAdV-4)
c/FAdV-11	FAdV-11 challenge control	adjuvant only	08–18926 (FAdV-11)
neg. contr.	negative control	–	–

[a] not applicable

ficed and submitted to necropsy at 3, 5, 7 and 14 dpc, and individuals that were euthanized due to severe clinical affection were necropsied and sampled immediately. During necropsy, the organ-body weight (BW) ratio of liver, spleen, thymus and bursa of Fabricius was recorded in order to determine the presence and degree of hepatomegaly, splenomegaly and atrophy of the lymphoid organs.

All the procedures were discussed and approved by the institutional ethics committee and licensed by the Austrian government (license number GZ 2020–0.220.316).

2.4. Clinical chemistry

Immediately before euthanasia, blood was collected from the jugular vein of each bird from the infected groups and negative control into heparin tubes (VACUETTE®, Greiner Bio-One, Kremsmünster, Austria) to investigate the aspartate transaminase (AST) content in plasma as previously described [25].

2.5. Quantitative polymerase chain reaction (qPCR) from tissues of target organs

Tissue samples for quantification of viral load, including liver, spleen and bursa of Fabricius, were collected from birds of the infected groups and negative control, and stored at −20 °C until processing. DNA extraction was performed with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturers protocol, and the DNA was subsequently analyzed with an adapted qPCR assay based on the 52 K gene [26].

2.6. Chimeric fiber enzyme-linked immunosorbent assay (ELISA) and virus neutralization test (VNT)

Sera collected during the experiments were tested on ELISA plates coated with the vaccination antigen crecFib-4/11, and their OD value was determined following the protocol described by Feichtner *et al.* [27]; the cut-off OD value was calculated from the sera of the negative control birds by computing the arithmetic mean plus three times the standard deviation. Samples from 21, 27 and 29-day-old birds (respectively: pre-challenge, 5 dpc and 7 dpc) were also investigated for neutralizing antibodies (nAbs) as earlier described [7], with VNT against the strains that served as a template for fiber expression (template strains) and the field isolates used as challenge strains during the protection studies (challenge strains).

2.7. Statistical analyses

Significance between the clinical signs observed within the groups challenged with FAdV-4 (v/c/FAdV-4 vs. c/FAdV-4) and the ones challenged with FAdV-11 (v/c/FAdV-11 vs. c/FAdV-11) was assessed comparing the numbers of symptomatic birds per group through chi-square test. For the numeric datasets, a preliminary analysis was carried out using Shapiro-Wilk test together with a visual inspection of histograms and normal Q-Q plots in order to verify the normal distribution assumption. The mean values for organ-BW ratios, plasma AST and viral load in target organs of vaccinated groups were compared with the negative control and their respective challenge control group via unpaired Student's *t*-test. Mann-Whitney *U* test was used for the datasets that did not meet normality assumptions. In each case, p values ≤ 0.05 were considered statistically significant. Statistical analyses were performed with the software package SPSS Version 26 (IBM SPSS Statistics; IBM Corp., Armonk, New York, USA).

3. Results

3.1. Clinical protection

Out of twenty birds, sixteen individuals belonging to the FAdV-4 challenge control showed clinical affection after challenge (Table 2). In this group, clinical signs started at 2 dpc with mild depression in two birds, and progressed across the group with 13 affected birds at 3 dpc, three of which showing severe depression; at 4 dpc there were nine affected birds, and two of them had to be euthanized due to their inability to move and take feed; at 5 dpc, three birds were still showing milder clinical signs. In the FAdV-11 challenge control, only one bird was affected with mild depression at 4 dpc. Among the vaccinated groups, only one individual challenged with FAdV-4 showed signs of depression (3 dpc), marking a strongly significant difference compared to the respective challenge control ($p < 0.001$). No clinical signs were observed in the groups v/crecFib-4/11, v/c/FAdV-11, and negative control throughout the whole experiment. The recorded parameters for the birds euthanized at 4 dpc were included in the 5 dpc cluster for statistical analyses.

Mean liver-BW ratio was significantly increased from 3 to 7 dpc in c/FAdV-4 compared to the negative control, and from 3 to 5 dpc for the v/c/FAdV-4 group, whereas the spleen-BW ratio remained affected up to 7 dpc in both groups (Fig. 1i, 1ii). Group c/FAdV-4 also showed a significant decrease in the organ-BW ratio of bursa of Fabricius, compared to the negative control, at 3 and 5 dpc, and of the thymus-BW ratio at 5 and 7 dpc, both differently from the respective vaccinated group (Fig. 1iii, 1iv). Group c/FAdV-11 showed a significant increase of the liver-BW ratio at 3 and 7 dpc, and of the spleen-BW ratio from 3 to 7 dpc, whereas group v/c/FAdV-11 only showed significant affection of the spleen-BW ratio at 5 dpc.

3.2. Plasma AST

Plasma AST was significantly increased compared to the negative control from 3 to 7 dpc in c/FAdV-4, and from 3 to 5 dpc in v/c/FAdV-4, although the values of the vaccinated birds were significantly lower compared to c/FAdV-4 at these time points (Fig. 2). At 7 dpc, AST values from c/FAdV-11 birds were significantly increased compared to those from the negative control, whereas values from v/c/FAdV-11 were significantly increased at 14 dpc.

3.3. Viral load in target organs

The mean viral load of v/c/FAdV-4 birds was significantly lower than in c/FAdV-4 in liver at 5 dpc, in spleen at 3 dpc, and in bursa of Fabricius at both time points (Fig. 3). No significant differences were recorded in the analyzed organs between v/c/FAdV-11 and c/FAdV-11 at any time point. At 14 dpc, it was possible to detect viral DNA exclusively in the spleen of birds from FAdV-4-infected groups.

3.4. Antibody response: ELISA and VNT

The cut-off value for crecFib-4/11 ELISA was calculated at OD 0.12. At 14-day-old (7 dpb), the mean OD of all vaccinated birds (n = 60: from v/crecFib-4/11, v/c/FAdV-4, v/c/FAdV-11) was OD 0.48 ± 0.48, with 81.7% (49/60) of the birds above the cut-off (Fig. 4). The day before challenge (21-day-old, 14 dpb), the mean OD value of vaccinated birds raised up to 2.43 ± 0.87 with all the birds being above the cut-off. Adjuvant-injected birds (n = 40: from c/FAdV-4 and c/FAdV-11) had mean OD values of 0.07 ± 0.03 and

Table 2
Daily clinical signs for each experimental group following challenge. The number of affected individuals is indicated in brackets. No clinical signs were recorded before 2 dpc and after 5 dpc.

group	clinical signs				symptomatic/ total birds
	2 dpc	3 dpc	4 dpc	5 dpc	
v/crecFib-4/11	–[a]	–	–	–	0/20
v/c/FAdV-4	–	depression (n = 1)	–	–	1/20[c]
v/c/FAdV-11	–	–	–	–	0/20
c/FAdV-4	mild depression (n = 2)	mild depression (n = 9) depression (n = 1) severe depression (n = 3)	mild depression (n = 5) depression (n = 2) severe depression, bird unable to stand or take feed (n = 2)[b]	mild depression (n = 2) depression (n = 1)	16/20
c/FAdV-11	–	–	mild depression (n = 1)	–	1/20
neg. contr.	–	–	–	–	0/20

[a] no clinical signs observed
[b] birds were euthanized
[c] significant difference ($p \leq 0.001$) compared to the respective challenge control

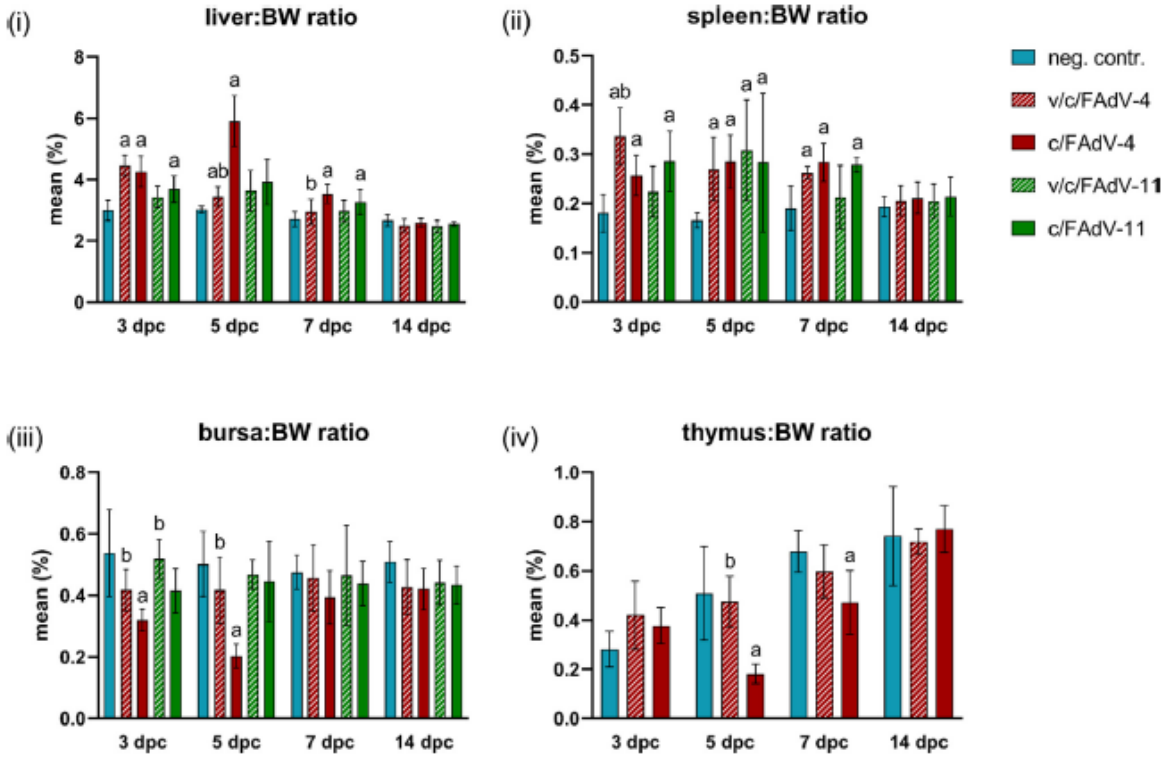

Fig. 1. Organ-BW ratios post-challenge. Mean and standard deviation of liver-BW ratio (i), spleen-BW ratio (ii), bursa-BW ratio (iii) and thymus-BW ratio (iv) for each experimental group in the time period after challenge. Significant difference against negative control is indicated with letter "a", whereas significant difference against respective challenge control is indicated with letter "b". Significance was assessed at $p \leq 0.05$. No significant difference was observed for thymus-BW ratio between v/c/FAdV-11 and c/FAdV-11 at any measured time point.

0.05 ± 0.01 at 14- and 21-day-old respectively. Mean OD of the v/crecFib-4/11 group suffered a decrease at 25-day-old (1.70 ± 1.09) before continuously increasing throughout the experiment, reaching an OD of 3.46 ± 0.01 at 36-day-old (29 dpb). After challenge, mean OD of v/c/FAdV-4 group raised to 3.09 ± 0.42 at 3 dpc and reached the highest end of measurable values at 5 dpc (3.32 ± 0.28), remaining then consistent in its plateau until the end of the experiment. In comparison, a sharp rise in ELISA-measured antibodies was observed in c/FAdV-4 birds from 5 to 14 dpc (mean OD 0.25 ± 0.15 to 2.57 ± 0.44), although they remained lower than the vaccinated group. Birds from v/c/FAdV-11 quickly reached the plateau after challenge as well (mean OD 3.24 ± 0.13 at 3 dpc to 3.39 ± 0.13 at 14 dpc), whereas c/FAdV-11 birds showed low levels of antibody development (peak at 14 dpc with mean OD 0.28 ± 0.13). Sera of negative control birds remained below the cut-off value throughout the whole experiment.

The day prior challenge, only two vaccinated birds displayed some degree of nAbs against the FAdV-11 template strain, one of them reacting against both template and challenge strain (titers: 3 and 8 $\log_2$ respectively), one only against the template strain (titers: 5 $\log_2$), and two against the challenge strain (titers: 3 and 9 $\log_2$) (Fig. 5). In contrast, there was no neutralizing activity against any of the FAdV-4 strains, and the vaccine-only group saw no nAbs development at any of the tested time points. The rise

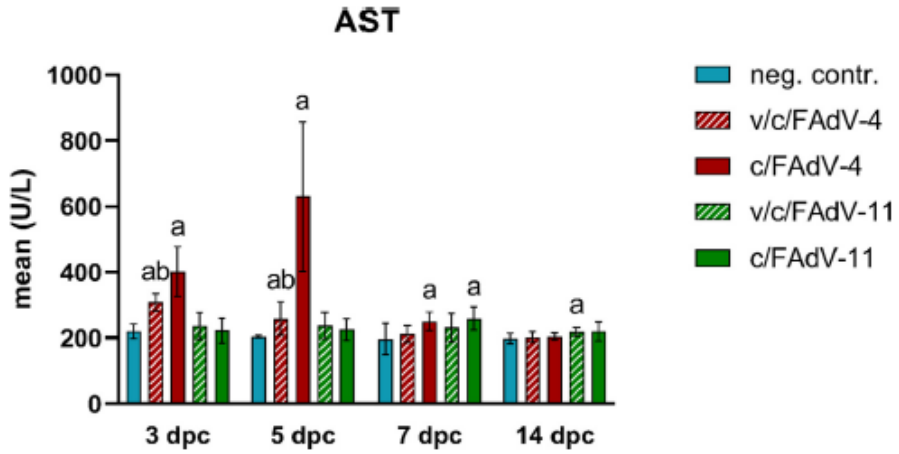

Fig. 2. AST mean values post-challenge. Mean and standard deviation of plasma AST for each experimental group in the time period after challenge. Significant difference against negative control is indicated with letter "a", whereas significant difference against respective challenge control is indicated with letter "b". Significance was assessed at $p \leq 0.05$.

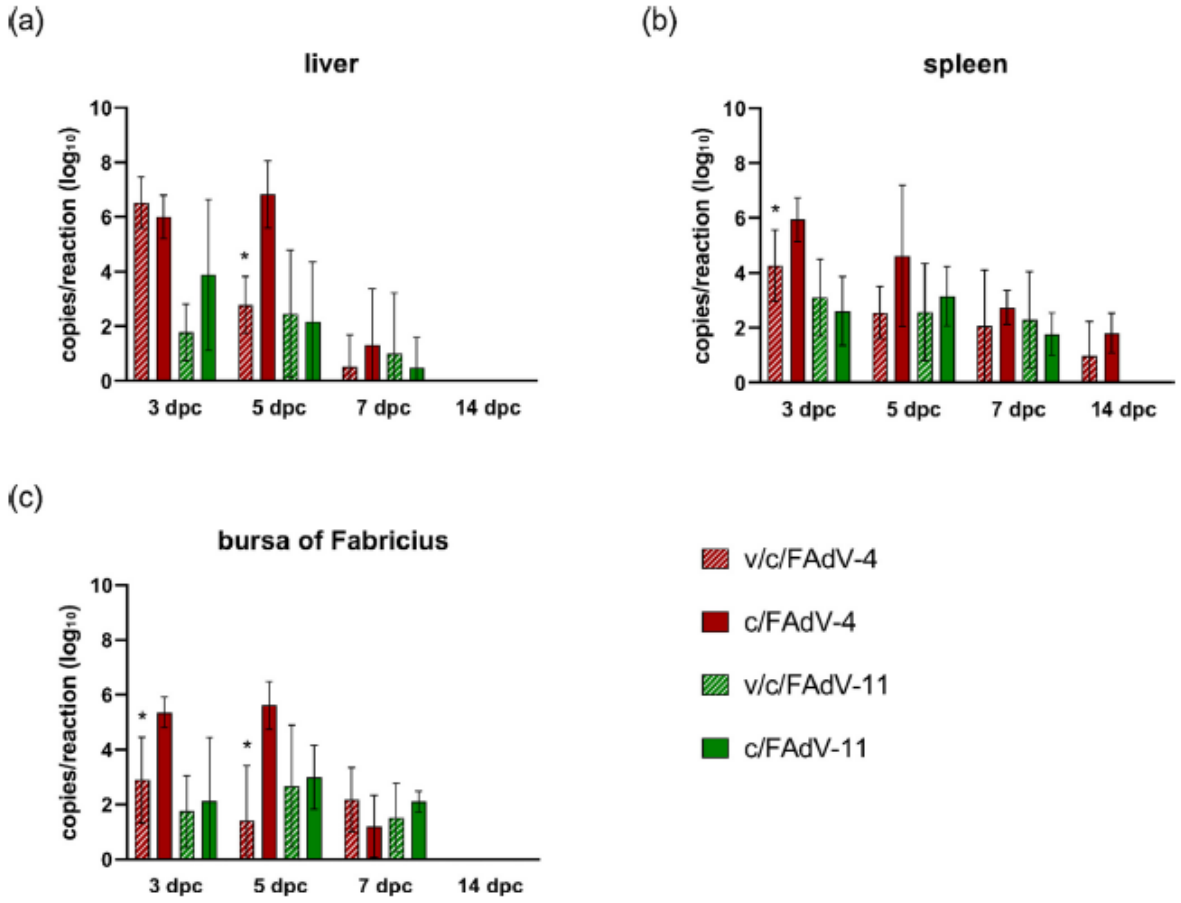

Fig. 3. Viral load in main target organs post-challenge. Mean and standard deviation of viral load (expressed in $\log_{10}$) for each experimental group in the time period after challenge in liver (a), spleen (b) and bursa of Fabricius (c). Asterisks indicate significant difference compared to the respective challenge control. Significance was assessed at $p \leq 0.05$.

in nAbs against FAdV-4 started after challenge in v/c/FAdV-4 and c/FAdV-4, showing similar values against both the challenge strain (mean titer: 5.07 ± 2.09 $\log_2$ vs. 4.57 ± 1.83 $\log_2$ at 5 dpc, and 7.56 ± 1.51 $\log_2$ vs. 6.90 ± 0.99 $\log_2$ at 7 dpc) and the template strain (2.53 ± 2.56 $\log_2$ vs. 3.64 ± 2.37 $\log_2$ at 5 dpc, and 6.67 ± 1.87 $\log_2$ vs. 6.10 ± 0.99 $\log_2$ at 7 dpc). Experimental groups infected with FAdV-11 showed a similar pattern against the challenge strain, although titers in sera from v/c/FAdV-11 birds were slightly lower than in c/FAdV-11 (mean: 3.79 ± 2.78 $\log_2$ vs. 5.36 ± 2.68 $\log_2$ at 5 dpc, and 5.90 ± 1.10 $\log_2$ vs. 7.00 ± 1.63 $\log_2$

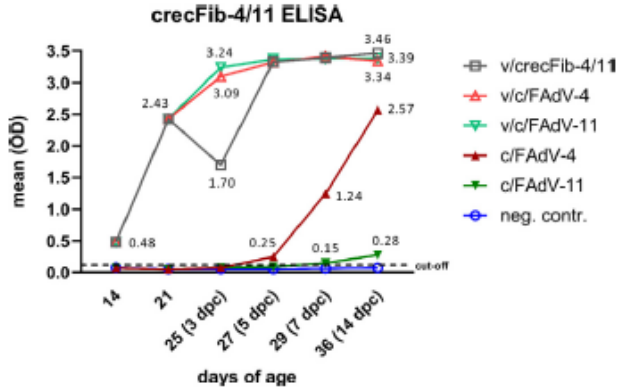

Fig. 4. Antibody development post vaccination. Mean titers (OD) for experimental groups tested on crecFib-4/11 ELISA throughout the protection study. Values given for the time points prior challenge (14 and 21 days of age) are cumulative for the three vaccinated groups (v/crecFib-4/11, v/c/FAdV-4, v/c/FAdV-11) and the two challenge control groups (c/FAdV-4, c/FAdV-11). The cut-off value is marked in black.

at 7 dpc), and mean titers were generally lower against the template strain (0.29 ± 1.07 log_2 vs. 0.50 ± 1.29 log_2 at 5 dpc, and 2.80 ± 2.10 log_2 vs. 2.30 ± 2.11 log_2 at 7 dpc). Interestingly, a small number of sera from v/c/FAdV-4 birds developed nAbs against FAdV-11 after challenge, with three individuals displaying nAbs against both strains and one only against the template strain between 5 and 7 dpc. Data on the number of individuals exhibiting nAbs for each group are shown in Fig. 5.

4. Discussion

FAdV diseases, with discrete clinical pictures caused by particular serotypes, are an economic burden for the poultry industry worldwide, with an important increase of outbreaks observed in recent years asking for new protection strategies [28]. As a general paradigm, the immunity developed against a specific FAdV type does not confer protection against other types. This conclusion is supported by observed shifts towards outbreaks with other viral types after implementation of a vaccination regimen against the previously dominating ones [29,30,31,32]. An increasing awareness of antigenically diverse FAdV types co-circulating in the field, and the demand for their control, explains why much research has been dedicated lately to elucidating cross-protectivity of candidate antigens, with less promising results so far in regards to recombinant proteins. On the contrary, heterotypic efficacy was demonstrated within the FAdV-E species with live vaccines [33]. Furthermore, inactivated FAdV-C and -E strains were able to induce broad serotype coverage, extending the protection spectrum outside species boundaries as well [34,35]. Obviously, whole virus formulations are more likely to produce a synergistic effect from different antigenic components that cannot be replicated in the same way by subunit vaccines.

Subunit vaccines based on the fiber protein from FAdV types causing hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH) were repeatedly shown to ensure robust protection against the respective disease conditions, although only in settings limited to homologous vaccination/challenge systems [7,8,19,36,37]. In fact, the FAdV fiber is a surface antigen that features high levels of diversification between FAdV species and sero-

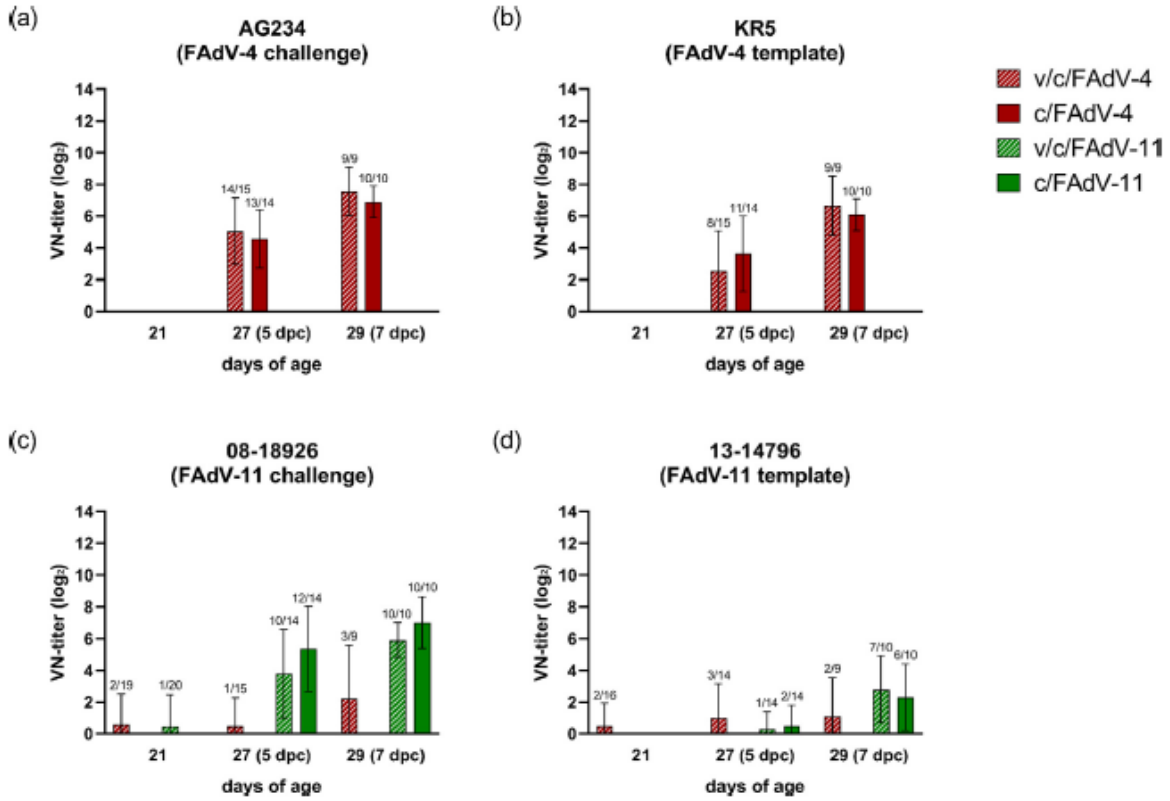

Fig. 5. Neutralizing antibodies development post vaccination. Mean and standard deviation of VNT titers (log_2) before challenge (21 days of age) and at 5–7 dpc: v/c/FAdV-4, v/c/FAdV-11 and c/FAdV-4 against the FAdV-4 challenge (a) and template (b) strain; v/c/FAdV-4, v/c/FAdV-11 and c/FAdV-11 against the FAdV-11 challenge (c) and template (d) strain. Headers show the number of birds exhibiting nAbs (some individuals could not be tested due to a lack of serum volume). No nAbs were observed in sera from v/crecFib-4/11 birds at any measured time point.

types, and type-specificity of fiber-induced neutralization was recently shown to be linked with failure to cross-protect [16,19,20]. Whereas this is not an issue for HHS and its monotype etiology, the diverse etiological nature of IBH requires the pursue of a more broad-protective immunization strategy. Recently, cross-protection between different IBH-causing serotypes was demonstrated with the introduction of a novel chimeric fiber protein merging putative epitopes from both FAdV-8a and -8b fibers [21]. This represents a promising concept towards broad-protection from FAdV-related diseases, but it has to be clarified whether this strategy can be applied to simultaneously protect chickens against IBH and HHS, despite their differences in etiology, pathogenesis and related immune mechanisms. In order to tackle this issue, a novel chimeric fiber retaining molecular characteristics of both FAdV-4 and -11 (crecFib-4/11) was designed for this study. This represents the first instance where a recombinant chimeric protein presenting combined epitopes from two different FAdV species was successfully expressed, despite the important structural differences between fibers of FAdV-C and -D. The new construct was then tested *in vivo* for its efficacy to immunize chickens against different FAdV-associated diseases, such as HHS and IBH.

Vaccination of birds with crecFib-4/11 was able to prevent clinical signs and limit pathological affection upon FAdV-4 and -11 infection, in contrast with the challenge control groups. The reduced clinical signs noticed in the FAdV-4 challenge control mark a substantial difference compared to a previous study by Schachner *et al.* [7], which utilized the same challenge strain analogously to the present work. This may be due to underlying differences in the batches of SPF chickens between the two experiments, keeping in mind that IBH and HHS are strongly affecting the metabolism of the birds [38]. Various FAdV protection studies faced a scarcity of severe clinical signs upon challenge, especially given the wide variability in pathogenicity among different strains, even within the same serotype [19,21,34,35,39]. In general, it must be considered that repeated passaging on embryonic eggs or cell culture, necessary for plaque purification and viral propagation, can result in virus attenuation, which could explain discrepancies between field observations and experimental trials [40,41]. In the present study, no mortality was noticed in the FAdV-11 challenge control, which is in agreement with a previous study using the same strain [42]. In fact, results from other vaccination studies indicate that susceptibility to FAdV-11 varies according to the age and type of the birds, as well as route and dose of infection, similarly to other FAdVs, as mentioned above [18,35,43]. For these reasons, a panel of parameters was taken into account to assess protection in the present study, especially in order to counter the mild effects of the FAdV-11 challenge: organ-body weight ratios, viral load in target organs, and plasma AST, with the latter confirmed as a valuable indicator of hepatic health in the course of IBH infection [25].

Clinical protection provided by crecFib-4/11 against both HHS and IBH was linked to the induction of high and uniform levels of systemic antibodies against the antigen used for vaccination, measured with in-house fiber ELISA. However, a consistent lack of neutralizing antibodies (nAbs) was observed in the vaccinated groups before challenge, as a very small number of birds exhibited neutralization against FAdV-11, and none against FAdV-4. In contrast, challenge control birds consistently showed neutralizing activity against the respective challenge virus, although antibodies from the FAdV-11 challenge control showed a low level of reaction with the crecFib-4/11 antigen on ELISA. It is therefore concluded that antibodies produced in response to the FAdV-11 virus recognize the chimeric fiber to a lesser degree compared to antibodies directed against the FAdV-4 challenge. This could be due to the conformation of the vaccine antigen, such as the folding of the recombinant protein, and/or the predominance of epitopes with FAdV-4 specific identity on crecFib-4/11.

Clinical protection in absence of vaccine-elicited nAbs has been described before within the HHS system: FAdV-4 fiber vaccines have been shown to provide full coverage against HHS despite lack of neutralizing activity [7], a feature that was observed even upon vaccination with live-attenuated FAdV-4 [22]. On the other hand, immunization against IBH has been linked to stimulation of the humoral immune response with subsequent production of nAbs for whole virus vaccines, either live or inactivated, and subunit antigens as well [18,19,21,36,43]. However, it must be considered that vaccine-induced nAbs against FAdV-11 were only noticed after bivalent vaccination with live or inactivated vaccines, whereas data for recombinant subunits only refer to the FAdV-8a/8b system [18,43]. In another study, a vaccine based on inactivated FAdV-8a elicited detectable ELISA antibodies and was found protective against FAdV-11 challenge despite the lack of cross-reacting nAbs in most of the experimental birds [35]. These data, together with the results of the present work, pose questions over the role of non-neutralizing antibodies (non-nAbs), which are already considered crucial for immunization against certain viruses affecting chickens, such as specific subtypes of Avian Influenza [44]. Since non-nAbs are known to contribute in conferring coverage against viral diseases [45,46], our results, together with the reported lack of nAbs after immunization against HHS, suggest that they may play an important role against FAdV-associated diseases as well, depending on the serotype. Moreover, recent efforts have highlighted the importance of vaccine-dependent cellular immunity when it comes to subunit antigens against both HHS and IBH, marking it as another possible major player in the protection from FAdV-related diseases [8,19,36]. Therefore, the present work highlights the need of further investigations to dissect the mechanisms of humoral and cellular immune responses associated with FAdV vaccines, not only systemically, but also on a local level in regards to target and lymphoid organs.

In conclusion, subunit vaccination with crecFib-4/11 protected chickens from clinical signs and severe outcome of HHS, while at the same time limiting pathological affection from both HHS and IBH. Therefore, chimeric fibers are confirmed as an efficient protection strategy to provide broad coverage against FAdVs, not only on heterotypic level, as previously demonstrated, but also across the species boundary. On this basis, now that the merging of such diverse molecular structure into a unique chimeric fiber was successfully achieved, the possibility of designing new chimeras including even more diverse epitopes and further extending the spectrum of protection has been unlocked. Although more investigations are needed to elucidate the immune mechanisms behind said protection, these findings represent a fundamental step towards reaching comprehensive cross-protection against the FAdV-mediated diseases affecting the poultry industry worldwide.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michael Hess and Anna Schachner have patent "Fowl adenovirus subunit vaccine and production method thereof" pending to European Patent Office.

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Ethics approval

All the procedures on experimental animals were discussed and approved by the institutional ethics committee and licensed by the Austrian government (license number GZ 2020-0.220.316).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.01.060.

References

[1] Harrach B, Benkö M, Both GW, Brown M, Davison AJ, Echavarría M, et al. Family *Adenoviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Amsterdam, The Netherlands: Elsevier Academic Press; 2012. p. 125–41.
[2] Hess M. Detection and differentiation of avian adenoviruses: a review. Avian Pathol 2000;29(3):195–206.
[3] Schachner A, Matos M, Grafl B, Hess M. Fowl adenovirus-induced diseases and strategies for their control – a review on the current global situation. Avian Pathol 2018;47(2):111–26.
[4] Hess M. Aviadenovirus infections. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. Diseases of Poultry. Hoboken, NJ: Wiley-Blackwell; 2020. p. 322–31.
[5] Norrby E. The relationship between the soluble antigens and the virion of adenovirus type 3. IV. Immunological complexity of soluble components. Virology 1969;37(4):565–76.
[6] Shah MS, Ashraf A, Rahman M, Khan MI, Qureshi JA. A subunit vaccine against hydropericardium syndrome using adenovirus penton capsid protein. Vaccine 2012;30(50):7153–6.
[7] Schachner A, Marek A, Jaskulska B, Bilic I, Hess M. Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis-hydropericardium syndrome (HHS). Vaccine 2014;32(9):1086–92.
[8] Chen Li, Yin L, Zhou Q, Li Q, Luo Y, Xu Z, et al. Immunogenicity and protective efficacy of recombinant fiber-2 protein in protecting SPF chickens against fowl adenovirus 4. Vaccine 2018;36(9):1203–8.
[9] Wang X, Tang Q, Qiu Li, Yang Z. Penton-dodecahedron of fowl adenovirus serotype 4 as a vaccine candidate for the control of related diseases. Vaccine 2019;37(6):839–47.
[10] Meulemans G, Boschmans M, van den Berg TP, Decaesstecker M. Polymerase chain reaction combined with restriction enzyme analysis for detection and differentiation of fowl adenoviruses. Avian Pathol 2001;30(6):655–60.
[11] Singh A, Oberoi MS, Grewal GS, Hafez HM, Hess M. The use of PCR combined with restriction enzyme analysis to characterize fowl adenovirus field isolates from northern India. Vet Res Commun 2002;26:577–85.
[12] Rahul S, Kataria JM, Senthilkumar N, Dhama K, Sylvester SA, Uma R. Association of fowl adenovirus serotype 12 with hydropericardium syndrome of poultry in India. Acta Virol 2005;49:139–43.
[13] Gomis S, Goodhope R, Ojkic D, Willson P. Inclusion body hepatitis as a primary disease in broilers in Saskatchewan, Canada. Avian Dis 2006;50(4):550–5.
[14] Kaján GL, Kecskeméti S, Harrach B, Benkő M. Molecular typing of fowl adenoviruses, isolated in Hungary recently, reveals high diversity. Vet Microbiol 2013;167(3-4):357–63.
[15] Mittal D, Jindal N, Tiwari AK, Khokhar RS. Characterization of fowl adenoviruses associated with hydropericardium syndrome and inclusion body hepatitis in broiler chickens. Virusdisease 2014;25(1):114–9.
[16] Schachner A, Marek A, Grafl B, Hess M. Detailed molecular analyses of the hexon loop-1 and fibers of fowl aviadenoviruses reveal new insights into the antigenic relationship and confirm that specific genotypes are involved in field outbreaks of inclusion body hepatitis. Vet Microbiol 2016;186:13–20.
[17] Niu Y, Sun Q, Zhang G, Sun W, Liu X, Xiao Y, et al. Epidemiological investigation of outbreaks of fowl adenovirus infections in commercial chickens in China. Transbound Emerg Dis 2018;65(1):e121–6.
[18] Gupta A, Popowich S, Ojkic D, Kurukulasuriya S, Chow-Lockerbie B, Gunawardana T, et al. Inactivated and live bivalent fowl adenovirus (FAdV8b + FAdV11) breeder vaccines provide broad-spectrum protection in chicks against inclusion body hepatitis (IBH). Vaccine 2018;36:744–50.
[19] De Luca C, Schachner A, Mitra T, Heidl S, Liebhart D, Hess M. Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH) provides type-specific protection guided by humoral immunity and regulation of B and T cell response. Vet Res 2020;51:143.
[20] Schachner A, Gonzalez G, Endler L, Ito K, Hess M. Fowl adenovirus (FAdV) recombination with intertypic crossovers in genomes of FAdV-D and FAdV-E, displaying hybrid serological phenotypes. Viruses 2019;11(12):1094. https://doi.org/10.3390/v11121094.
[21] Schachner A, De Luca C, Heidl S, Hess M. Recombinantly expressed chimeric fibers demonstrate discrete type-specific neutralizing epitopes in the *Fowl aviadenovirus E* (FAdV-E) fiber, promoting the optimization of FAdV fiber subunit vaccines towards cross-protection *in vivo*. Microbiol Spectr 2022;10:e02123–e2221.
[22] Schonewille E, Jaspers R, Paul G, Hess M. Specific-pathogen-free chickens vaccinated with a live FAdV-4 vaccine are fully protected against a severe challenge even in the absence of neutralizing antibodies. Avian Dis 2010;54(2):905–10.
[23] Schat K, Sellers HS. Cell culture methods. In: Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR, editors. A laboratory manual for the isolation and identification of avian pathogens. Madison, WI, USA: OmniPress Inc; 2008. p. 195–203.
[24] Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493–7.
[25] Matos M, Grafl B, Liebhart D, Schwendenwein I, Hess M. Selected clinical chemistry analytes correlate with the pathogenesis of inclusion body hepatitis experimentally induced by fowl aviadenoviruses. Avian Pathol 2016;45(5):520–9.
[26] Günes A, Marek A, Grafl B, Berger E, Hess M. Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). J Virol Methods 2012;183(2):147–53.
[27] Feichtner F, Schachner A, Berger E, Hess M. Development of sensitive indirect enzyme-linked immunosorbent assays for specific detection of antibodies against fowl adenovirus serotypes 1 and 4 in chickens. Avian Pathol 2018;47(1):73–82.
[28] Schachner A, Grafl B, Hess M. Spotlight on avian pathology: fowl adenovirus (FAdV) in chickens and beyond – an unresolved host-pathogen interplay. Avian Pathol 2021;50(1):2–5.
[29] Steer PA, ÓRourke D, Ghorashi SA, Noormohammadi AH. Application of high-resolution melting curve analysis for typing of fowl adenovirus in field cases of inclusion body hepatitis. Aust Vet J 2011;89:184–92.
[30] Venne D. Field data of the changing clinical picture over time of inclusion body hepatitis in Canada with an emphasis on diagnosis, prevention and trials on supportive treatments. In: Proceedings of the 24th Annual Australian Poultry Science Symposium, Sydney, New South Wales, Australia, February 2013. The Poultry Research Foundation and the Worlds Poultry Science Association, 2013, p 222-9.
[31] Wang J, Wang S, Zou K, Zhang Yi, Xu S, Yin Y. Variant serotypes of fowl adenovirus isolated from commercial poultry between 2007 and 2017 in some regions of China. Avian Dis 2018;62(2):171–6.
[32] Mo J. Historical investigation of fowl adenovirus outbreaks in South Korea from 2007 to 2021: a comprehensive review. Viruses 2021;13(11):2256. https://doi.org/10.3390/v13112256.
[33] Pallister JA, Erny KM, Fahey KJ. Serological relationships within the group E fowl adenoviruses. Intervirology 1993;36(2):84–90.
[34] Kim M-S, Lim T-H, Lee D-H, Youn H-N, Yuk S-S, Kim B-Y, et al. An inactivated oil-emulsion fowl adenovirus serotype 4 vaccine provides broad cross-protection against various serotypes of fowl adenovirus. Vaccine 2014;32(28):3564–8.
[35] Steer-Cope PA, Sandy JR, O'Rourke D, Scott PC, Browning GF, Noormohammadi AH. Vaccination with FAdV-8a induces protection against inclusion body hepatitis caused by homologous and heterologous strains. Avian Pathol 2019;48(5):396–405.
[36] Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya S, Goonewardene K, et al. Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. Vaccine 2017;35(20):2716–22.
[37] Wang X, Tang Q, Chu Z, Wang P, Luo C, Zhang Y, et al. Immune protection efficacy of FAdV-4 surface proteins fiber-1, fiber-2, hexon and penton base. Virus Res 2018;245:1–6.
[38] Matos M, Grafl B, Liebhart D, Hess M. The outcome of experimentally induced inclusion body hepatitis (IBH) by fowl aviadenoviruses (FAdVs) is crucially influenced by the genetic background of the host. Vet Res 2016;47:69.
[39] Grafl B, Berger E, Wernsdorf P, Hess M. Successful reproduction of adenoviral gizzard erosion in 20-week-old SPF layer-type chickens and efficacious prophylaxis due to live vaccination with apathogenic fowl adenovirus serotype 1 strain (CELO). Vaccine 2020;38:143–9.
[40] Schonewille E, Singh A, Göbel TW, Gerner W, Saalmüller A, Hess M. Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid

organs in specific pathogen-free chickens following experimental infection. Vet Immunol Immunopathol 2008;121(1-2):130–9.
[41] Sohaimi NM, Bejo MH, Omar AR, Ideris A, Isa NM. Hexon and fiber gene changes in an attenuated fowl adenovirus isolate from Malaysia in embryonated chicken eggs and its infectivity in chickens. J Vet Sci 2018;19(6):759. https://doi.org/10.4142/jvs.2018.19.6.759.
[42] Matos M, Dublecz K, Grafl B, Liebhart D, Hess M. Pancreatitis is an important feature of broilers suffering from inclusion body hepatitis leading to dysmetabolic conditions with consequences for zootechnical performance. Avian Dis 2018;62(1):57. https://doi.org/10.1637/11755-092717-Reg.1\10.1637/11755-092717-Reg.1.s1.
[43] Popowich S, Gupta A, Chow-Lockerbie B, Ayalew L, Ambrose N, Ojkic D, et al. Broad spectrum protection of broiler chickens against inclusion body hepatitis by immunizing their broiler breeder parents with a bivalent live fowl adenovirus vaccine. Res Vet Sci 2018;118:262–9.
[44] Hu Z, Zhao J, Zhao Y, Fan X, Hu J, Shi L, et al. Hemagglutinin-specific non-neutralizing antibody is essential for protection provided by inactivated and viral-vectored H7N9 Avian Influenza vaccines in chickens. Front Vet Sci 2020;6. https://doi.org/10.3389/fvets.2019.00482.
[45] Henry Dunand C, Leon P, Huang M, Choi A, Chromikova V, Ho I, et al. Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. Cell Host Microbe 2016;19(6):800–13.
[46] Stadlbauer D, Rajabhathor A, Amanat F, Kaplan D, Masud A, Treanor JJ, et al. Vaccination with a recombinant H7 hemagglutinin-based influenza virus vaccine induces broadly reactive antibodies in humans. MSphere 2017;2(6). https://doi.org/10.1128/mSphere.00502-17.

Supplementary Table 1. Design of the recombinant chimera crecFib-4/11. Overhangs at the 5´-termini of primer sequences with the flanking vector sequence (FP1 and RP2 primers) or with the counterpart template sequence (RP1 and FP2 primers) are represented by underlined nucleotides.

Designation of chimeric construct	Fragment (position in template sequence)	Template strain (GenBank accession number)	Primer sequences	Expression vector (restriction sites used for cloning)
crecFib-4/11	I (nt1-1221[a])	KR5, FAdV-4 reference strain (HE608152)	FP1-KR5: 5´- AAA CCT GTA TTT TCA GGG CAT GCT CCG AGC CCC TA-3´ RP1-KR5: 5´- AAG TTT GCA CGG ATC CGC TGG ATG GTT GAT AGT AT-3´	pFAST BAC HTb (*EheI/StuI*)
	II (nt1468-1713)	13/14796, FAdV-11 field strain (n.a.)	FP2-13/14796: 5´-TCC AGC GGG TCC GTG CAA ACT TTC CAA CCC GTA TT-3´ RP2-13/14796: 5´-GTG AGC TCG TCG ACG TAG GTT AGG GTT GTG TTA AT	

[a] position refers to fiber-2 gene

8.4. Local cellular immune response plays a key role in protecting chickens against hepatitis-hydropericardium syndrome (HHS) by vaccination with a recombinant fowl adenovirus (FAdV) chimeric fiber protein

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Local cellular immune response plays a key role in protecting chickens against hepatitis-hydropericardium syndrome (HHS) by vaccination with a recombinant fowl adenovirus (FAdV) chimeric fiber protein

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Fowl adenovirus (FAdV)-induced diseases hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH) have been affecting the poultry industry with increasing severity in the last two decades. Recently, a subunit vaccine based on a chimeric fiber protein with epitopes from different fowl adenovirus serotypes (named crecFib-4/11) has been shown to confer simultaneous protection against both HHS and IBH. However, the underlying immune mechanisms in chickens are still enigmatic, especially because of frequently absent neutralizing response despite high levels of protection. In this study, we investigated the kinetics of the humoral and cellular immune responses in specific pathogen-free chickens after vaccination with crecFib-4/11 and/or challenge with a HHS-causing strain, on a systemic level, as well as locally in target and lymphoid organs. The humoral response was assessed via enzyme-linked immunosorbent assay (ELISA) and virus neutralization test in serum, while the cellular immune response was determined by phenotyping using flow cytometry. Although vaccination induced serum antibodies, as confirmed by ELISA, such antibodies exhibited no pre-challenge neutralizing activity against FAdV-4. Nevertheless, immunized birds experienced a significant B cell increase in the liver upon challenge, remaining high throughout the experiment. Furthermore, vaccination stimulated the proliferation of cytotoxic T lymphocytes, with earlier circulation in the blood compared to the challenge control and subsequent increase in liver and spleen. Overall, these findings imply that protection of chickens from HHS after

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crecFib-4/11 vaccination relies on a prominent local immune response in the target organs, instead of circulating neutralizing antibodies.

KEYWORDS

fowl adenovirus, FAdV-4, chimeric fiber vaccine, humoral immunity, cellular immunity, hepatitis-hydropericardium syndrome

1 Introduction

Fowl adenoviruses (FAdVs) are non-enveloped dsDNA viruses belonging to the family *Adenoviridae*, genus *Aviadenovirus* (1). They are classified into five species (*Fowl aviadenovirus A* to *Fowl aviadenovirus E*, FAdV-A to FAdV-E) based on genomic features, and 12 serotypes (FAdV-1 to -8a, -8b to -11) defined by cross-neutralization (2). Different FAdV species are responsible for three disease complexes affecting chickens: adenoviral gizzard erosion (AGE), caused by FAdV-A (serotype 1), hepatitis-hydropericardium syndrome (HHS), caused by FAdV-C (serotype 4), and inclusion body hepatitis (IBH), caused by FAdV-D (serotypes 2 and 11) and -E (serotypes 8a and 8b) with increasing importance in recent years (2, 3). Among these diseases, HHS and IBH share common pathogenesis and immunological features, as opposed to AGE (2, 4). Despite the worldwide occurrence of FAdVs and their growing economic impact, the lack of broadly protective and commercially accessible immunization strategies has led to extensive investigations on experimental vaccine antigens (3–7). In regards to subunit vaccines, the fiber protein, one of the major structural capsid components and surface antigens, has proven highly effective against HHS- and IBH-causing strains (8–15). Recently, the novel concept of chimeric fiber protein (crecFib) was formulated by designing a recombinant construct mimicking the full fiber structure incorporating epitopes from two distinct serotypes, in order to protect chickens from the diverse etiology of IBH (16). The concept was subsequently extended across FAdV species to obtain a singular vaccination antigen against IBH and HHS. The relevant chimeric construct containing epitopes from FAdV-4 and -11 fibers (named crecFib-4/11) provided coverage against both diseases (17). Even so, the immune mechanisms underlying such protection remain unclear. In fact, discrepancies between the potently neutralizing protective response against crecFib-8b/8a and an absent neutralizing activity of the crecFib-4/11 response, despite eliciting high protection, further complicate the matter (16, 17). It has been shown previously that live and fiber-based vaccines against FAdV-4 do not always lead to the development of neutralizing antibodies (nAbs), which suggests the involvement of alternative immune pathways (8, 17, 18). Recent studies have indicated a more substantial role of the cellular immune response not only in regards to live vaccines (18, 19), but for recombinant fiber antigens as well (9, 10, 13). Despite some preliminary data, investigations of the cellular immunity in FAdV subunit vaccines are, however, limited to peripheral blood mononuclear cells (PBMCs), and only to a few cell populations. Consequently, the local response within target and/or lymphoid organs is still entirely unknown. For this reason, we investigated the kinetics of a comprehensive panel of major immune cell populations in specific pathogen-free (SPF) chickens vaccinated with the novel crecFib-4/11, with or without subsequent FAdV-4 challenge. The investigation was performed on both circulating cells in blood, and locally in primary (thymus and bursa of Fabricius) and secondary (spleen) lymphoid organs, which are also infected by the virus, with the further addition of one of the major target organs of FAdV-4, the liver.

Abbreviations: AGE, adenoviral gizzard erosion; AST, aspartate transaminase; dpb, days post booster; dpc, days post challenge; ELISA, enzyme-linked immunosorbent assay; FAdV, fowl adenovirus; FBS, fetal bovine serum; FCM, flow cytometry; HHS, hepatitis-hydropericardium syndrome; IBH, inclusion body hepatitis; nAbs, neutralizing antibodies; non-nAbs, non neutralizing antibodies; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SPF, specific pathogen-free; $TCID_{50}$, 50% tissue culture infective dose; TCR, T cell receptor; VN, virus neutralization.

2 Materials and methods

2.1 Chimeric fiber vaccine and virus preparation

A chimeric fiber protein retaining epitopes from FAdV-4 and FAdV-11, named crecFib-4/11, was designed and expressed as previously described (16, 17). Briefly, the open reading frames of fibers from FAdV-4 (fib-2) (reference strain KR5, GenBank accession no. HE608152) and FAdV-11 (field isolate 13/14796) were divided into an amino- and a carboxy-distal segment, and assembled via Gibson assembly cloning. The recombinant protein crecFib-4/11 was expressed in *Spodoptera frugiperda* Sf9 cells using baculovirus system, and purified via polyhistidine

tag on affinity chromatography columns (His GraviTrap, GE Healthcare, Freiburg, Germany) as described by Schachner et al. (8).

The FAdV-4 field isolate AG234 (GenBank accession no. MK572849) was applied as challenge strain for the animal experiment after being 3-fold plaque purified and propagated on primary chicken-embryo liver cells (20). Viral titers were determined by endpoint titration (21).

2.2 Animal experiment

The protection study was described previously in detail (17). Briefly, 80 SPF layer-type chicks were hatched, individually tagged and divided into four groups (n = 20) designed as: vaccinated only, vaccinated+challenged (vaccinated with crecFib-4/11 and challenged with FAdV-4), challenge control, and negative control (Table 1). Each group was housed separately in isolator units (HM2500, Montair, The Netherlands). One-day-old chicks received a 0.5 ml intramuscular injection of the vaccine containing 50 µg of crecFib-4/11 homogenized in a 40% (wt/vol) antigen oil-based adjuvant phase in the *Musculus iliotibialis lateralis*, whereas challenge control birds were injected with phosphate buffered saline (PBS) mixed with adjuvant, and the negative control group was administered sterile PBS only. An analogous procedure was repeated for all groups at 7-day-old for a booster vaccination. The challenge was performed intramuscularly at 22 days of life (15 days post booster, dpb) for the vaccinated+challenged and challenge control groups with 200 µl of 10^7 tissue culture infectious dose 50 ($TCID_{50}$)/ml of FAdV-4 strain AG234. After challenge, the birds were monitored daily for clinical signs. At 3, 5, 7 and 14 days post challenge (dpc), up to five birds per group were euthanized and submitted to necropsy, during which data and samples were collected to assess several protection endpoints as detailed below. Animals that had to be euthanized due to severe clinical signs were examined and sampled immediately. All procedures were discussed and approved by the institutional ethics and welfare committee and the national authority according to §§26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 20212 (license number: BMBWF GZ 68.205/0116-V/3b/2019).

2.3 Protection endpoints and antibody development

Investigations to assess the protective efficacy of the vaccine included the measurement of aspartate transaminase (AST) in blood, organ-body weight ratio and viral load quantification in target and lymphoid organs through qPCR. The antibody development was investigated in serum with ELISA plates coated with the vaccination antigen and via virus neutralization (VN) test. All the methods have been described previously in detail (17). For the purpose of the present study, the viral load in thymus was investigated in addition to the data already available.

2.4 Histopathology

Samples from liver, spleen, thymus and bursa of Fabricius of birds euthanized at 4-5 dpc were collected during necropsy from the vaccinated+challenged, challenge control and negative control groups. The tissues were fixed in 4% neutral buffered formalin before being dehydrated and embedded in paraffin. Tissue sections of 5 µm thickness were cut with a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany) and mounted on glass slides before undergoing hematoxylin-eosin staining. Examination was performed using the Olympus BX53 microscope and documented with an Olympus DP72 camera (Olympus Corporation, Tokyo, Japan).

2.5 Cellular response

Blood for flow cytometry (FCM) analyses on PBMCs was collected from five birds per group before challenge, at 21-day-old (14 dpb). At different time points after challenge (3, 5, 7 and 14 dpc), FCM analyses were performed from mononuclear cells from blood, liver, spleen, thymus and bursa of Fabricius from three birds per group.

2.5.1 Blood collection and preparation

For separation of PBMCs, 2 ml of blood were collected from the wing vein of each bird in a syringe containing 10% heparin

TABLE 1 Experimental groups of the protection study.

group	vaccination (1 + 7-day-old)	challenge (22-day-old)
vaccinated only	crecFib-4/11	-[1]
vaccinated+challenged	crecFib-4/11	FAdV-4
challenge control	adjuvant only	FAdV-4
negative control	-	-

[1]not applied.

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(Serva, Heidelberg, Germany) at 21-day-old, and 4 ml from the jugular vein into a 10% heparin tube for the post-challenge time points. The blood was mixed with an equal volume of cold PBS, pH 7.4 (ThermoFisher Scientific, Vienna, Austria) with 2% fetal bovine serum (FBS) (ThermoFisher Scientific). The prepared suspension was then slowly layered above a double volume of Histopaque®-1077 (Sigma-Aldrich, Vienna, Austria) for density gradient centrifugation performed at room temperature, 350 x g for 30 min, without brake. Afterwards, the cells from the interphase layer were collected and washed three times through centrifugation, 400 x g for 10 min at 4°C, with full brake. Finally, the pellet was dissolved in 1 to 5 ml of cold PBS + 2% FBS.

2.5.2 Preparation of liver, spleen, thymus and bursa of Fabricius

Single cell suspensions from the four organs were obtained by mechanical dissection (22). Briefly, isolation of lymphocytes was performed by squeezing the liver with the end of the plunger of a 20 ml syringe, and for the other organs by tearing apart the tissue with the help of two forceps in petri dishes containing up to 30 ml cold PBS + 2% FBS. The cells were then separated from the remaining tissue through a 40 µm nylon cell strainer (BD Falcon, BD Bioscience, San Jose, CA, USA) in a 50 ml tube. Following that, centrifugation was performed at room temperature, 350 x g for 10 min. The pellet was resuspended in 5 ml cold PBS + 2% FBS and separated by density gradient before washing as described for PBMCs. Mononuclear cells were finally resuspended in 5-10 ml cold PBS + 2% FBS.

2.5.3 FCM staining protocol

Mononuclear cells from the blood and the analyzed organs were examined for their viability and quantity using Nexcelom cellometer X2 fluorescent viability cell counter system (Nexcelom Bioscience, Manchester, UK). A concentration of 2 x 10^7 live cells/ml of PBS + 2% FBS was adjusted before further processing. Two different sets of monoclonal antibodies were used for immunophenotyping of live B cells ($CD45^+Bu1^+$), monocytes/macrophages ($CD45^+Kul01^+$), $CD4^+$ T cells ($CD45^+CD4^+CD8\alpha^-$), $CD8\alpha^+$ T cells ($CD45^+CD4^-CD8\alpha^+$), and $CD8\alpha^+TCR\delta\gamma^+$ T cells ($CD45^+CD4^-CD8\alpha^+TCR\delta\gamma^+$) from the isolated cells. The gating strategy is given as additional figure (Supplementary File 1). A uniform gating hierarchy was used throughout all sampling days. Detailed information on antibody combinations, their fluorescence labelling strategy, manufacturers and catalogue number is given in Supplementary File 2. The final concentration of every antibody was determined by titration. The respective isotype controls and live cells without antibody staining were included for every group.

For staining of mononuclear cells, 25 µl of 2 x 10^7 live cells/ml were transferred into each well of 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) together with the respective primary antibodies for incubation for 20 min at 4°C. Afterwards, cell pellets obtained by centrifugation with full brake at 4°C, 450 x g for 4 min, were washed two times with cold PBS + 2% FBS. For biotinylated antibodies, the secondary reagent Brilliant Violet 421™ Streptavidin (BioLegend, San Diego, CA, USA) was applied. Following another incubation step for 20 min at 4°C, further washing was performed. The cells were fixed with BD fixation buffer (BD Biosciences) according to the manufacturer's protocol. Finally, the pellets were resuspended in 100 µl cold PBS + 2% FBS and kept at 4°C until FCM analysis the following day.

2.5.4 FCM analysis

FCM assay was conducted on the stained cells with a FACSCanto II (BD Biosciences) flow cytometer equipped by FACSDiva Software version 9.0 (BD Biosciences). At least 40,000 lymphocytes per sample were recorded. Analysis of FCM raw data was performed by FlowJo_V10 software (BD Biosciences).

2.6 Statistical analyses

A preliminary analysis of the datasets was carried out using Shapiro-Wilk test together with a visual inspection of histograms and normal Q-Q plots to assess normal distribution. Unpaired Student's *t*-test was applied to compare the mean values for each cell population of the different experimental groups to the negative control, in addition to the previously published pathology parameters (17). Mann-Whitney *U* test was used for the datasets that did not meet the normality assumptions. In each case, p values ≤ 0.05 were considered statistically significant. Statistical analyses were performed with the software package SPSS Version 27 (IBM SPSS Statistics; IBM Corp., Armonk, New York, USA).

3 Results

3.1 Protection endpoints and antibody development

Vaccination with crecFib-4/11 prevented clinical signs as opposed to the challenge control, and significantly limited hepatomegaly, atrophy of the lymphoid organs, and pathological rise of AST caused by HHS. Details on the values of each parameter for every experimental group have been previously published (17). Vaccination also reduced the viral load in liver, spleen, and bursa of Fabricius compared to the challenge control. However, no significant differences were observed in thymus between the vaccinated+challenged and the challenge control group at any time point. Mean viral copies/reaction values in thymus were the highest between 3 dpc (2.91 ± 1.98 $\log_{10}$ in vacc.+chall. vs. 2.09 ± 1.28 $\log_{10}$ in chall. contr.) and 5 dpc (2.05 ± 2.38 $\log_{10}$ vs. 3.33 ± 1.36

log_{10}), before decreasing at 7 dpc (0.87 ± 1.95 log_{10} vs. 0.45 ± 0.63 log_{10}). No virus was detected in thymus at 14 dpc for any of the investigated groups. As reported in our previous study, the vaccine induced a high and uniform antibody response measured on crecFib-4/11 ELISA before challenge, with the vaccinated groups plateauing at the highest measurable OD values by 25 days of age (corresponding to 3 dpc), independently from the challenge (17). Nevertheless, nAbs against FAdV-4 were only detectable after challenge in both challenged groups. The antibody development is summarized in Figure 1.

3.2 Histopathology

The histological lesions highlighting differences between experimental groups are exemplarily shown in Figure 2. At 4-5 dpc, birds from the challenge control group presented extensive and severe microscopic lesions in the liver such as lymphocytic infiltration, necrotic areas and degeneration of hepatocytes with vacuolization. Furthermore, lymphocytic depletion was observed in spleen, thymus and bursa of Fabricius of challenge control birds, together with necrotic areas in the follides of the bursa. Histological lesions were also observed in livers of vaccinated+challenged birds, although substantially reduced compared to only-challenged birds. In samples from vaccinated+challenged birds, lymphocytic depletion was not recorded in the spleen and thymus but it was sporadically noticed in their bursa of Fabricius. No lesions were observed in the organs of negative control birds.

3.3 Cellular immune response

3.3.1 B cells

In the PBMCs, B cell frequencies were significantly decreased before challenge compared to basal levels (always defined by the negative control) in the challenge control, which at this point comprised birds injected with adjuvant only (Figure 3A). After challenge, the priming effect of the vaccination was noticed at 3 and 5 dpc, with a significant rise of circulating B lymphocyte frequencies in the vaccinated +challenged group, before a drop was observed for both challenged groups at 14 dpc. The same priming effect of the vaccine was recorded in the liver, with a significant increase of B cells at 3, 7 and 14 dpc in the vaccinated+challenged group, whereas challenge control birds experienced a rise only at 5 and 14 dpc (Figure 3B). In the same organ, there was a significant decrease of hepatic B lymphocytes in the vaccinated only group from 3 to 7 dpc (corresponding to 18 to 22 dpb). The frequencies

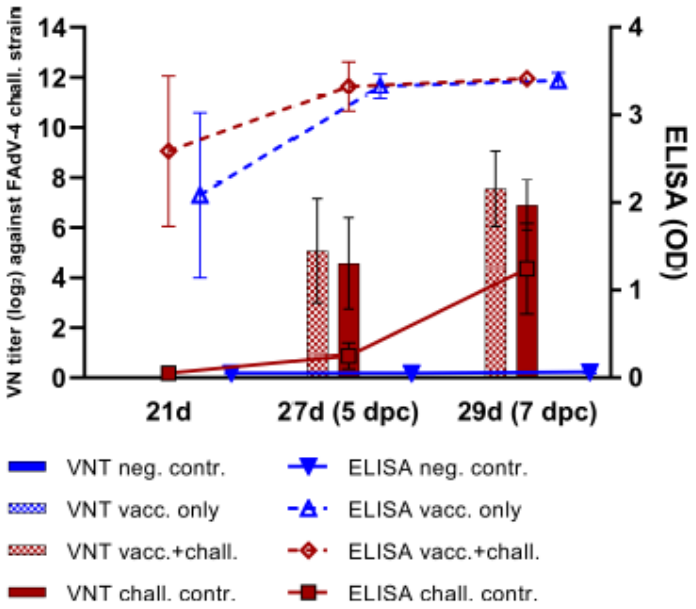

FIGURE 1
Antibody development following crecFib-4/11 vaccination in each experimental group of the animal study. The lines represent mean values of antibodies measured on ELISA plates coated with crecFib-4/11, whereas the bars indicate VN titers against the FAdV-4 challenge strain. No neutralizing antibodies were detected in the vaccinated only and the negative control groups at any time point. Reprinted from: Vaccination with a fowl adenovirus chimeric fiber protein (crecFib-4/11) simultaneously protects chickens against hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH), Vaccine (2022) 40, De Luca C, Schachner A, Heidl S, Hess M, pages no. 1837–45, Copyright © 2022 Elsevier B.V. or its licensors or contributors, with permission from Elsevier (17).

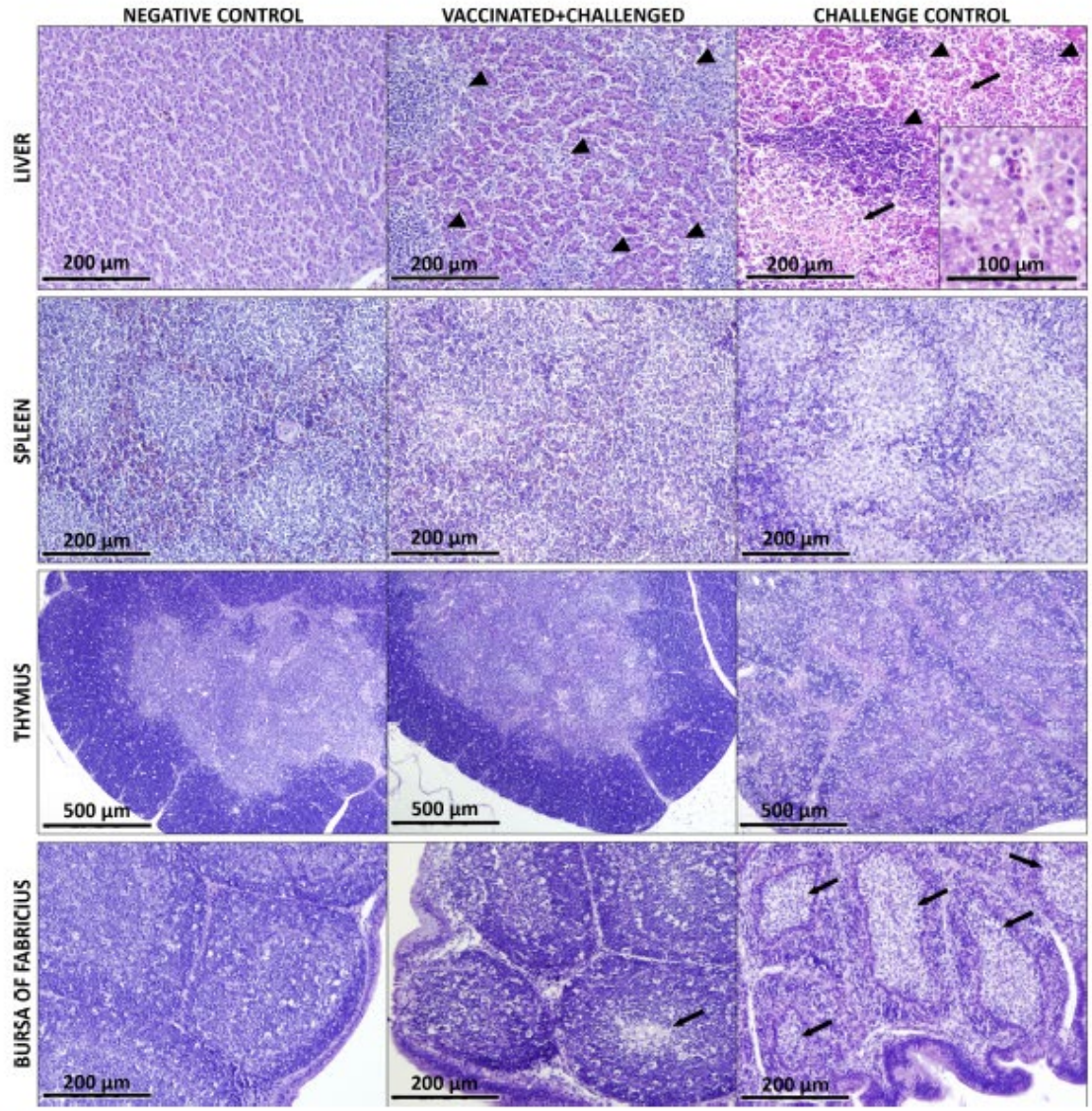

FIGURE 2
Main histological lesions observed in the analyzed organs of birds from negative control, vaccinated+challenged, and challenge control group. The liver of vaccinated+challenged group presents lymphocytic infiltration (marked by arrow heads) and small areas of degeneration (arrows), which can be seen more severe in the challenge control. Vacuolization of the hepatocytes is shown in a magnified field for the challenge control. Lymphocytic depletion is visible in the spleen and thymus of the challenge control group. The bursa of Fabricius of the vaccinated+challenged group shows mild lymphocyte depletion and few necrotic areas (arrows) in the medulla of the follicles, whereas the same lesions are much more pronounced and widespread in the challenge control.

of splenic B cells significantly rose at 5 dpc in vaccinated only and challenge control birds, whereas a significant decrease was noticed in thymus at 3 dpc for the challenge control, and at 7 dpc in the vaccinated+challenged birds (Figures 3C, D). No relevant changes were recorded in the bursa of Fabricius (Figure 3E)

3.3.2 Monocytes/macrophages

The only significant variations in the frequencies of circulating monocytes/macrophages were limited to an increase in the challenge control and the vaccinated+challenged group at 3 and 5 dpc, respectively (Figure 4A). Subsequently, monocytes/macrophages

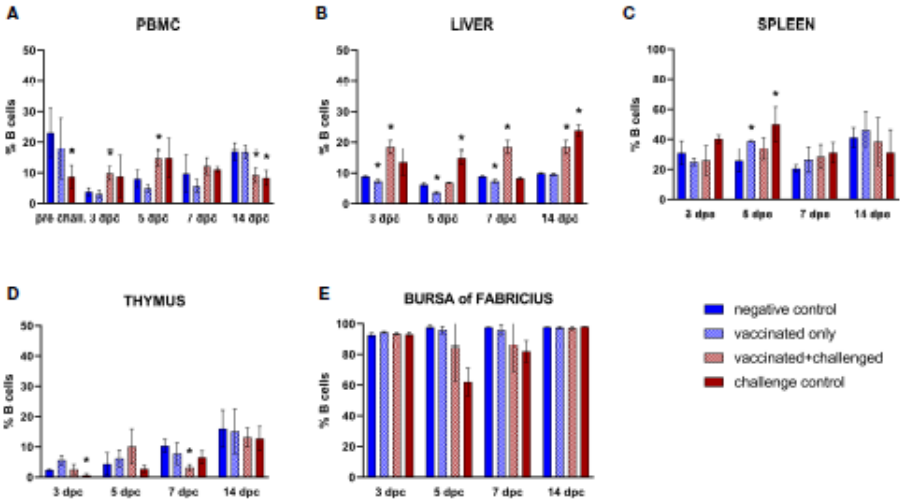

FIGURE 3
Relative number of B cells within total CD45$^+$ cells measured via FCM for each experimental group throughout the study in PBMC (**A**), liver (**B**), spleen (**C**), thymus (**D**), and bursa of Fabricius (**E**). Data for the two vaccinated groups (vaccinated only and vaccinated+challenged) are merged to determine values in PBMCs pre-challenge. Asterisks represent significant difference compared to the negative control at each time point ($p \leq 0.05$).

frequencies consistently rose in the liver of challenge control birds at 3, 5 and 14 dpc, whereas vaccinated+challenged birds only experienced an increase in the early phase of infection (3 dpc) (Figure 4B). On the other hand, splenic monocytes/macrophages frequencies rose at 3 dpc and dropped below basal levels immediately after, at 5 dpc, in both challenged groups, before increasing once again in the vaccinated+challenged group (7 dpc), whereas they were still found significantly low in the challenge

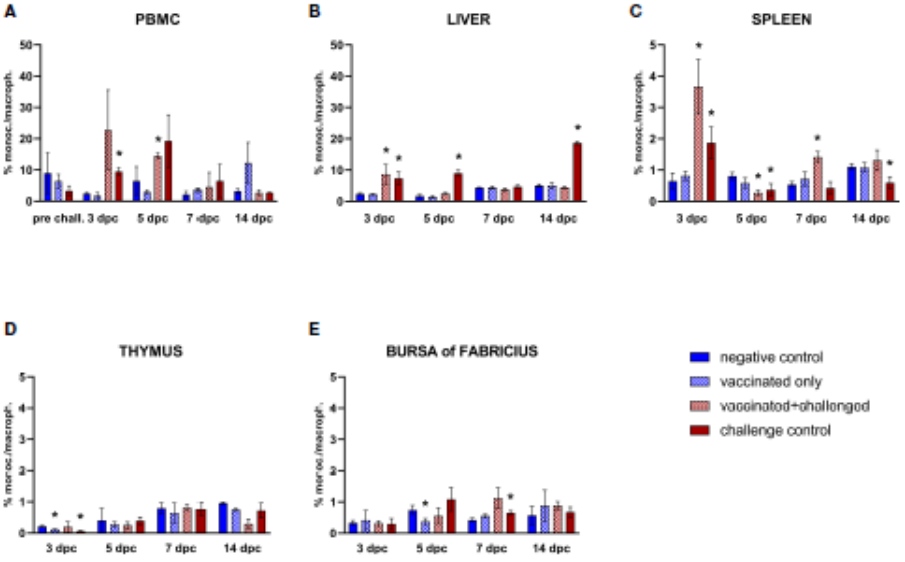

FIGURE 4
Relative number of monocytes/macrophages within total CD45$^+$ cells measured via FCM for each experimental group throughout the study in PBMC (**A**), liver (**B**), spleen (**C**), thymus (**D**), and bursa of Fabricius (**E**). Data for the two vaccinated groups (vaccinated only and vaccinated+challenged) are merged to determine values in PBMCs pre-challenge. Asterisks represent significant difference compared to the negative control at each time point ($p \leq 0.05$).

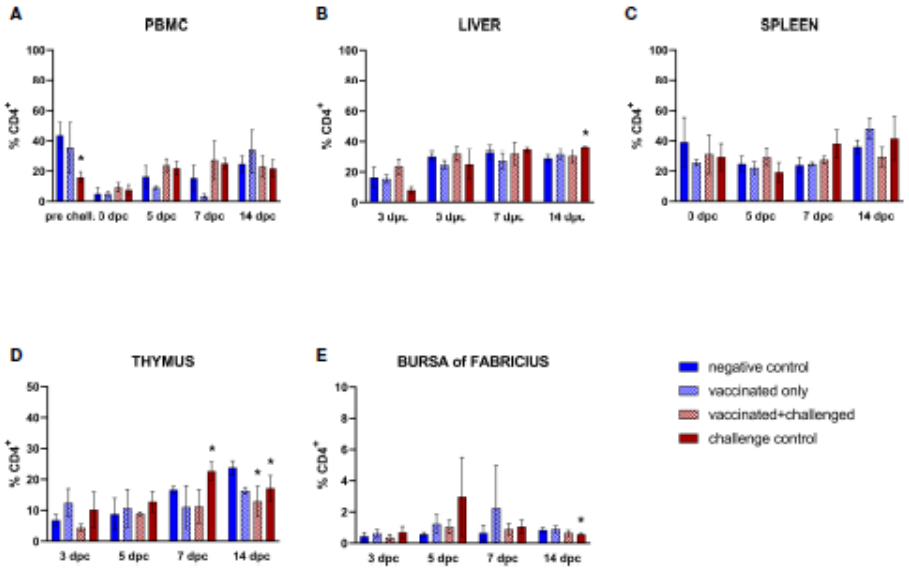

FIGURE 5
Relative number of CD4$^+$ T lymphocytes within total CD45$^+$ cells measured via FCM for each experimental group throughout the study in PBMC (**A**), liver (**B**), spleen (**C**), thymus (**D**), and bursa of Fabricius (**E**). Data for the two vaccinated groups (vaccinated only and vaccinated+challenged) are merged to determine values in PBMCs pre-challenge. Asterisks represent significant difference compared to the negative control at each time point ($p \leq 0.05$).

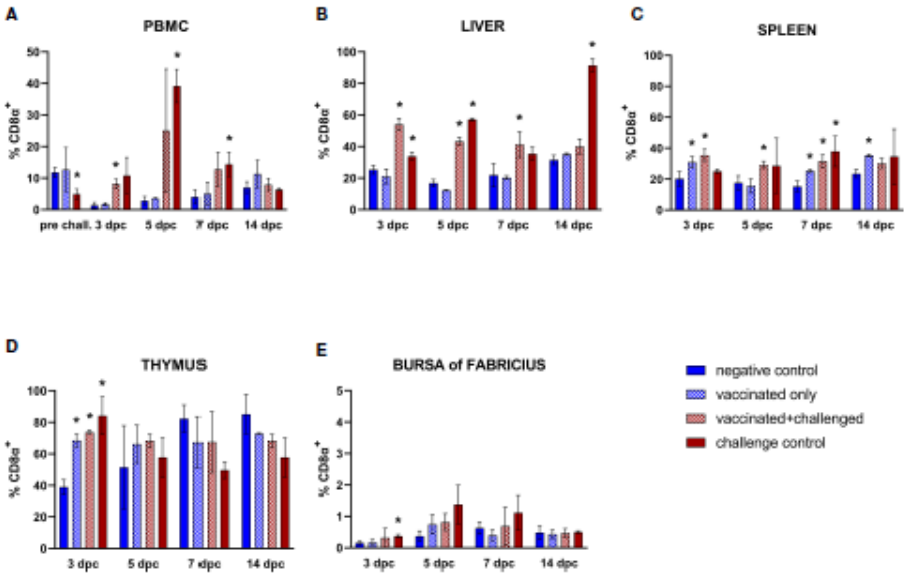

FIGURE 6
Relative number of CD8α^+ T lymphocytes within total CD45$^+$ cells measured via FCM for each experimental group throughout the study in PBMC (**A**), liver (**B**), spleen (**C**), thymus (**D**), and bursa of Fabricius (**E**). Data for the two vaccinated groups (vaccinated only and vaccinated+challenged) are merged to determine values in PBMCs pre-challenge. Asterisks represent significant difference compared to the negative control at each time point ($p \leq 0.05$).

control at 14 dpc (Figure 4C). The vaccinated only and challenge control groups showed a significant decrease of monocytes/macrophages frequencies in thymus at 3 dpc compared to the negative control, and a drop was also observed in the bursa of Fabricius for vaccinated only birds (5 dpc, corresponding to 20 dpb), whereas challenge control birds showed a rise (7 dpc) (Figures 4D, E).

3.3.3 CD4$^+$ T cells

Similarly to the trend observed in the B cells, circulating CD4$^+$ T lymphocyte frequencies of challenge control birds were significantly decreased compared to basal levels in PBMCs before challenge (Figure 5A). Aside from that, helper T cells (CD45$^+$CD4$^+$CD8α$^-$) were only sporadically affected throughout the study. A late increase (14 dpc) was noticed in the frequencies of hepatic CD4$^+$ T cells in the challenge control (Figure 5B). No changes were observed in the spleen (Figure 5C). Thymus and bursa of Fabricius presented a drop of this cell population in the challenge control at 14 dpc, after an earlier rise in the thymic population at 7 dpc (Figures 5D, E). A late significant decrease of CD4$^+$ T cell frequencies was also observed in the thymus of vaccinated +challenged birds (14 dpc).

3.3.4 CD8α$^+$ T cells

Frequencies of circulating CD8α$^+$ T cells suffered from a pre-challenge decrease in the challenge control (15 dpb) (Figure 6A). After challenge, the priming effect of vaccination was strongly recorded in PBMC CD8α$^+$ T lymphocytes, whose frequency rose significantly in the vaccinated+challenged group at 3 dpc, before immediately returning to basal levels, whereas the challenge control responded with an increase that came later and lasted longer (5-7 dpc). In the liver, this trend was reflected with an early and prolonged rise of CD8α$^+$ T lymphocytes of both challenged groups, although the challenge bird control still showed significantly higher hepatic cytotoxic T cells (CD45$^+$CD4$^-$ CD8α$^+$) at 14 dpc (Figure 6B). Both the vaccination and the challenge showed the tendency to stimulate CD8α$^+$ T cells in the spleen, with a significant rise in the vaccinated+challenged group through the whole first week after challenge, and in the vaccinated only group at 3, 7, and as late as 14 dpc, whereas the challenge control group only showed an increase of splenic CD8α$^+$ T lymphocytes at 7 dpc (Figure 6C). These rises were accompanied by an increase of this lymphocyte population frequency in the thymus of birds from all the three groups early after infection (3 dpc), which was also observed in the bursa of Fabricius of the challenge control birds (Figures 6D, E).

3.3.5 CD8α$^+$TCRγδ$^+$ T cells

A significant increase of CD8α$^+$TCRγδ$^+$ T cell frequencies was observed in the PBMCs of challenged birds at 5 dpc, followed by a late drop at 14 dpc in the vaccinated+challenged group (Figure 7A). A decrease was also observed in the

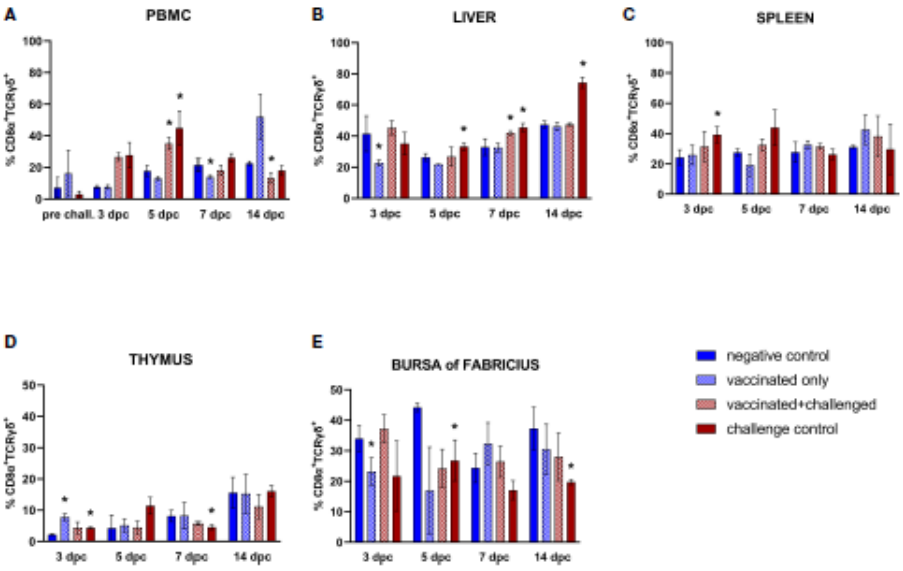

FIGURE 7
Relative number of CD8α$^+$TCRγδ$^+$ T lymphocytes within total CD8α$^+$ T lymphocytes measured via FCM for each experimental group throughout the study in PBMC (**A**), liver (**B**), spleen (**C**), thymus (**D**), and bursa of Fabricius (**E**). Data for the two vaccinated groups (vaccinated only and vaccinated+challenged) are merged to determine values in PBMCs pre-challenge. Asterisks represent significant difference compared to the negative control at each time point ($p \leq 0.05$).

vaccinated only group in PBMCs at 7 dpc (corresponding to 22 dpb) and, before, in the liver at 3 dpc (corresponding to 18 dpb). Hepatic CD8α$^+$TCRγδ$^+$ T lymphocyte frequencies were significantly elevated in the challenge control from 5 to 14 dpc, whereas such increase was only observed at 7 dpc for the vaccinated+challenged group (Figure 7B). The challenge control was the only group showing increased CD8α$^+$TCRγδ$^+$ T cells in the spleen (3 dpc) (Figure 7C). A proliferation of these cells was noticed in the thymus of vaccinated only and challenge control birds at 3 dpc (corresponding to 18 dpb), before a drop was recorded at 7 dpc in the challenge control (Figure 7D). A decrease of CD8α$^+$TCRγδ$^+$ T cell frequencies in the bursa of Fabricius was observed in the vaccinated only group at 3 dpc, and in the challenge control at 5 and 14 dpc (Figure 7E).

4 Discussion

FAdV-related diseases such as HHS and IBH have proven to be particularly vexing to the poultry industry in the last two decades (3, 4). Recently, a novel concept based on recombinant chimeric fiber proteins, merging epitopes from different FAdV serotypes, was able to achieve broad coverage against both HHS and IBH, with a new construct retaining epitopes from FAdV-4 and -11 (crecFib-4/11) (17). However, the immune pathways associated with said protection are not fully elucidated. Whereas immunization from FAdV-8a and -8b is usually linked to a potent humoral response associated to the development of serotype-specific nAbs (9, 13, 16, 23–25), neutralizing activity seems to be dispensable when it comes to vaccines against FAdV-4 (8, 18, 19). This led to speculations over the potentially crucial role of the cellular immune response against HHS infections. Despite the numerous studies describing cytokines development over the course of FAdV-4 infection, which confirmed an important involvement of the cellular immune response (26), there is very little information available over the kinetics of the immune cell populations themselves. In particular, to date, no studies have focused on unravelling the development of both systemic and local cellular response related to FAdV-4 fiber vaccines before and after challenge. Therefore, in the present work, we investigated the kinetics of the major immune cell populations after vaccination with crecFib-4/11 and/or HHS challenge, in not only PBMCs, but also taking into account target and lymphoid organs of the birds over the course of the disease.

Vaccination with crecFib-4/11 resulted into high levels of systemic ELISA-measured antibodies against the vaccine antigen, but no nAbs against FAdV-4. Nevertheless, immunized birds challenged with virulent FAdV-4 showed a significant reduction of clinical signs and pathological lesions (17). This marks a substantial difference compared to fiber vaccines developed from FAdV-8a and -8b fibers, whose protection against IBH correlates with the presence of serotype-specific nAbs (9, 13, 16). High levels of antibodies after FAdV-8a fiber vaccination have been linked to a significant increase of B cells in blood before challenge (13), whereas in the present study, the increase of circulating B cells compared to basal levels (defined by the negative control) was noted in vaccinated birds only after challenge. A prominent rise of B lymphocytes was also observed in the liver of crecFib-4/11-vaccinated birds throughout the whole period after challenge, conforming with the hepatic lymphoid infiltration observed in histological analyses. The immediate increase of these cells in the blood of vaccinated+challenged birds, and subsequently in one of the major target organs, the liver, highlights the priming effect of vaccination. In fact, the kinetics of B lymphocytes in challenge control birds evolved differently, with an increase in the hepatic and splenic cell population after an initial depletion in the thymus, without a rise in circulating B cell frequencies. A decrease in thymus was also observed in the present study for immunized birds a week after challenge, possibly due to the proliferation of other immune cell populations in response to infection. Furthermore, a drop in the frequency of circulating B lymphocytes was noted in both challenged groups two weeks after challenge, independently from previous vaccination, possibly due to the migration of these cells in the liver. Notably, even with histological investigations highlighting the lymphocyte depletion in challenged groups, no significant changes were noticed in B cell frequencies of the bursa of Fabricius for any experimental group, confirming an earlier study published by Schonewille et al. (19) in birds infected with the same challenge strain. This may be because the relative count of B lymphocytes, which in the bursa of Fabricius constitute at least 98% of the whole lymphocyte population (27), did not suffer significant changes despite the overall depletion.

Monocytes/macrophages were also reduced in thymus of challenge control birds in the early phase of infection, while they rose in blood, liver and spleen of both vaccinated+challenged and challenge control birds. This marks a difference compared to FAdV-8a fiber vaccine, which did not elicit such rise in the blood of SPF broilers in response to an IBH infection, as opposed to the SPF layers utilized in the present work (13). In the challenge control, levels of hepatic monocytes/macrophages remained elevated longer than in the vaccinated+challenged group. At the same time, after an early increase, the infection caused a depletion of these antigen-presenting cells in the spleen, which was quickly resolved only in previously immunized birds.

No significant differences were observed in levels of CD4$^+$ T lymphocytes in blood or spleen of vaccinated and/or challenged birds, contrary to previous studies, where both FAdV-4 (fib-2) and FAdV-8a fiber vaccines were shown to promote the proliferation of helper T cells in the blood of chickens (10, 13). Apart from the differences in the vaccination antigens, this discrepancy may also be explained by divergences between the adjuvant used, the age and, in particular, the type of birds. However, the same challenge strain

(AG234) caused a drop in splenic $CD4^+$ T cells in unvaccinated chickens in a previous study (19). This may be ascribable to the inconsistencies in pathogenicity already observed between *in vivo* experiments utilizing said challenge strain, possibly due to underlying differences between the batches of SPF birds (8, 17). Nevertheless, the crecFib-4/11 vaccine was able to prevent a late drop of $CD4^+$ T lymphocytes in the bursa of Fabricius, as observed in the challenge control, although a decrease in thymus was recorded in both challenged groups two weeks after infection, despite the absence of viral load in the organ at this time point. Atrophy and lymphocyte depletion were repeatedly reported in the thymus and bursa of Fabricius in relation to HHS infection as a result of the immunosuppressive nature of the virus (17, 19, 28–33). However, in the present study, histological analyses showed that the crecFib-4/11 vaccine was able to limit HHS-caused lymphocyte depletion in immunized birds, a feature shared among successful experimental vaccines against FAdV-4 (19, 28, 33). Furthermore, cytotoxic $CD8\alpha^+$ T cell frequencies were never found significantly decreased compared to basal levels in any of the experimental groups after challenge. On the contrary, the inclusion of a vaccinated only group allowed us to detect the proliferation of $CD8\alpha^+$ T lymphocytes in thymus at 18 dpb, and in spleen as late as 29 dpb, which conforms to previous observations related to FAdV-E fiber vaccines inducing an increase of cytotoxic T lymphocytes, albeit in PBMCs (9, 13). In fact, the present work highlights the pivotal role of $CD8\alpha^+$ T cells in protecting chickens against HHS, thanks to the immediate rise observed in the thymus and liver of challenged birds, and the earlier increase of circulating and splenic cytotoxic T cells in vaccinated birds compared to the challenge control. On the other hand, frequencies of $CD8\alpha^+TCR\gamma\delta^+$ lymphocyte subpopulation increased in the blood and liver of both challenged groups, although vaccinated+challenged birds did not show a previous proliferation in thymus and spleen, nor suffered a drop in primary lymphoid organs, as opposed to the challenge control. Prolonged exposure to an antigen can result in T cell exhaustion, which is characterized by the loss of T cell effector function (34). In fact, we demonstrated that FAdV-4 can persist up to two weeks after infection in the spleen (17). These findings may also relate to the downregulation of certain splenic cytokines, such as IL-18 and INF-γ, during the late phase of infection (35), confirming that FAdV-4 viruses reflecting different pathogenicity can persist in this organ even after the acute phase of infection, thus extending a condition of subclinical immunosuppression in the birds.

Interestingly, many of the analyzed cell populations were affected by the adjuvant with a significant decrease in the blood after booster vaccination (14 dpb) compared to basal levels, as observed in the challenge control group that, at this point, had been injected with adjuvant only. This may be due to the adjuvant-induced recruitment of these immune cells to the injection site (36). Furthermore, the process was possibly enhanced by the local inflammation at the site of injection, which was still detectable during necropsy in the inoculated birds. Sporadic drops in the frequencies of B cells, monocytes/macrophages and $CD8\alpha^+TCR\gamma\delta^+$ T lymphocytes were also recorded in various organs of the vaccinated only group such as PBMCs, liver, thymus and bursa of Fabricius. Although the reason behind this phenomenon is not clear, we hypothesize that the vaccine/adjuvant combination tailored the immune responsiveness towards cytotoxic $CD8\alpha^+$ T cells. Contrary to FAdV-8a-induced IBH in broilers, HHS infection in layers did not cause a prolonged decrease of monocytes/macrophages, nor a significant rise of $CD4^+$ T lymphocytes in the blood over the course of the infection (13). It is known, in fact, that differences in the genetic background of the birds greatly influence their susceptibility to FAdVs (37). However, these contrasts may also be linked to FAdV-specific immune pathways, stressing the importance of broad-protective vaccines such as crecFib-4/11.

Overall, the observed priming and proliferation of immune cells after crecFib-4/11 immunization highlight the need for further investigations over the role of vaccine-induced antigen-specific antibodies. In fact, the importance of non-neutralizing antibodies (non-nAbs) has already been proven for vaccines against avian influenza virus (38–41). Therefore, it is possible that the non-nAbs induced by fiber vaccination may constitute a bridge between humoral and cellular immune response through mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), reflected by proliferation of macrophages and cytotoxic T cells in the present study.

In conclusion, despite the lack of circulating nAbs against FAdV-4, protection against HHS provided by crecFib-4/11 was characterized by a prominent and prolonged increase of B lymphocytes in one of the major target organs, the liver. Furthermore, the vaccine-induced increase of cytotoxic T lymphocytes, both circulating and in target organs, such as liver, spleen and thymus, highlights for the first time the pivotal role of local, cell-mediated immune response involved in protection against HHS after priming by FAdV fiber vaccine.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by institutional ethics and welfare committee and the national authority according to §§26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 20212 (license number: BMBWF GZ 68.205/0116-V/3b/2019).

Author contributions

TM, MH, DL, CDL, and AS conceived and designed the work. CDL and AS performed the animal trial. CDL, TM, and SH performed the laboratory analysis. CDL, TM, DL, AS, and MH interpreted the data. CDL drafted the manuscript. TM, DL, AS, and MH revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MH and AS declare the following financial interest that may be considered as potential competing interests: Patent "Fowl adenovirus subunit vaccine and production method thereof" pending to European Patent Office.

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

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1026233/full#supplementary-material

References

1. Benkő M, Aoki K, Arnberg N, Davison AJ, Echavarría M, Hess M, et al. ICTV virus taxonomy profile: Adenoviridae 2022. *J Gen Virol* (2022) 103:001721. doi: 10.1099/jgv.0.001721

2. Hess M. *Diseases of poultry, 14th ed*. Hoboken, NJ: Wiley-Blackwell (2020).

3. Schachner A, Grafl B, Hess M. Spotlight on avian pathology: fowl adenovirus (FAdV) in chickens and beyond – an unresolved host-pathogen interplay. *Avian Pathol* (2021) 50:2–5. doi: 10.1080/03079457.2020.1810629

4. Schachner A, Matos M, Grafl B, Hess M. Fowl adenovirus-induced diseases and strategies for their control – a review on the current global situation. *Avian Pathol* (2018) 47:111–26. doi: 10.1080/03079457.2017.1385724

5. Li PH, Zheng PP, Zhang TF, Wen GY, Shao HB, Luo QP. Fowl adenovirus serotype 4: epidemiology, pathogenesis, diagnostic detection, and vaccine strategies. *Poult Sci* (2017) 96:2630–40. doi: 10.3382/ps/pex087

6. Shah MS, Ashraf A, Khan MI, Rahman M, Habib M, Chughtai MI, et al. Fowl adenovirus: history, emergence, biology and development of a vaccine against hydropericardium syndrome. *Arch Virol* (2017) 162:1833–43. doi: 10.1007/s00705-017-3313-5

7. Liu A, Zhang Y, Cui H, Wang X, Gao Y, Pan Q. Advances in vaccine development of the emerging novel genotype fowl adenovirus 4. *Front Immunol* (2022) 13:916290. doi: 10.3389/fimmu.2022.916290

8. Schachner A, Marek A, Jaskulska B, Bilic I, Hess M. Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis-hydropericardium syndrome (HHS). *Vaccine* (2014) 32:1086–92. doi: 10.1016/j.vaccine.2013.12.056

9. Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya R, Ojkic D, et al. Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. *Vaccine* (2017) 35:2716–22. doi: 10.1016/j.vaccine.2017.03.075

10. Chen L, Yin L, Zhou Q, Li Q, Luo Y, Xu Z, et al. Immunogenicity and protective efficacy of recombinant fiber-2 protein in protecting SPF chickens against fowl adenovirus 4. *Vaccine* (2018) 36:1203–8. doi: 10.1016/j.vaccine.2018.01.028

11. Wang X, Tang Q, Chu Z, Wang P, Luo C, Zhang Y, et al. Immune protection efficacy of FAdV-4 surface proteins fiber-1, fiber-2, hexon and penton base. *Virus Res* (2018) 245:1–6. doi: 10.1016/j.virusres.2017.12.003

12. Wang X, Tang Q, Qiu L, Yang Z. Penton-dodecahedron of fowl adenovirus serotype 4 as a vaccine candidate for the control of related diseases. *Vaccine* (2019) 37:839–47. doi: 10.1016/j.vaccine.2018.12.041

13. De Luca C, Schachner A, Mitra T, Heidl S, Liebhart D, Hess M. Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH) provides type-specific protection guided by humoral immunity and regulation of B and T cell response. *Vet Res* (2020) 51:143. doi: 10.1186/s13567-020-00869-8

14. Hu J, Li G, Wang X, Cai L, Rong M, Li H, et al. Development of a subunit vaccine based on fiber2 and hexon against fowl adenovirus serotype 4. *Virus Res* (2021) 305:198552. doi: 10.1016/j.virusres.2021.198552

15. Yin D, He L, Zhu E, Fang T, Yue J, Wen M, et al. A fowl adenovirus serotype 4 (FAdV-4) Fiber2 subunit vaccine candidate provides complete protection against challenge with virulent FAdV-4 strain in chickens. *Vet Microbiol* (2021) 263:109250. doi: 10.1016/j.vetmic.2021.109250

16. Schachner A, De Luca C, Heidl S, Hess M. Recombinantly expressed chimeric fibers demonstrate discrete type-specific neutralizing epitopes in the *Fowl aviadenovirus E* (FAdV-E) fiber, promoting the optimization of FAdV fiber subunit vaccines towards cross-protection *in vivo*. *Microbiol Spectr* (2022) 10:e02123–21. doi: 10.1128/spectrum.02123-21

17. De Luca C, Schachner A, Heidl S, Hess M. Vaccination with a fowl adenovirus chimeric fiber protein (crecFib-4/11) simultaneously protects chickens against

hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH). *Vaccine* (2022) 40:1837–45. doi: 10.1016/j.vaccine.2022.01.060

18. Schonewille E, Jaspers R, Paul G, Hess M. Specific-pathogen-free chickens vaccinated with a live FAdV-4 vaccine are fully protected against a severe challenge even in the absence of neutralizing antibodies. *Avian Dis* (2010) 54:905–10. doi: 10.1637/8999-072309-Reg.1

19. Schonewille E, Singh A, Göbel TW, Gerner W, Saalmüller A, Hess M. Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid organs in specific pathogen-free chickens following experimental infection. *Vet Immunol Immunop* (2008) 121:130–9. doi: 10.1016/j.vetimm.2007.09.017

20. Schat K, Sellers HS. *A laboratory manual for the isolation and identification of avian pathogens, 5th ed*. Madison, WI, USA: OmniPress Inc (2008).

21. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* (1938) 27:493–7. doi: 10.1093/oxfordjournals.aje.a118408

22. Mitra T, Gerner W, Kidane FA, Wernsdorf P, Hess M, Saalmüller A, et al. Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens. *Vaccine* (2017) 35:4184–241. doi: 10.1016/j.vaccine.2017.06.035

23. Gupta A, Popowich S, Ojkic D, Kurukulasuriya S, Chow-Lockerbie B, Gunawardana T, et al. Inactivated and live bivalent fowl adenovirus (FAdV8b + FAdV11) breeder vaccines provide broad-spectrum protection in chicks against inclusion body hepatitis (IBH). *Vaccine* (2018) 36:744–50. doi: 10.1016/j.vaccine.2017.12.047

24. Popowich S, Gupta A, Chow-Lockerbie B, Ayalew L, Ambrose N, Ojkic D, et al. Broad spectrum protection of broiler chickens against inclusion body hepatitis by immunizing their broiler breeder parents with a bivalent live fowl adenovirus vaccine. *Res Vet Sci* (2018) 118:262–9. doi: 10.1016/j.rvsc.2018.03.003

25. Lu H, Xie Q, Zhang W, Zhang J, Wang W, Lian M, et al. A novel recombinant FAdV-4 virus with fiber of FAdV-8b provides efficient protection against both FAdV-4 and FAdV-8b. *Viruses* (2022) 14:376. doi: 10.3390/v14020376

26. Wang B, Guo H, Zhao J. The pros and cons of cytokines for fowl adenovirus serotype 4 infection. *Arch Virol* (2022) 167:281–92. doi: 10.1007/s00705-021-05318-1

27. Abdul-Aziz T, Fletcher OJ. *Avian histopathology, 4th ed*. Jacksonville, FL: American Association of Avian Pathologists, Inc (2016).

28. Pan Q, Yang Y, Gao Y, Qi X, Liu C, Zhang Y, et al. An inactivated novel genotype fowl adenovirus 4 protects chickens against the hydropericardium syndrome that recently emerged in China. *Viruses* (2017) 9:216. doi: 10.3390/v9080216

29. Yu G, Wang Y, Zhang M, Lin Y, Tang Y, Diao Y. Pathogenic, phylogenetic, and serological analysis of group I fowl adenovirus serotype 4 SDSX isolated from Shandong, China. *Front Microbiol* (2018) 9:2772. doi: 10.3389/fmicb.2018.02772

30. Niu Y, Sun Q, Shi Y, Ding Y, Li Z, Sun Y, et al. Immunosuppressive potential of fowl adenovirus serotype 4. *Poult Sci* (2019) 98:3514–22. doi: 10.3382/ps/pez179

31. Ren G, Wang H, Yan Y, Liu F, Huang M, Chen R. Pathogenicity of a fowl adenovirus serotype 4 isolated from chickens associated with hydropericardium-hepatitis syndrome in China. *Poult Sci* (2019) 98:2765–71. doi: 10.3382/ps/pez042

32. Liu R, Zhang Y, Guo H, Li N, Wang B, Tian K, et al. The increased virulence of hypervirulent fowl adenovirus 4 is independent of fiber-1 and penton. *Res Vet Sci* (2020) 131:31–7. doi: 10.1016/j.rvsc.2020.04.005

33. Tian K, Guo H, Li N, Zhang Y, Wang Z, Wang B, et al. Protection of chickens against hepatitis-hydropericardium syndrome and Newcastle disease with a recombinant Newcastle disease virus vaccine expressing the fowl adenovirus serotype 4 fiber-2 protein. *Vaccine* (2020) 38:1989–97. doi: 10.1016/j.vaccine.2020.01.006

34. Yi JS, Cox MA, Zajac AJ. T-Cell exhaustion: characteristics, causes and conversion. *Immunology* (2010) 129:474–81. doi: 10.1111/j.1365-2567.2010.03255.x

35. Grgić H, Poljak Z, Sharif S, Nagy É. Pathogenicity and cytokine gene expression pattern of a serotype 4 fowl adenovirus isolate. *PloS One* (2013) 8:1–10. doi: 10.1371/journal.pone.0077601

36. Awate S, Babiuk LA, Mutwiri G. Mechanisms of action of adjuvants. *Front Immunol* (2013) 4:114. doi: 10.3389/fimmu.2013.00114

37. Matos M, Grafl B, Liebhart D, Hess M. The outcome of experimentally induced inclusion body hepatitis (IBH) by fowl aviadenoviruses (FAdVs) is crucially influenced by the genetic background of the host. *Vet Res* (2016) 47:69. doi: 10.1186/s13567-016-0350-0

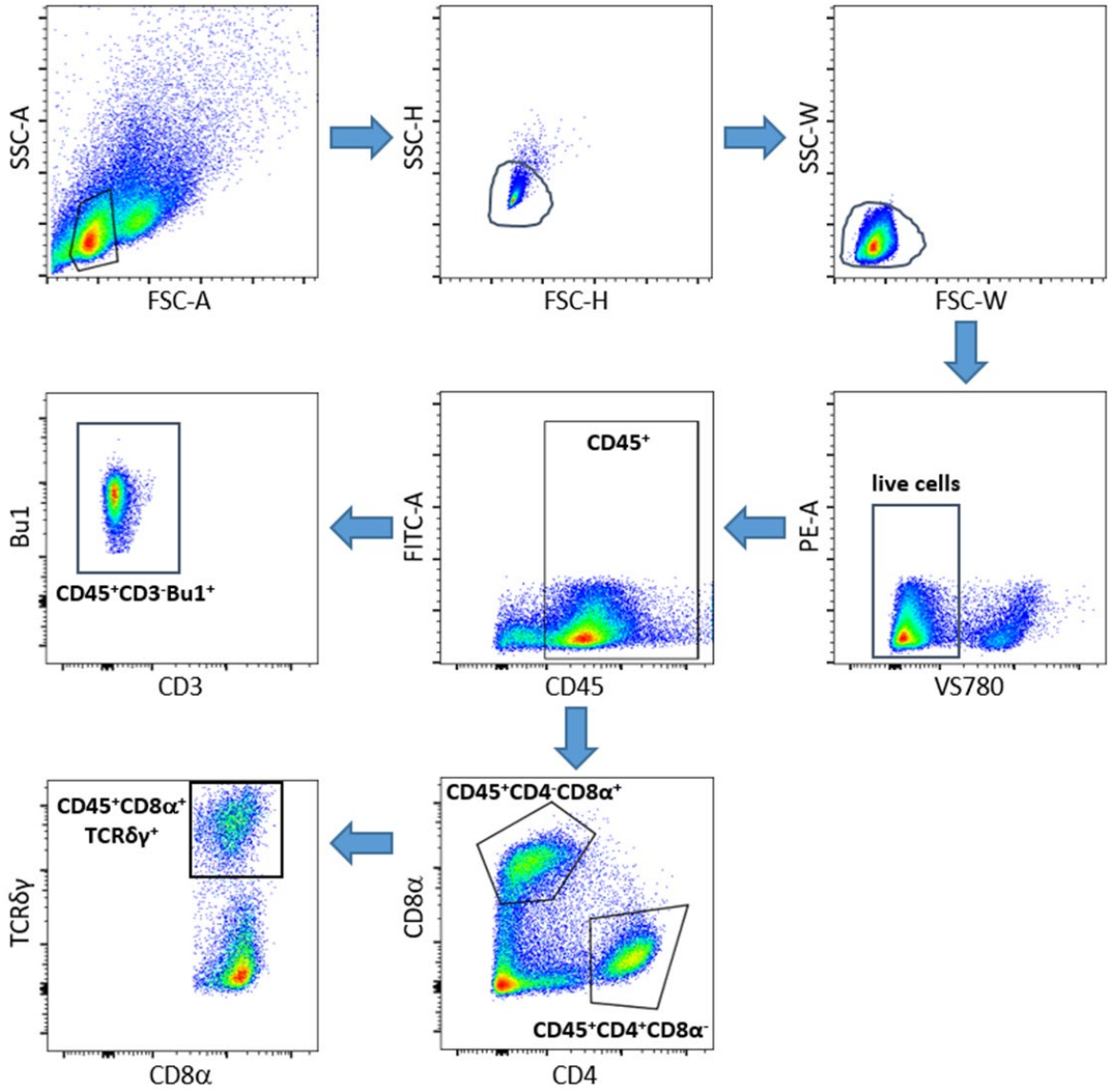
38. Henry Dunand CJ, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, et al. Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. *Cell Host Microbe* (2016) 19:800–13. doi: 10.1016/j.chom.2016.05.014

39. Stadlbauer D, Rajabhathor A, Amanat F, Kaplan D, Masud A, Treanor JJ, et al. Vaccination with a recombinant H7 hemagglutinin-based influenza virus vaccine induces broadly reactive antibodies in humans. *MSphere* (2017) 2:e00502–17. doi: 10.1128/mSphere.00502-17

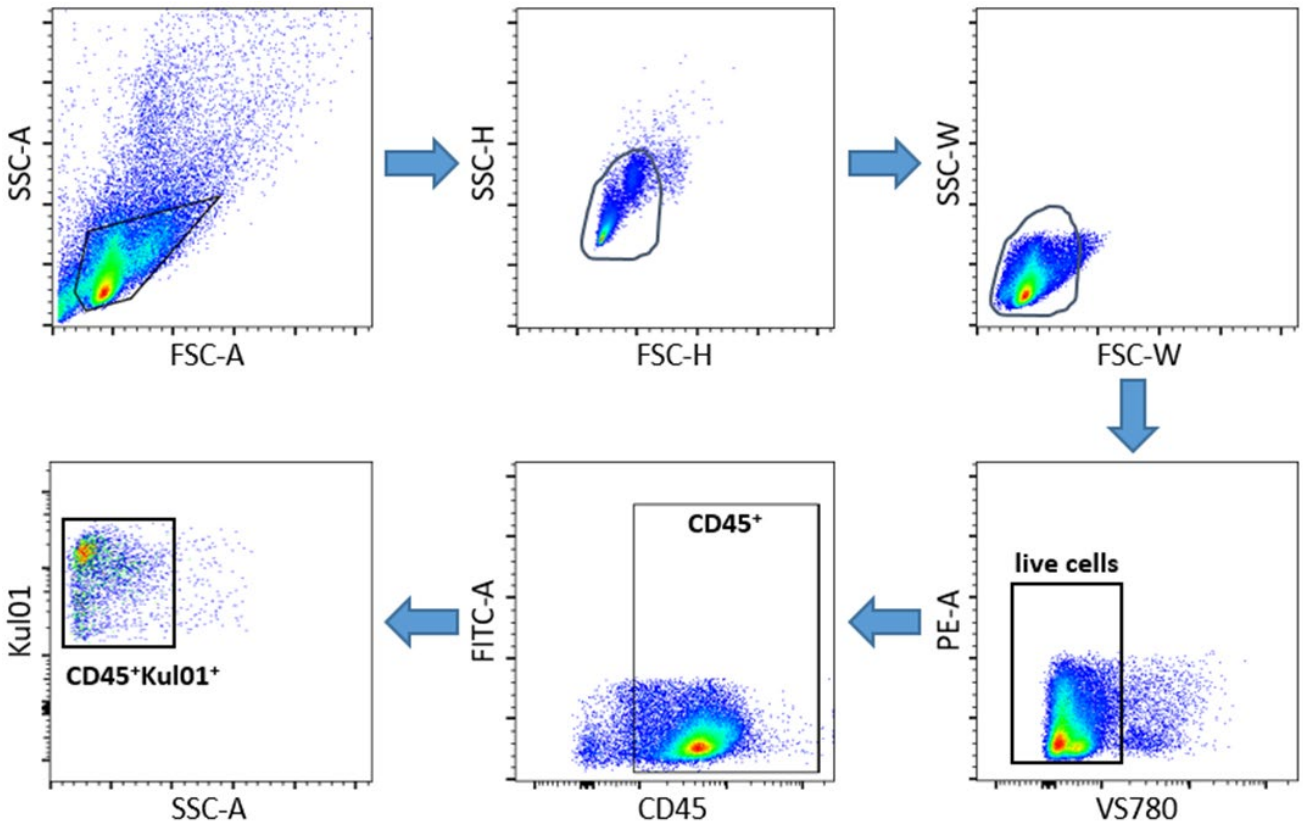
40. Sicca F, Neppelenbroek S, Huckriede A. Effector mechanisms of influenza-specific antibodies: neutralization and beyond. *Expert Rev Vaccines* (2018) 17:785–95. doi: 10.1080/14760584.2018.1516553

41. Hu Z, Zhao J, Zhao Y, Fan X, Hu J, Shi L, et al. Hemagglutinin-specific non-neutralizing antibody is essential for protection provided by inactivated and viral-vectored H7N9 avian influenza vaccines in chickens. *Front Vet Sci* (2020) 6. doi: 10.3389/fvets.2019.00482

Supplementary File 1a: Gating strategy of immune cell populations analysed by flow cytometry.
Lymphocyte subsets were determined through FSC-A/SSC-A plot, followed by doublet discrimination through FSC-H/SSC-H and FSC-W/SSC-W. Subsequently dead cells were excluded by using the BD Horizon™ Fixable Viability Stain 780. Live cells were then gated against $CD45^+$ marker. Within the $CD45^+$ cell population, $Bu1^+$, $CD4^+$ and $CD8\alpha^+$ were gated. From the total $CD8\alpha^+$ cells, the $CD8\alpha^+TCR\gamma\delta^+$ subpopulation was gated as indicated.



Supplementary File 1b: Gating strategy of immune cell populations analysed by flow cytometry.
Monocytes/macrophages were determined by applying an extended FSC-A/SSC-A plot (given the size of the cells) followed by doublet discrimination through FSC-H/SSC-H and FSC-W/SSC-W. Dead cells were excluded by using the BD Horizon™ Fixable Viability Stain 780. Live cells were then gated against $CD45^{+}$ marker, and subsequently for the monocytes/macrophage (Kul01) population.



Supplementary file 2. Complete panels of antibody combinations utilized in the study and their fluorescence labelling strategy

antigen	clone (isotype)	fluorochrome/label	labelling strategy	source of primary monoclonal antibody	catalogue number
panel 1					
CD45	LT40 (IgM)	APC	direct conjugation	Southern-Biotech	8270-11
CD4	CT4 (IgG1)	PE-cy7	direct conjugation	Southern-Biotech	8210-17
CD8α	3-298 (IgG2b)	PE	direct conjugation	Southern-Biotech	8405-09
TCR γδ	TCR1 (IgG1)	BIOTIN	biotin-streptavidin[a] conjugation	Southern-Biotech	8230-08
live/dead staining	-[b]	APC Cy7	direct conjugation	BD Horizon	565388
panel 2					
CD45	LT40 (IgM)	APC	direct conjugation	Southern-Biotech	8270-11
Bu1	AV20 (IgG1)	PacBlu	direct conjugation	Southern-Biotech	8395-26
Kul01	Kul01 (IgG1)	PE	direct conjugation	Southern-Biotech	8420-09
live/dead staining	-	APC Cy7	direct conjugation	BD Horizon	565388

[a]Brilliant Violet 421™ Streptavidin, BioLegend
[b]not applicable

9. Oral presentations

9.1. Divergent expression profiles in a genomically conserved virulent/attenuated fowl adenovirus serotype 4 (FAdV-4) strain pair identifies possible biomarkers for virulence modulation

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Purpose of work

FAdVs harbor huge diversity, with unknown virulence factors. Despite a link between specific pathologies and serotypes, virulence differences even between strains of the same serotype remain a conundrum. To address virulence modulation beyond the genetic level, we compared temporal proteomes of FAdVs of the same parental-genomic origin. Virulent FAdV-4 (*AG234*) and its *in vitro*-attenuated derivate (*INT4*) represent the first such strain pair, confirmed as distinctly pathogenic in animal trials.

Materials and methods

Full genomes were Illumina-sequenced. Protein samples were prepared from standardized LMH cell infections, harvested in 6 h-intervals. Viral proteomes were analyzed by quantitative time-course DIA-MS in a quadrupole Orbitrap mass spectrometer. Replication kinetics were monitored by titrating intra- and extracellular virus.

Results

Genomes of distinctly pathogenic FAdVs were widely conserved with main exceptions in non-coding regions, featuring manifold repeat variations.

Of 44 total ORFs, 24 (INT4) and 25 (AG234) proteins were identified by MS, several lacking expression evidence so far. Among structural proteins fiber-2, having few amenable fragmentation sites, remained undetected though later on confirmed in immunoblotting.

Avirulent INT4 had a severely impaired development of hexon, along with 100K, matching with a delayed *in vitro* replication. Yet this was accompanied by a steep rise of DNA pol, pTP and DBP which remained near-basal level in AG234. Further differences occurred in 3´-terminally encoded ORFs 2 and 13, spiking at 42/48 hpi in the attenuated proteome vs. basal or gradually progressive abundance in the virulent proteome.

Discriminatory 5´-terminal products were the two lipase homologues, with a converse trend: after initial synchronicity ORF19 inclined more rapidly in AG234, while ORF19A accumulated within 30-48 hpi in INT4. As the only non-shared protein, ORF16 was abundantly, but exclusively detected in AG234.

Conclusions

Temporally-quantitatively retarded structural protein synthesis reflects the inefficient replication of attenuated vs. virulent FAdV. Despite this, an overshooting recruitment of the replication machinery occurred, possibly as counteractive mechanism.
In absence of major genomic differences, progressively discordant proteomic signatures suggest terminal ORFs with already purported functions in transcriptional control or host interference as biomarkers for virulence modulation.

9.2. Local immune response provided by fowl adenovirus (FAdV) fiber vaccine plays a key role in protecting chickens from hepatitis-hydropericardium syndrome (HHS)

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Hepatitis-hydropericardium syndrome (HHS) is a disease affecting chickens caused by fowl adenovirus (FAdV) serotype 4. Subunit vaccines based on recombinant capsid proteins have demonstrated great potential, but their protection mechanisms need to be further unraveled. In this study, specific pathogen-free one-day-old chickens were vaccinated with a recombinant FAdV fiber and boosted at seven-day-old, whereas challenge and negative control birds were administered adjuvant and PBS, respectively. At 21-day-old, chickens were intramuscularly infected with a virulent FAdV-4 strain, while negative control and vaccine-only group received PBS. Endpoints for protection included clinical signs, pathological lesions and viral load in target organs. The systemic immune response was monitored before and after challenge via enzyme-linked immunosorbent assay (ELISA), virus neutralization and flow cytometry (FCM) in peripheral blood mononuclear cells (PBMCs); the local response was investigated in liver with FCM over the course of infection.
The vaccination reduced clinical signs, viral load in target organs and hepatomegaly. It also prevented atrophy in thymus and bursa of Fabricius. Despite the development of pre-challenge antibodies measured by ELISA, there was no neutralizing activity against FAdV-4 before challenge. However, B-cells significantly increased locally in the liver of vaccinated birds at all time points post-challenge. This trend was sporadically mirrored by monocytes/macrophages, $CD4^+$, $CD8\alpha^+$ and $CD8\alpha^+TCR\gamma\delta^+$ T-cells locally, whereas systemically, only monocytes/macrophages and $CD8\alpha^+TCR\alpha\beta^+$ T-cells significantly increased at selected time points post-challenge.

These results suggest that fiber vaccines protect chickens from HHS by priming a potent immune response on a local level, rather than exclusively relying on systemic neutralizing antibodies.

9.3. Fowl adenovirus (FAdV) induced diseases: vaccination strategies

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State-of-the-art taxonomy divides fowl adenoviruses (FAdVs) in five species (FAdV-A to -E) based on their genotypes, and twelve serotypes (FAdV-1 to -8a, and -8b to -11), based on their serological features. These viruses are responsible for three disease complexes affecting chickens: adenoviral gizzard erosion (AGE), associated with FAdV-A serotype 1, hepatitis-hydropericardium syndrome (HHS), associated with FAdV-C serotype 4, and inclusion body hepatitis (IBH), associated with FAdV-D serotypes 2 and 11, and FAdV-E serotypes 8a and 8b.
Over the past two decades, these diseases became an increasing burden for the poultry industry, resulting in substantial economical losses worldwide. Aside from biosecurity protocols, protection strategies applied in the field are so far limited to commercial live or inactivated vaccines against HHS and/or IBH, only available in selected countries, with Europe solely relying on autogenous formulations, and therefore farm-specific protection. Consequently, one prominent drawback of these vaccines is the lack of broad-spectrum coverage, which represents the ultimate goal to efficiently immunize chickens against FAdVs, given the diverse aetiology of FAdV-related diseases.
As a result, a plethora of studies investigating different kind of experimental vaccines emerged throughout the years, with a particular focus on subunit vaccines based on recombinant FAdV capsid proteins, which are cost- and time-effective.
In order to achieve a more comprehensive protection, subunit vaccines recently evolved from monotype antigen formulations toward the novel concept of chimeric recombinant proteins retaining epitopes from different FAdV serotypes, or even species, thus expanding their protective spectrum. This shift was mirrored in most recent studies on live and inactivated vaccines, which investigated the immunizing efficiency of recombinant hybrid viruses merging characteristics of different FAdVs, in some instances incorporating the VP2 protein from Infectious Bursal Disease Virus into FAdV-4, or the Newcastle virus modified as vector for the FAdV-4 fiber-2 protein.
In conclusion, the landscape of experimental vaccines against FAdV-related diseases is rapidly evolving to pursue extensive broad-spectrum immunization in order to fulfil the urgent need of an efficient and comprehensive protection strategy in the field.

10. Additional works

In addition to the published fiber-based vaccines investigated in the present thesis, two recombinant FAdV-E penton base (Pb) proteins were expressed and tested as antigens *in vivo* against IBH. Furthermore, in the process of optimizing the vaccine formulations, recombinant proteins of interest were analysed and compared via gel filtration chromatography. Additionally, one of the chimeric fiber proteins (crecFib-8b/8a) was also expressed via *E. coli* and its immunogenicity compared to the baculovirus-derived formulation.

10.1. Recombinant FAdV-E penton base against IBH

Two *in vivo* vaccination studies were performed to elucidate the potential of the penton base as protective antigen against IBH.

Material and methods

Cloning and expression of FAdV-E penton base proteins

Two penton base proteins from two different FAdV-E serotypes were expressed in the present study: Pb-7, from FAdV-7 (reference strain YR36, GenBank accession nr. KT862809), and Pb-8b, from FAdV-8b (field isolate 14-259, GenBank accession nr. MK572865). Briefly, the viral strains were propagated on primary chicken-embryo liver (CEL) cells and, after 3-fold plaque purification, the viral DNA was extracted from the supernatant using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The Pb gene was amplified via PCR, whereas cloning and expression were carried out via baculovirus Bac-to-Bac expression system (Invitrogen, Vienna, Austria) according to manufacturer's instruction. Purification of the recombinant proteins, visualization on

SDS-polyacrylamide gel and confirmation on western blot were performed as previously described (Schachner *et al*., 2014).

Protection studies

Two *in vivo* studies were carried out: a Pb-7 and a Pb-8b protection study.

Embryonated eggs from either SPF broilers (Animal Health Service, Deventer, The Netherlands) (Pb-7 protection study) or SPF layers (Valo BioMedia GmbH, Osterholz-Scharmbeck, Germany) (Pb-8b protection study) were hatched in our facilities and randomly divided into three different groups (up to 15 birds/group) to serve for vaccine/challenge, challenge control and negative control. The birds were vaccinated during their first day of life with 50 µg of either Pb-7 (Pb-7 study) or Pb-8b (Pb-8b study) mixed 1:1 with GERBU adjuvant P (GERBU Biotechnik GmbH, Heidelberg, Germany) in a 500 µl intramuscular injection. For the Pb-8b protection study, a booster vaccination containing 100 µg of Pb-8b was administered at 25 days of life using Freund incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) to ensure a better antibody development prior challenge. Birds in the challenge and negative control received only adjuvant and sterile phosphate buffered saline (PBS), respectively. The viral challenge was carried out at 21 (Pb-7 study) and 33 days old (Pb-8b study) with an intramuscular injection consisting in $10^{6.2}$ tissue culture infective dose 50 ($TCID_{50}$) of FAdV-8b (field isolate 13-18153, GenBank accession nr. MK572862). The challenge strain was prepared via 3-fold plaque purification, propagated on primary CEL cells, as described by Schat & Sellers (2008); viral titres were determined by endpoint titration (Reed & Muench, 1938) and the strain was subjected to full genome sequencing and analysed as described by Schachner *et al.* (2019). Birds in the negative control received a sterile PBS injection instead. Blood samples for antibody investigation were collected weekly from all the birds from their third week of life. At 3, 5 and 7 dpc (days

post challenge) blood was collected from all the birds and up to five individuals per group were sacrificed and submitted to necropsy. Birds that died due to infection were analysed immediately. Endpoints for protection included clinical signs from daily monitoring in the time period after challenge, gross pathology and liver-body weight (BW) ratio assessment. Statistical analyses were performed with the software package SPSS Version 26 (IBM SPSS Statistics; IBM Corp., Armonk, New York, USA); *p* values ≤ 0.05 were considered significant.

All procedures were discussed and approved by the institutional ethics and welfare committee and the national authority according to §§26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 20212 (license number: GZ. 68.205/0048-V/3b/2019).

Assessment of the humoral response

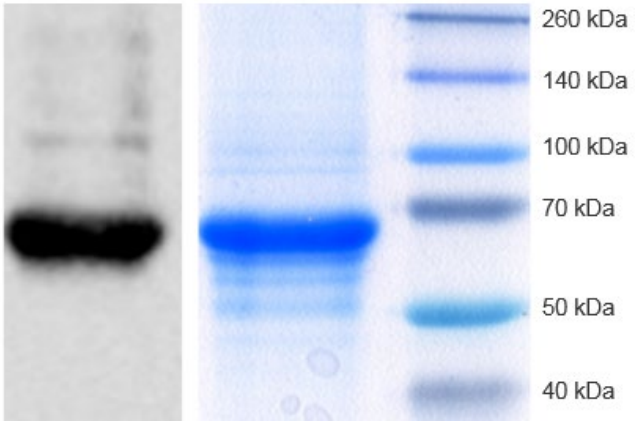
Sera collected during the experiments were tested on ELISA plates coated with 2 µg/well of the respective vaccination antigen following the protocol described by Feichtner *et al.* (2018a; 2018b). Virus neutralization test was carried out as described by Schachner *et al.* (2014).

Results

Cloning and expression of FAdV-E penton base proteins

The two recombinant penton base proteins, Pb-7 and Pb-8b, were successfully expressed and purified. A visualization on western blot and SDS-polyacrylamide gel is exemplarily shown for Pb-8b (Fig.2).

Fig. 2. The recombinant penton base protein Pb-8b shown on western blot and SDS-polyacrylamide gel after purification

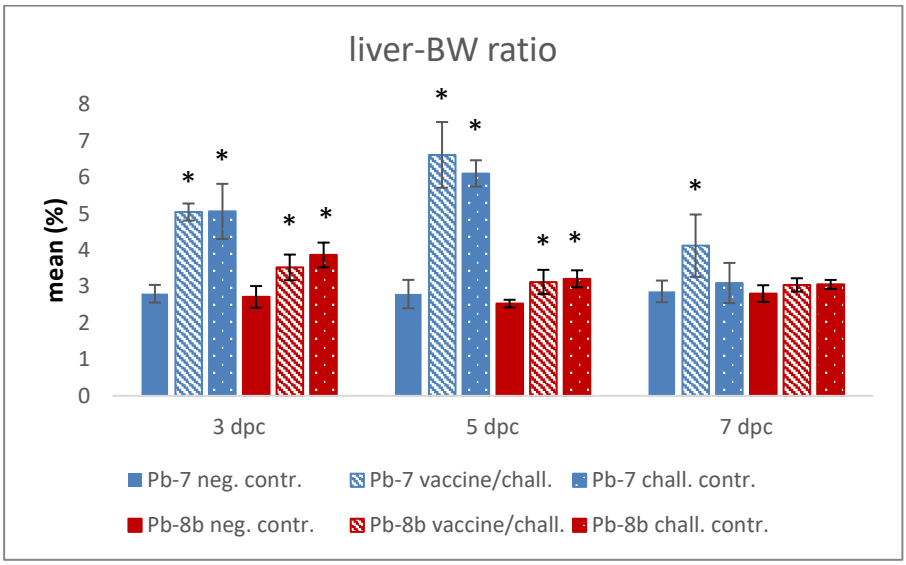


Protection studies

In the Pb-7 animal experiment, mortality occurred between 2 and 5 dpc, with a rate of 35.7% (5/14) in the vaccine/challenge group and 40% (6/15) in the challenge control. The surviving birds showed clinical affection up to 7 dpc, with only two exceptions in the vaccine/challenge group and one in the challenge control. In the Pb-8b animal experiment, only one bird showed mild clinical affection from 1 to 3 dpc in the challenge control. In both protection studies, the majority of the infected birds presented hepatic lesions compatible with IBH, including hepatomegaly, marbled appearance, focal necrosis and haemorrhages. Only 3/14 birds in Pb-7 vaccine/challenge group, and 1/15 bird in Pb-8b challenge control did not show hepatic affection. Other gross lesions were occasionally observed in kidneys, pancreas and muscles. No clinical signs or lesions were recorded in any of the negative control groups at any time point.

The liver-BW ratio was significantly increased at 3 and 5 dpc in all the infected groups from both protection studies, whereas at 7 dpc, only the vaccine/challenge group of the Pb-7 animal experiment was still elevated (Fig.3).

Fig. 3. Mean liver-BW ratio of each experimental group of both Pb-7 (marked in blue) and Pb-8b (marked in red) protection studies. The asterisk indicates significant differences compared to the respective negative control ($p \leq 0.05$)

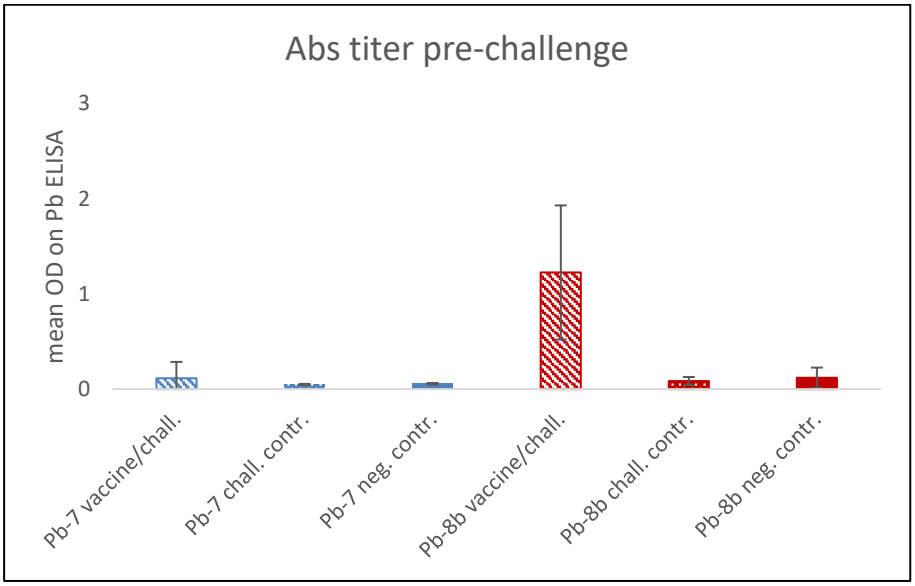


ELISA and virus neutralization test (VNT)

In the Pb-7 protection study, vaccinated birds did not develop high antibody titers, with an ELISA-measured mean OD of 0.11±0.17 at 21 days old, prior challenge (20 dpv, days post vaccination), compared to OD 0.05±0.01 of adjuvant-injected birds, and OD 0.06±0.01 of the negative control (Fig.4).

In the Pb-8b protection study, a similar observation was made at the same time point (20 dpv, prior challenge), with a mean OD of 0.09±0.14 in the vaccinated group (vs. 0.05±0.02 and 0.06±0.01 of the adjuvant-injected birds and negative control, respectively). The antibody titers rose after booster, with the mean OD of the vaccinated group reaching 1.23±0.70 at 6 dpb (days post booster) and immediately prior challenge (vs. 0.08±0.04 and 0.12±0.11 of the adjuvant-injected birds and negative control, respectively). However, this did not translate in neutralizing activity, since none of the pre-challenge sera showed detectable nAbs against the FAdV-8b challenge strain on VNT.

Fig. 4. Pre-challenge Abs titers measured on homologous-coated ELISA for both Pb-7 (marked in blue) and Pb-8b (marked in red) protection studies



10.2. Optimization of vaccine formulations

10.2.1. Gel-filtration chromatography

The purity and molecular size of FAdV recombinant proteins such as FAdV-8a and -8b single fibers (Fib-8a and Fib-8b) were analysed and compared via size-exclusion chromatography in the Institute of Virology, Vetmeduni Vienna.

Materials and methods

The recombinant proteins Fib-8a and Fib-8b were expressed and purified as previously described (Schachner *et al*., 2014; De Luca *et al*., 2020). Eluates obtained from protein purification were subsequently analysed through gel-filtration chromatography via Superose 6 10/300 GL column, and the results were visualized with the software UNICORN 5.0, according to manufacturer's protocol. The presence of corresponding

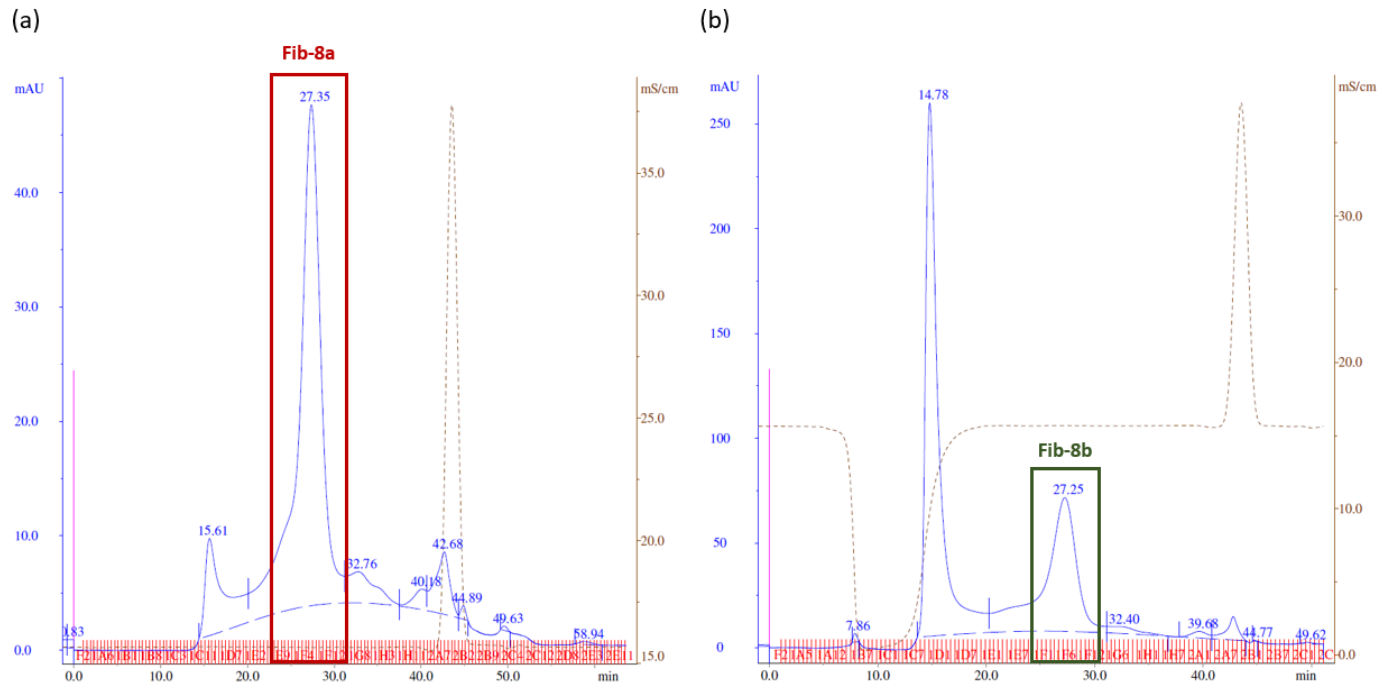
recombinant proteins in the eluted fractions was confirmed via western blot, and their molecular weight was determined using β-amylase (223 kDa), apoferritin (480 kDa) and thyroglobulin (670 kDa) as molecular weight markers.

Results

The eluted fraction corresponding to Fib-8a represented the highest peak visualized during the gel-filtration chromatography run, indicating a high degree of purity (Fig. 5a). On the other hand, Fib-8b was identified in a distinct, similar peak, albeit in this case a predominant peak was recorded in the preliminary exclusion volume of the run, which contains particles too big to be processed during filtration (Fig. 5b).

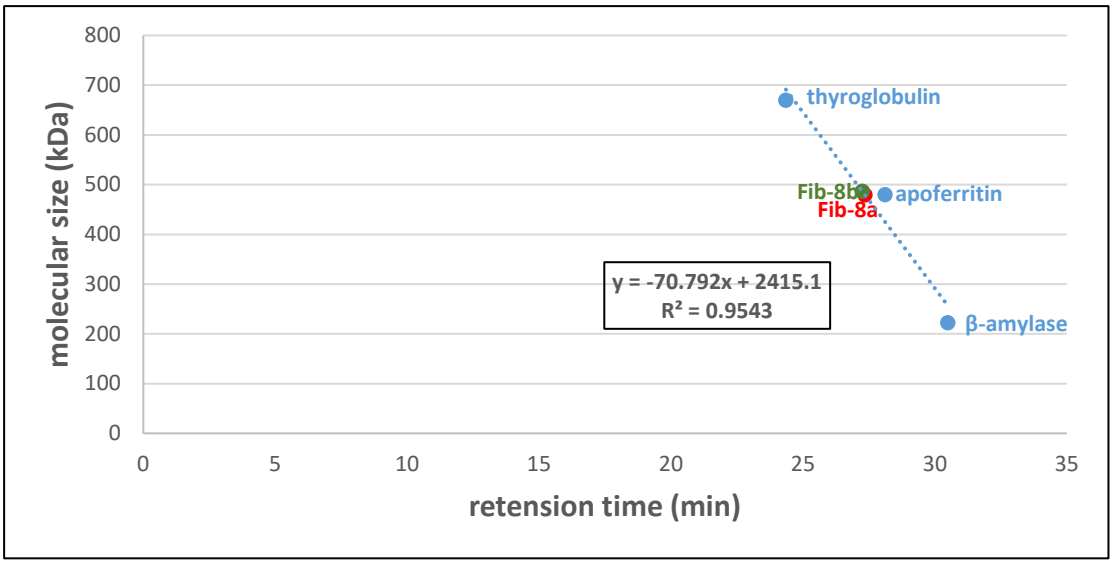
The calibration run with β-amylase, apoferritin and thyroglobulin was used to define the measurable range and the function to calculate the molecular weight of the recombinant proteins (Fig. 6). Fib-8a and -8b were 478.94 kDa and 486.02 kDa, respectively, which is ~8 times the size of the fiber monomer (60 kDa).

Fig. 5. Gel-filtration chromatography of Fib-8a (a) and Fib-8b (b) on a Superose 6 10/300 column



superose 10 300007:10_UV
superose 10 300007:10_Cond
superose 10 300007:10_Conc
superose 10 300007:10_Fractions
superose 10 300007:10_Inject
superose 10 300007:10_UV@01,BASEM

Fig. 6. Determination of the molecular mass of Fib-8a and -8b according to calibration with β-amylase (223 kDa), apoferritin (480 kDa) and thyroglobulin (670 kDa)



10.2.2. Baculovirus vs. *E. coli*-expressed recombinant chimeric fiber

One of the relevant recombinant FAdV proteins used as subunit vaccine in the present work (crecFib-8b/8a) was selected because most favourable in protecting chickens against IBH, and was expressed to evaluate feasibility and efficiency of crecFib expression in the prokaryotic *E. coli*, compared to the eukaryotic baculovirus expression system.

Material and methods

The baculovirus-expression of crecFib-8b/8a was carried out in house as previously described (Schachner *et al.*, 2022), whereas expression and purification of the *E. coli* crecFib-8b/8a was conducted by Vaxxinova Münster, Germany. Briefly, upon cloning into the pET28 expression vector (Novagen, Merck Millipore, Darmstadt, Germany), the construct was transformed into BL21 (DE3) competent cells. The protein was purified from inclusion bodies with 8M urea, followed by resuspension with 2%

sarcosine (designated batch 1) or by ultrafiltration and removal of lipopolysaccharides with a column (batch 4).

Immunogenicity of the *E. coli*- vs. the baculovirus-expressed crecFib-8b/8a was comparatively assessed by immunizing 1-day-old SPF broiler chicks (Animal Health Service, Deventer, The Netherlands) with 50 µg of baculovirus-expressed crecFib-8b/8a (n = 8), *E. coli*-expressed crecFib-8b/8a-batch 1 (n = 7), or *E. coli*-expressed crecFib-8b/8a-batch 4 (n = 8), in a formulation with oil-based adjuvant prepared by Vaxxinova Nijmegen, The Netherlands. Sera were collected in weekly intervals, and antibody development was investigated with ELISAs coated with each of the three antigen fractions (Feichter *et al.*, 2018a), and VNT against virulent FAdV-8a and -8b field isolates (11-16629 and 13-18153, respectively).

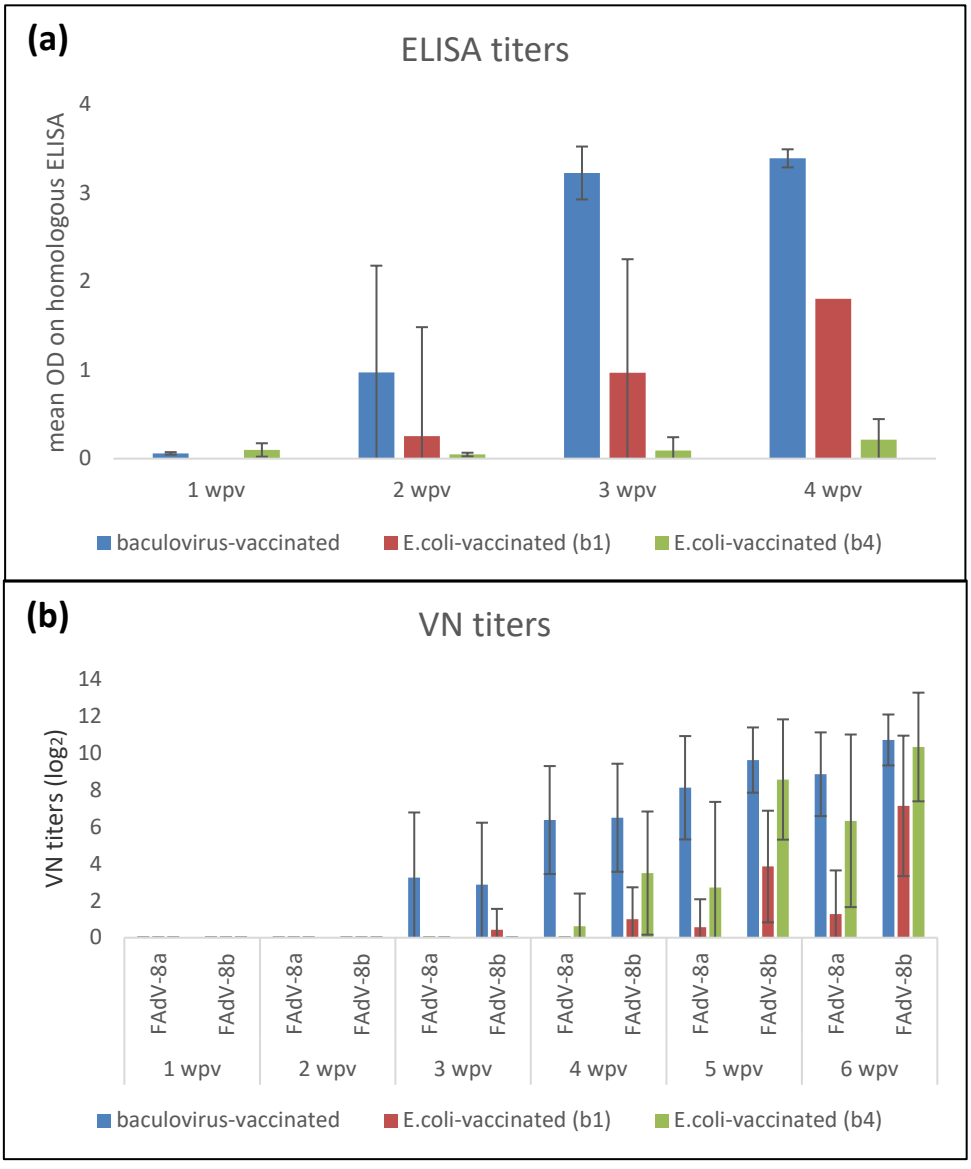
All procedures were discussed and approved by the institutional ethics and welfare committee and the national authority according to §§26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 20212 (license number: GZ. 68.205/0006-V/3b/2019).

Results

For the baculovirus-expressed construct, antibody development was first noted at 2 weeks post vaccination (wpv) on the ELISA coated with the homologous antigen fraction (Fig. 7a). Sera of birds immunized with the baculovirus-expressed construct also reacted to some extent on ELISA coated with *E. coli*-expressed crecFib-8b/8a-batch 1, although more scattered and unrelated in magnitude compared to the strictly homologous ODs. In birds immunized with the baculovirus-expressed construct, nAbs were first detected at 3 wpv, and some individuals already exhibited bilateral activity against FAdV-8a and FAdV-8b (Fig. 7b). At the last two time points (5-6 wpv), 8/8 birds had developed bilateral neutralization with neutralizing titers ranging from 3 to 14 $\log_2$.

The *E. coli*-crecFib-8b/8a immunized birds showed first indicative ODs on the homologous ELISA at 3 wpv (batch 1). Development of nAbs was overall more reluctant and heterogeneous in these birds, but at 6 wpv, 2/7 birds showed bilateral neutralizing activity and an additional 4 birds presented nAbs against FAdV-8b. The *E. coli*-crecFib-8b/8a (batch 4) induced bilateral nAbs in 5/8 birds by 6 wpv, and nAbs against FAdV-8b in one bird, although it generally failed as coating antigen on the ELISA. This discrepancy, however, might be explained by prolonged storage of the protein between immunization and use as coating antigen, and possible degradation processes.

Fig. 7. Mean Abs titers expressed in OD measured on homologous-coated ELISA (a), and VN titers (b) for each experimental group



11. Discussion

Since their discovery in the second half of the last century, FAdVs have been isolated worldwide and, over the last two decades, they became an increasing burden for the poultry industry (Helmboldt & Frazier, 1963; Cheema *et al*., 1989; Gjevre *et al*., 2013; Schachner *et al*., 2021). In particular, HHS (caused by FAdV-4) and IBH (caused by FAdV-2, -8a, -8b, -11) are currently causing substantial economic losses on a global level (Schachner *et al*., 2021). On top of vertical transmission, confirmed by the isolation of FAdVs from embryonated eggs (Olson, 1950; Yates & Fry, 1957; Grafl *et al*., 2012), the virus can be horizontally transmitted among chickens via oral-faecal route, and airborne transmission has also been recently described for FAdV-4 adding to the data supplied by earlier studies (Clemmer, 1972; Cook, 1983; Jones & Georgiou, 1984; Li *et al*., 2019). Information over possible treatments against FAdV-induced diseases is so far limited. As chickens have been shown to undergo hypoglycaemia during IBH infection, supportive treatment with sugar and sodium bicarbonate helps reducing the metabolic acidosis (Venne, 2013; Matos *et al*., 2018). More recently, an antiviral therapy has been successfully tested against HHS under experimental conditions, and the use of supportive supplements such as arginine is being investigated as well (Xiang *et al*., 2022; Yin *et al*., 2022). Despite these efforts, there is no effective treatment against FAdV-induced diseases available in the field to date, which stresses the importance of a solid prevention, a general paradigm in poultry production.

Commercial vaccines against these viruses are only available in selected countries and they are limited to live or inactivated vaccines against FAdV-4 and/or FAdV-8b. Currently, European countries solely resort to autogenous formulations to prevent outbreaks of IBH, as HHS is not reported yet, and therefore rely on farm-specific protection. In fact, one of the major pitfall of these vaccines is the lack of a broadly

protective spectrum, which represents the ultimate goal to efficiently immunize chickens against FAdVs. The observed shifts toward outbreaks with other viral types after implementation of a certain vaccination regimen against the previously dominating ones in the field has led to the conclusion that immunity developed against a specific FAdV type does not necessarily confer protection against other types (Steer *et al*., 2011; Venne, 2013; Wang *et al*., 2018c; Bertran *et al*., 2021; Mo, 2021). Furthermore, the occurrence of natural recombination between different serotypes has also been reported in field isolates (Schachner *et al*., 2019). This contributes to increase the variability of the dominant serotypes in the field, which outlines an epidemiologic picture that is very fragile and highly prone to shifts. Therefore, the situation calls for an urgent need of a comprehensive immunization strategy against the diverse aetiology of FAdV-related diseases. As a result, experimental vaccines have been widely investigated throughout the last decade, including live and inactivated whole virus formulations, and single component antigens based on FAdV capsid proteins. Subunit vaccines based on FAdV-4 penton base and fiber-2 were effective in protecting chickens against HHS (Shah *et al*., 2012; Schachner *et al*., 2014; Wang *et al*., 2018b; Chen *et al*., 2018; Wang *et al*., 2019; Hu *et al*., 2021; Yin *et al*., 2021). However, differently from HHS and its monotype aetiology, subunit vaccines against IBH need to overcome the issue of a greater diversity of causative strains, especially in regards to the fiber, which is the most type-specific among the FAdV antigenic domains (Schachner *et al*., 2016; 2019). A FAdV-8b fiber formulation was successfully used to induce nAbs in breeders, which translated in protection of the progeny against a virulent strain from homologous serotype (Gupta *et al*., 2017). However, information over potential cross-protection from fiber vaccines against different IBH-causing serotypes, in either horizontal or vertical setting, was still lacking. In order to clarify this crucial point, a FAdV-8a fiber vaccine was used to immunize SPF

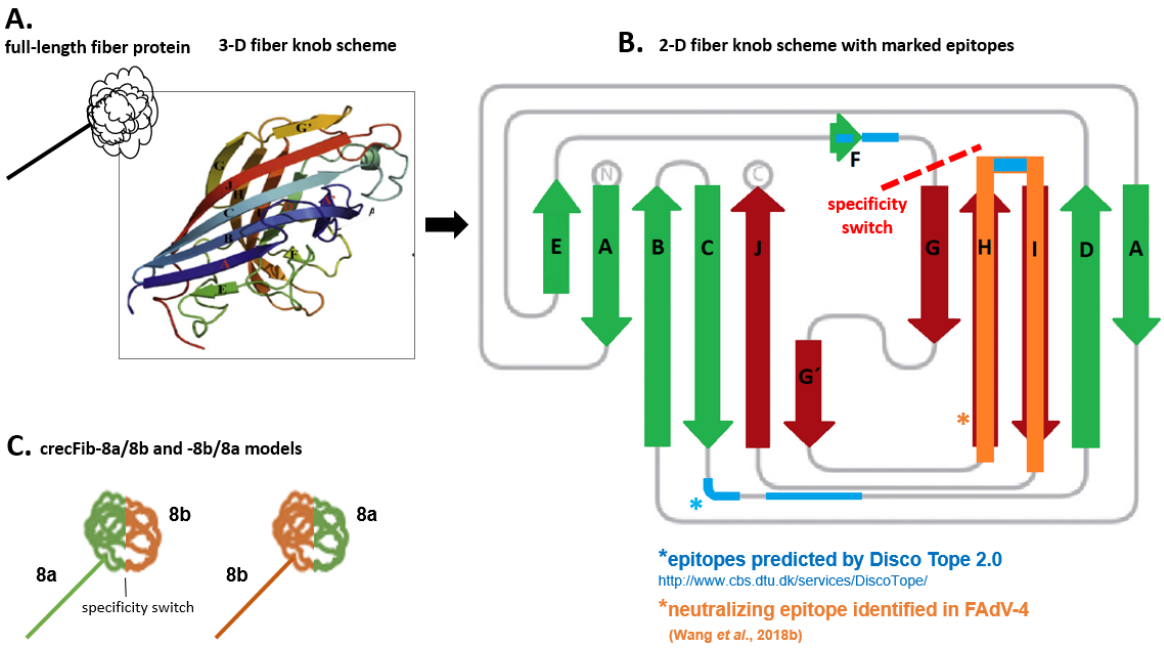
broiler chicks against IBH, which were subsequently challenged with a FAdV-8a or -8b virulent strain (De Luca *et al*., 2020). Taking into account the pronounced antigenic specificity of the fiber protein and the occurrence in the field of recombinant strains with exchanges in their antigenic domains, such as hexon and fiber (Schachner *et al*., 2019), all the strains utilized in the study were fully typed through whole-genome sequencing, in order to avoid intrinsic biases when addressing cross-protection. The results, reported in the first experiment of the present thesis, demonstrated that the protection provided by a single fiber vaccine is in fact serotype-specific, and it fails to provide broad coverage against the diverse aetiology of IBH due to the induction of specific nAbs. Whereas promising results were obtained with live and inactivated whole virus formulations, able to protect chickens from multiple FAdVs serotypes (Pallister *et al*., 1993; Kim *et al*., 2014; Steer-Cope *et al*., 2019), the single-component nature of subunit vaccines makes them less promising candidates to reach wide-spectrum protection, since they lack the synergistic effect obtained from different antigens. In fact, *in vitro* findings in human adenoviruses (HAdVs) suggest that neutralization may be a result of interactions between antibodies against different capsid antigens (Wohlfart, 1988; Gahery-Segard *et al*., 1998). Nevertheless, further efforts to achieve cross-protective immunization were made by testing additional recombinant capsid proteins other than the FAdV fiber as vaccination antigens. In particular, the penton base protein had proved efficient within the framework of HHS, by stimulating the development of ELISA-measured antibodies and protecting chickens from FAdV-4 challenge (Shah *et al*., 2012). Furthermore, the amino acidic structure of the penton base is relatively conserved (Schachner *et al*., 2019), and it can therefore be hypothesized as a promising broad-spectrum vaccination antigen. To investigate such hypothesis, penton base proteins derived from two FAdV-E strains (FAdV-7 and -8b: Pb-7 and Pb-8b, respectively) were recombinantly expressed and used in two

different protection studies to immunize chickens against IBH caused by a virulent FAdV-8b strain. In the first study, Pb-7 failed to elicit high antibody titers before challenge, which might well be the reason why survival rate and macroscopic lesions of vaccinated/challenged birds did not differ from challenge control. In order to overcome the low immunogenicity observed for the penton base protein, a prime-booster immunization regimen was applied in the second study, utilizing Pb-8b as vaccine antigen, and detectable antibody titers were ensured via ELISA before challenge. However, this did not translate in protection, since severe hepatic lesions were present in both vaccinated and challenge control birds in a comparable measure. A possible reason for this outcome may be the absence of neutralizing activity in the vaccine-induced antibodies measured before challenge, which would support the hypothesis of nAbs being necessary to protect chickens against IBH (Gupta *et al*., 2017; 2018; Popowich *et al*., 2018; Steer-Cope *et al*., 2019; De Luca *et al*., 2020; Lu *et al*., 2022; Schachner *et al*., 2022). Therefore, no protection was observed with penton base vaccines utilized in the present work. Other studies reached similar outcomes, even within the framework of HHS, leading to somewhat contradictory results upon the efficacy of vaccines based on the penton base (Wang *et al*., 2018b; 2019; Aziz *et al*., 2019). In fact, to date, only one linear epitope was identified for this protein and tested for its protectiveness against FAdV-4 and, despite the induction of antigen-specific antibodies on ELISA, it only ensured a 50% survival rate against FAdV-4 (Aziz *et al*., 2019). Thus it is possible that linear penton base epitopes, although being among the most conserved between FAdVs, are insufficient to immunize birds against the disease, because unsuitable to elicit nAbs development. In this case, the folding of the protein may be crucial to maintain the functionality of conformational epitopes, possibly more relevant for protection, which may be an explanation for the discordant outcomes among penton base studies. These results

call for more in-depth investigations to clarify the role of this antigen in protection against FAdVs.

In order to design a broad-protective strategy against IBH, the present work introduced the novel concept of chimeric fiber proteins (crecFib): recombinant constructs that retain the full-length fiber structure and merge epitopes from different FAdV serotypes, by the engineering of a specificity switch in the terminal domain, the fiber knob (Fig. 8a, 8b) (Schachner *et al*., 2022). In the second study of the present thesis, two crecFib were expressed retaining epitopes from FAdV-8a and -8b (crecFib-8a/8b and crecFib-8b/8a) (Fig. 8c). However, only crecFib-8b/8a was able to successfully protect birds from both IBH-causing serotypes through the induction of bilateral nAbs (Schachner *et al*., 2022).

Fig. 8. Schematic representation of crecFib: A) full-length FAdV fiber protein with a magnified 3-D scheme of the terminal part of the fiber, the knob. B) 2-D scheme of the fiber knob with marked specificity switch introduced in the crecFib and highlight of epitopes previously described (Wang *et al*., 2018a) or predicted *in silico*. C) schematic models of crecFib-8a/8b and -8b/8a with different colours marking the identity switch between the two serotypes in the knob



The reason for the failure of crecFib-8a/8b in inducing adequate nAbs development may be due to structural differences between the two constructs, specifically in a region that merges residues from opposite sides of the specificity switch, each expressing separate epitopes in the linear sequences (Fig. 8b). The conformational variations lead to the identification of a major neutralizing epitope in the relevant area, possibly located to the right of the specificity switch for the FAdV-8a fiber, and to the left for the FAdV-8b fiber (Schachner *et al*., 2022). These findings are supported by serotype-specificity of nAbs induced by single fiber antigens from FAdV-8a and -8b (Gupta *et al*., 2017; De Luca *et al*., 2020). Therefore, the successful construct crecFib-8b/8a was able to achieve broad coverage against IBH by condensing these serotype-specific neutralizing epitopes into a single-antigen component. Differently from most of the research groups working on FAdV subunit particles, all the recombinant capsid proteins utilized in the present studies were expressed via the baculovirus system. Comparisons between crecFib-8b/8a expressed via baculovirus and the more widespread *E. coli* system revealed higher immunogenicity of the baculovirus-derived construct crecFib-8b/8a. Furthermore, gel-filtration chromatography of the baculovirus-expressed FAdV-8a and -8b fibers showed a high degree of purity after his-tag purification, albeit different between the two proteins. These data are in line with previous studies highlighting the validity of the baculovirus system for other recombinant proteins with a relevance for human medicine, such as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) domains, and human glutamic acid decarboxylase (hGAD65), which also turned out favourable in comparison to *E. coli* expression (Victor *et al*., 2010; Gecchele *et al*., 2015).

As a follow-up to the promising results obtained with the chimeric fiber protein against IBH, the concept was extended to expand protection across the boundary of FAdV diseases, by designing a crecFib retaining epitopes from FAdV-4 and -11 (crecFib-

4/11). This construct was tested as vaccination antigen in the third study of the present thesis, and it was able to provide simultaneous protection against HHS and IBH (De Luca *et al*., 2022a). More recently, a different approach was used to increase the broadness of coverage provided by whole-virus antigens by designing recombinant hybrid viruses merging characteristics of different FAdVs, such as FAdV-4 and -8b (Lu *et al*., 2022). This concept was also applied to incorporate viral proteins from distinct chicken pathogens into FAdV, such as the VP2 protein from IBDV into FAdV-4, and simultaneously protect chickens against both IBD and HHS (Pan *et al*., 2021; Zhang *et al*., 2022a). On the other hand, further studies utilized other viruses or bacteria as vectors to deliver FAdV-4 subunits, such as hexon and fiber-2, successfully immunizing chickens against HHS (Tian *et al*., 2020; Jia *et al*., 2021; 2022; Cao *et al*., 2022). However, the immune mechanisms behind such protection remained largely unexplored. In the present study, serological analyses conducted on birds vaccinated with crecFib-4/11 showed that the antibodies measured on ELISA plates coated with the same antigen were lacking any neutralizing activity against FAdV-4 (De Luca *et al.*, 2022a). This marks an important difference compared to the mechanisms of protection against IBH, which appears to be linked to the induction of nAbs for live, inactivated and subunit vaccines (Gupta et al., 2017; 2018; Popowich *et al*., 2018; De Luca *et al*., 2020; Schachner *et al*., 2022). Full coverage against HHS has already been described in the absence of nAbs after vaccination with FAdV-4 fiber-2, and even upon immunization with a live attenuated FAdV-4 strain (Schonewille *et al*., 2010; Schachner *et al*., 2014). Nevertheless, differently from the *in vivo* protection studies on the penton base antigen, the presence of antigen-specific antibodies observed on ELISA after vaccination with crecFib-4/11 correlates with protection against the relevant serotypes. It is known that non-nAbs are important against viral diseases (Henry Dunand *et al*., 2016; Stadlbauer *et al*., 2017), and they are considered crucial to protect chickens

against specific subtypes of Avian Influenza (Hu *et al*., 2020). Therefore, they may be important in vaccine-induced immunity against FAdV diseases, especially in the absence of nAbs. In particular, non-nAbs are responsible for immune mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent complement-mediated lysis (ADCML), and antibody-dependent cellular phagocytosis (ADCP), although their role against viral diseases in chickens has yet to be investigated. The vaccine-related cellular immune response, upon which limited data are available to date, is another relevant factor to be considered. In the first study of the present thesis, the kinetics of the cellular immune response was investigated in the blood of birds immunized with FAdV-8a fiber and subsequently challenged with a virulent strain of homologous serotype (De Luca *et al*., 2020). In particular, it was shown that the production of serotype-specific nAbs following fiber vaccination was linked to the priming of B and $CD4^+$ T lymphocyte populations, highlighting the importance of both humoral and cellular immune response. At the same time, a proliferation of $CD4^+$ and $CD8\alpha^+$ T cells was also documented after FAdV-4 fiber-2 and FAdV-8b fiber vaccines, respectively (Gupta *et al*., 2017; Chen *et al.*, 2018). In order to broaden the comprehension of the fiber-induced cellular immunity, especially when linked to the absence of nAbs, the investigation was extended to crecFib-4/11-vaccinated birds, with or without subsequent HHS challenge, taking into exam not only the systemic response, but also analysing several immune cells populations in target and lymphoid organs (De Luca *et al*., 2022b). The findings revealed that crecFib-4/11 vaccination stimulated a prolonged proliferation of hepatic B cells and promoted the circulation of cytotoxic T lymphocytes, with earlier rise in peripheral blood mononuclear cells compared to the challenge control and consequent increase in liver and spleen, two of the major target organs of the disease. These novel data contribute to broaden

the knowledge behind the immunopathogenic pathways associated to the protection from HHS provided by FAdV subunit vaccines.

12. Conclusions and outlook

After demonstrating that protection associated to FAdV fiber vaccines is serotype-specific, the present study introduced the novel concept of chimeric fiber proteins, and described the first successful attempt at merging epitopes from different FAdV serotypes, and even viruses from distantly related species, into single-unit recombinant constructs despite the phylogenetic distance of the template strains. The induction of bilateral nAbs after vaccination with crecFib-8b/8a, as opposed to the poor immunogenicity of the opposite construct, crecFib-8a/8b, confirms the presence of different neutralizing epitopes in the fiber protein, which was not yet known. This concept was able to overcome the limited cross-protective potential of subunit vaccines, providing broad-coverage not only against different IBH-causing serotypes, but even including concurrent protection against HHS by designing the novel crecFib-4/11. Furthermore, for the first time, several major immune cell populations were investigated after FAdV fiber vaccination not only on a systemic level, but also locally in target and lymphoid organs over the course of a protection study, including monitoring before and after challenge. This provided novel data over the so far poorly understood role of the cellular immune response associated to protection from FAdV-related diseases, highlighting the importance of such response especially on a local level. Interestingly, no neutralizing activity was associated with the development of antigen-specific antibodies against crecFib-4/11, which poses questions over the role of non-neutralizing antibodies in protecting chickens against HHS. In fact, the non-nAbs induced by fiber vaccination may be directly involved in triggering the cellular immune response, and this hypothesis poses the premises for the subsequent studies aimed to clarify the intricacies of the immune response after FAdV vaccination. Further investigations are also needed to characterize the protective epitopes present on FAdV

antigens, and fully comprehend the immune mechanisms underlying subunit vaccines based on FAdV capsid proteins.

13. References

1. Afzal M, Ahmad I (1990) Efficacy of an inactivated vaccine against hydropericardium syndrome in broilers. Vet Rec 126:59-60
2. Ali S, Mahmood MS, Hussain I, Khan MN (2015) Preparation and evaluation of lyophilized live attenuated vaccine of inclusion body hepatitis hydro-pericardium syndrome (IBH-HPS) against challenge in broiler chickens. Int J Agric Biol 17:658-662
3. Alvarado IR, Villegas P, El-Attrache J, Jensen E, Rosales G, Perozo F, Purvis LB (2007) Genetic characterization, pathogenicity, and protection studies with an avian adenovirus isolate associated with inclusion body hepatitis. Avian Dis 51:27-32
4. Anjum AD (1990) Experimental transmission of hydropericardium syndrome and protection against it in commercial broiler chickens. Avian Pathol 19:655-660
5. Asrani RK, Gupta BK, Sharma SK, Singh SP, Katoch RC (1997) Hydropericardium-hepatopathy syndrome in Asian poultry. Vet Rec 141:271-273
6. Aziz F, Tufail S, Shah MA, Shah MS, Habib M, Mirza O, Iqbal M, Rahman M (2019) *In silico* epitope prediction and immunogenic analysis for penton base epitope-focused vaccine against hydropericardium syndrome in chicken. Virus Res 273:197750
7. Benkő M, Aoki K, Arnberg N, Davison AJ, Echavarría M, Hess M, Jones MS, Kaján GL, Kajon AE, Mittal SK, Podgorski II, San Martín C, Wadell G, Watanabe H, Harrach B and ICTV Report Consortium. ICTV virus taxonomy profile: Adenoviridae 2022. J Gen Virol 2022;103:001721.

8. Bertran K, Blanco A, Antilles N, Nofrarías M, Valle RM, Cobos À, Ramis A, Biarnés M, Majó N (2021) A 10-year retrospective study of inclusion body hepatitis in meat-type chickens in Spain (2011-2021). Viruses 13:2170
9. Cao H, Hua D, Zhang H, Zhang H, Liu N, Feng Z, Li H, Zhao B, Zhang L, Guo Y, Huang J, Zhang L (2022) Oral immunization of recombinant *Saccharomyces cerevisiae* expressing fiber-2 of fowl adenovirus serotype 4 induces protective immunity against homologous infection. Vet Microbiol 271:109490
10. Changjing L, Haiying L, Dongdong W, Jingjing W, Youming W, Shouchun W, Jida L, Ping L, Jianlin W, Shouzhen X, Shangjin C, Yi Z, Yanbo Y (2016) Characterization of fowl adenoviruses isolated between 2007 and 2014 in China. Vet Microbiol 197:62-67
11. Cheema AH, Ahmad J, Afzal M (1989) An adenovirus infection of poultry in Pakistan. Revue Scientifique et Technique de l´Office International des Epizooties 8:789-795
12. Chen H, Dou Y, Zheng X, Tang Y, Zhang M, Zhang Y, Wang Z, Diao Y (2015) Hydropericardium hepatitis syndrome emerged in Cherry Valley ducks in China. Transbound Emerg Dis 64:1262-1267
13. Chen L, Yin L, Peng P, Zhou Q, Du Y, Zhang Y, Xue C, Cao Y (2019) Isolation and characterization of a novel fowl adenovirus serotype 8a strain from China. Virol Sin 35:517-527
14. Chen L, Yin L, Zhou Q, Li Q, Luo Y, Xu Z, Zhang Y, Xue C, Cao Y (2018) Immunogenicity and protective efficacy of recombinant fiber-2 protein in protecting SPF chickens against fowl adenovirus 4. Vaccine 36:1203-1208
15. Chishti MA, Afzal M, Cheema AH (1989) Preliminary studies on the development of vaccine against the “hydropericardium syndrome” of poultry.

Revue Scientifique et Technique de l´Office International des Epizooties 8:797-801

16. Clemmer DI (1972) Age-associated changes in fecal excretion patterns of strain 93 chick embryo lethal orphan virus in chicks. Infect Immun 5:60-64
17. Cook JK (1983) Fowl adenoviruses: studies on aspects of the pathogenicity of six strains for 1-day-old chicks. Avian Pathol 12:35-43
18. Dar A, Tipu M, Townsend H, Potter A, Gerdts V, Tikoo S (2015) Administration of poly[di(sodium carboxylatoethylphenoxy) phosphazene] (PCEP) and avian beta defensin as adjuvants in inactivated inclusion body hepatitis virus and its hexon protein-based experimental vaccine formulations in chickens. Avian Dis 59:518-524
19. Das S, Fearniside K, Sarker S, Forwood JK, Raidal SR (2017) A novel pathogenic aviadenovirus from red-bellied parrots (*Poicephalus rufiventris*) unveils deep recombination events among avian host lineages. Virol 502:188-197
20. De Luca C, Schachner A, Heidl S, Hess M (2022a) Vaccination with a fowl adenovirus chimeric fiber protein (crecFib-4/11) simultaneously protects chickens against hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH). Vaccine 40:1837-1845
21. De Luca C, Schachner A, Heidl S, Hess M, Liebhart D, Mitra T (2022b) Local cellular immune response plays a key role in protecting chickens against hepatitis-hydropericardium syndrome (HHS) by vaccination with a recombinant fowl adenovirus (FAdV) chimeric fiber protein. Front Immunol 13:1026233
22. De Luca C, Schachner A, Mitra T, Heidl S, Liebhart D, Hess M (2020) Fowl adenovirus (FAdV) fiber-based vaccine against inclusion body hepatitis (IBH)

provides type-specific protection guided by humoral immunity and regulation of B and T cell response. Vet Res 51:143

23. Devlin JM, Vaz PK, Coppo MJC, Browning GF (2016) Impacts of poultry vaccination on viruses of wild bird. Curr Opin Virol 19:23-29
24. Du D, Zhang P, Li X, Tian H, Cheng Y, Sheng D, Han X, Shan Y, Li X, Yuan Y, Zhang H, Xue J, Liu W, Tian K (2017) Cell-culture derived fowl adenovirus serotype 4 inactivated vaccine provides complete protection for virus infection on SPF chickens. Virus Dis 28:182-188
25. Fadly AM, Winterfield RW, Olander HJ (1976) Role of the bursa of Fabricius in the pathogenicity of inclusion body hepatitis and infectious bursal disease viruses. Avian Dis 20:467-477
26. Feichtner F, Schachner A, Berger E, Hess M (2018a) Development of sensitive indirect enzyme-linked immunosorbent assays for specific detection of antibodies against fowl adenovirus serotypes 1 and 4 in chickens. Avian Pathol 47:73-82
27. Feichtner F, Schachner A, Berger E, Hess M (2018b) Fiber-based fluorescent microsphere immunoassay (FMIA) as a novel multiplex serodiagnostic tool for simultaneous detection and differentiation of all clinically relevant fowl adenovirus (FAdV) serotypes. J Immunol Methods 458:33-43
28. Fingerut E, Gutter B, Gallili G, Michael A, Pitcovski J (2003) A subunit vaccine against the adenovirus egg-drop syndrome using part of its fiber protein. Vaccine 21:2761-2766
29. Gahery-Segard H, Farace F, Godfrin D, Gaston J, Lengagne R, Tursz T, Boulanger P, Guillet JG (1998) Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. J Virol 72:2388-2397

30. Gecchele E, Merlin M, Brozzetti A, Falorni A, Pezzotti M, Avesani L (2015) A comparative analysis of recombinant protein expression in different biofactories: bacteria, insect cells and plant systems. J Vis Exp 97:e52459
31. Gelderblom H, Maichle-Lauppe I (1982) The fibers of fowl adenoviruses. Arch Virol 72:289-298
32. Gjevre AG, Kaldhusdal M, Eriksen GS (2013) Gizzard erosion and ulceration syndrome in chickens and turkeys: a review of causal or predisposing factors. Avian Pathol 42:297-303
33. Grafl B, Aigner F, Liebhart D, Marek A, Prokofieva I, Bachmeier J, Hess M (2012) Vertical transmission and clinical signs in broiler breeders and broilers experiencing adenoviral gizzard erosion. Avian Pathol 41:599-604
34. Grafl B, Berger E, Wernsdorf P, Hess M (2020) Successful reproduction of adenoviral gizzard erosion in 20-week-old SPF layer-type chickens and efficacious prophylaxis due to live vaccination with apathogenic fowl adenovirus serotype 1 strain (CELO). Vaccine 38:143-149
35. Grafl B, Liebhart D, Günes A, Wernsdorf P, Aigner F, Bachmeier J, Hess M (2013) Quantity of virulent fowl adenovirus serotype 1 correlates with clinical signs, macroscopical and pathohistological lesions in gizzards following experimental induction of gizzard erosion in broilers. Vet Res 44:38
36. Grafl B, Prokofieva I, Wernsdorf P, Steinborn R, Hess M (2014) Infection with an apathogenic fowl adenovirus serotype-1 strain (CELO) prevents adenoviral gizzard erosion in broilers. Vet Microbiol 172:177-185
37. Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya R, Ojkic D, Tikoo SK, Willson P, Gomis S (2017) Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. Vaccine 35:2716-2722

38. Gupta A, Popowich S, Ojkic D, Kurukulasuriya S, Chow-Lockerbie B, Gunawardana T, Goonewardene K, Karunarathna R, Ayalew LE, Ahmed KA, Tikoo SK, Willson P, Gomis S (2018) Inactivated and live bivalent fowl adenovirus (FAdV8b + FAdV11) breeder vaccines provide broad-spectrum protection in chicks against inclusion body hepatitis (IBH). Vaccine 36:744-750
39. Helmboldt CF, Frazier MN (1963) Avian hepatic inclusion bodies of unknown significance. Avian Dis 7:446-450
40. Henry Dunand CJ, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, Tan GS, Cruz J, Hirsh A, Zheng N, Mullarkey CE, Ennis FA, Terajima M, Treanor JJ, Topham DJ, Subbarao K, Palese P, Krammer F, Wilson PC (2016) Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. Cell Host Microbe 19:800-813
41. Hess M (2000) Detection and differentiation of avian adenoviruses: A review. Avian Pathol 29:195-206
42. Hess M (2017) Commensal or pathogen – a challenge to fulfil Koch's postulates. Br Poult Sci 58:1-12
43. Hess M. Aviadenovirus infections. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. Diseases of Poultry. Hoboken, NJ: Wiley-Blackwell; 2020. p. 322-31.
44. Hess M, Cuzange A, Ruigrok RWH, Chroboczek J, Jacrot B (1995) The avian adenovirus penton: two fibres and one base. J Mol Biol 252:379-385
45. Hess M, Prusas C, Vereecken M, De Herdt P (1998) Isolation of fowl adenoviruses serotype 4 from pigeons with hepatic necrosis. Berliner und Münchener Tierärztliche Wochenschrift 111:140-142

46. Hoffmann R, Wessling E, Dorn P, Dangschat H (1975) Lesions in chickens with spontaneous or experimental infectious hepato-myelopoietic disease (inclusion body hepatitis) in Germany. Avian Dis 19:224-236
47. Hu J, Li G, Wang X, Cai L, Rong M, Li H, Xie M, Zhang Z, Rong J (2021) Development of a subunit vaccine based on fiber2 and hexon against Fowl adenovirus serotype 4. Virus Res 305:198552
48. Hu Z, Zhao J, Zhao Y, Fan X, Hu J, Shi L, Wang X, Liu X, Hu S, Gu M, Cao Y, Liu X (2020) Hemagglutinin-specific non-neutralizing antibody is essential for protection provided by inactivated and viral-vectored H7N9 Avian Influenza vaccines in chickens. Front Vet Sci 6:482
49. Jia Z, Ma C, Yang X, Pan X, Li G, Ma D (2021) Oral immunization of recombinant *Lactococcus lactis* and *Enterococcus faecalis* expressing dendritic cell targeting peptide and hexon protein of Fowl adenovirus 4 induces protective immunity against homologous infection. Front Vet Sci 8:632218
50. Jia Z, Pan X, Zhi W, Chen H, Bai B, Ma C, Ma D (2022) Probiotics surface-delivering fiber2 protein of fowl adenovirus 4 stimulate protective immunity against hepatitis-hydropericardium syndrome in chickens. Front Immunol 13:919100
51. Jones RC, Georgiou K (1984) Experimental infection of chickens with adenoviruses isolated from tenosynovitis. Avian Pathol 13:13-23
52. Junnu S, Lertwatcharasarakul P, Jala S, Phattanakulanan S, Monkong A, Kulprasertsri S, Thivalai C, Chakritbudsabong W, Chaichoun K, Songserm T (2015) An inactivated vaccine for prevention and control of inclusion body hepatitis in broiler breeders. The Thai J Vet Med 45:55-62
53. Karunamoorthy G, Manickam R (1998) Hydropericardium syndrome in quails. Poultry Times of India 2:1-31

54. Kaur A, Oberoi MS, Singh A (1997) Neutralizing antibody and challenge response to live and inactivated avian adenovirus-1 in broilers. Trop Anim Health Prod 29:141-146
55. Kim M, Lim T, Lee D, Youn H, Yuk S, Kim B, Choi S, Jung C, Han J, Song C (2014) An inactivated oil-emulsion fowl adenovirus serotype 4 vaccine provides broad cross-protection against various serotypes of fowl adenovirus. Vaccine 32:3564-3568
56. Laver WG, Younghusband HB, Wrigley NG (1971) Purification and properties of chick embryo lethal orphan virus (an avian adenovirus). Virology 45:598-614
57. Li G, Yu G, Niu Y, Cai Y, Liu S (2019) Airborne transmission of a serotype 4 fowl adenovirus in chickens. Viruses 11:262
58. Liu J, Mei N, Wang Y, Shi W, Chen H (2021) Identification of a novel immunological epitope on hexon of fowl adenovirus serotype 4. AMB Expr 11:153
59. Lu H, Shao H, Chen H, Zhang J, Wang W, Li T, Xie Q, Qin A, Ye J (2019) Identification of novel B cell epitopes in the fiber protein of serotype 8 Fowl adenovirus. AMB Expr 9:172
60. Lu H, Xie Q, Zhang W, Zhang J, Wang W, Lian M, Zhao Z, Ren D, Xie S, Lin Y, Li T, Mu Y, Wan Z, Shao H, Qin A, Ye J (2022) A novel recombinant FAdV-4 virus with fiber of FAdV-8b provides efficient protection against both FAdV-4 and FAdV-8b. Viruses 14:376
61. Lucero MS, Zoth SC, Jaton J, Gravisaco MJ, Pinto S, Richetta M, Berinstein A, Gómez E (2021) Oral immunization with plant-based vaccine induces a protective response against infectious bursal disease. Front Plant Sci 12:741469

62. Mansoor MK, Hussain I, Arshad M, Muhammad G (2011) Preparation and evaluation of chicken embryo-adapted fowl adenovirus serotype 4 vaccine in broiler chickens. Trop Anim Health Prod 43:331-338
63. Marek A, Günes A, Schulz E, Hess M (2010) Classification of fowl adenoviruses by use of phylogenetic analysis and high-resolution melting-curve analysis of the hexon L1 gene region. J Virol Methods 170:147-154
64. Marek A, Nolte V, Schachner A, Berger E, Schlötterer C, Hess M (2012) Two fiber genes of nearly equal lengths are a common and distinctive feature of Fowl adenovirus C members. Vet Microbiol 156:411-417
65. Matos M, Dublecz K, Grafl B, Liebhart D, Hess M (2018) Pancreatitis is an important feature of broilers suffering from inclusion body hepatitis leading to dysmetabolic conditions with consequences for zootechnical performance. Avian Dis 62:57-64
66. Matos M, Grafl B, Liebhart D, Hess M (2016a) The outcome of experimentally induced inclusion body hepatitis (IBH) by fowl aviadenoviruses (FAdVs) is crucially influenced by the genetic background of the host. Vet Res 47:69
67. Matos M, Grafl B, Liebhart D, Schwendenwein I, Hess M (2016b) Selected clinical chemistry analytes correlate with the pathogenesis of inclusion body hepatitis experimentally induced by fowl aviadenoviruses. Avian Pathol 45:520-529
68. Mazaheri A, Prusas C, Voß M, Hess M (1998) Some strains of serotype 4 fowl adenoviruses cause inclusion body hepatitis and hydropericardium syndrome in chickens. Avian Pathol 27:269-276
69. Mazaheri A, Prusas C, Voß M, Hess M (2003) Vertical transmission of fowl Adenovirus serotype 4 investigated in specified pathogen-free birds after experimental infection. Archiv für Geflügelkunde 67:6-10

70. McFerran JB, Adair BMcC (1977) Avian adenoviruses – a review. Avian Pathol 6:189-217
71. Meng K, Yuan X, Yu J, Zhang Y, Ai W, Wang Y (2019) Identification, pathogenicity of novel fowl adenovirus serotype 4 SDJN0105 in Shandong, China and immunoprotective evaluation of the newly developed inactivated oil-emulsion FAdV-4 vaccine. Viruses 11:627
72. Mittal D, Jindal N, Tiwari AK, Khokhar RS (2014) Characterization of fowl adenoviruses associated with hydropericardium syndrome and inclusion body hepatitis in broiler chickens. Virusdisease 25:114-119
73. Mo J (2021) Historical investigation of fowl adenovirus outbreaks in South Korea from 2007 to 2021: a comprehensive review. Viruses 13:2256
74. Mu Y, Xie Q, Wang W, Lu H, Lian M, Gao W, Li T, Wan Z, Shao H, Qin A, Ye J (2021) A novel *Fiber-1*-edited and highly attenuated recombinant serotype 4 Fowl adenovirus confers efficient protection against lethal challenge. Front Vet Sci 8:759418
75. Naeem K, Akram HS (1995) Hydropericardium syndrome outbreak in a pigeon flock. Vet Rec 136:296-297
76. Naeem K, Niazi T, Malik SA, Cheema AH (1995b) Immunosuppressive potential and pathogenicity of an avian adenovirus isolate involved in hydropericardium syndrome in broilers. Avian Dis 39:723-728
77. Naeem K, Rabbani M, Hussain M, Cheema AH (1995a) Development of cell culture vaccine against hydropericardium syndrome in poultry. Pakistan Vet J 15:150-151
78. Okuda Y, Ono M, Yazawa S, Imai Y, Shibata I, Sato S (2001b) Pathogenicity of serotype 1 fowl adenovirus in commercial broiler chickens. Avian Dis 45:819-827

79. Okuda Y, Ono M, Yazawa S, Shibata I, Sato S (2001a) Experimental infection of specific-pathogen-free chickens with serotype-1 fowl adenovirus isolated from a broiler chicken with gizzard erosions. Avian Dis 45:19-25
80. Olson NO (1950) A respiratory disease (bronchitis) of quail caused by a virus. In: Proceedings of the 54th Annual Meeting of the United States Livestock Sanitary Association, Phoenix, AZ, USA, 1950, pp 171-174.
81. Ono M, Okuda Y, Yazawa S, Imai Y, Shibata I, Sato S, Okada K (2003a) Adenoviral gizzard erosion in commercial broiler chickens. Vet Pathol 40:294-303
82. Ono M, Okuda Y, Yazawa S, Shibata I, Sato S, Okada K (2003b) Outbreaks of adenoviral gizzard erosion in slaughtered broiler chickens in Japan. Vet Rec 153:775-779
83. Pallister JA, Erny KM, Fahey KJ (1993) Serological relationships within the group E fowl adenoviruses. Intervirology 36:84-90
84. Pan Q, Yang Y, Gao Y, Qi X, Liu C, Zhang Y, Cui H, Wang X (2017) An inactivated novel genotype fowl adenovirus 4 protects chickens against the hydropericardium syndrome that recently emerged in China. Viruses 9:216
85. Pan Q, Wang J, Gao Y, Cui H, Liu C, Qi X, Zhang Y, Wang Y, Wang X (2018) Identification of two novel fowl adenovirus C-specific B cell epitopes using monoclonal antibodies against the capsid hexon protein. Appl Microbiol Biotechnol 102:9243-9253
86. Pan Q, Zhang Y, Liu A, Cui H, Gao Y, Qi X, Liu C, Zhang Y, Li K, Gao L, Wang X (2021) Development of a novel avian vaccine vector derived from the emerging Fowl adenovirus 4. Front Microbiol 12:780978
87. Philippe C, Grgić H, Ojkić D, Nagy É (2007) Serologic monitoring of a broiler breeder flock previously affected by inclusion body hepatitis and testing of the

progeny for vertical transmission of fowl adenoviruses. Cad J Vet Res 71:98-102

88. Pitcovski J, Fingerut E, Gallili G, Eliahu D, Finger A, Gutter B (2005) A subunit vaccine against haemorrhagic enteritis adenovirus. Vaccine 23:4697-4702
89. Popowich S, Gupta A, Chow-Lockerbie B, Ayalew L, Ambrose N, Ojkic D, Gunawardana T, Kurukulasuriya S, Willson P, Tikoo SK, Gomis S (2018) Broad spectrum protection of broiler chickens against inclusion body hepatitis by immunizing their broiler breeder parents with a bivalent live fowl adenovirus vaccine. Res Vet Sci 118:262-269
90. Rani V, Kumar R, Upadhyay AK, Kumar M (2012) Therapeutic efficacy of chicken egg yolk immunoglobulins against hydropericardium syndrome in broiler chickens. Indian J Anim Sci 82:552-556
91. Raue R, Hess M (1998) Hexon based PCRs combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenoviruses and egg drop syndrome virus. J Virol Methods 73:211-217
92. Reece R, Barr D, Grix D (1987) Pathogenicity studies with a strain of fowl adenovirus serotype 8 (VRI-33) in chickens. Aust Vet J 64:365-367
93. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Hyg 27:493-497
94. Rosenberger JK, Klopp S, Eckroade RJ, Krauss WC (1975) The role of the infectious bursal agent and several avian adenoviruses in the haemorrhagic-aplastic-anaemia syndrome and gangrenous dermatitis. Avian Dis 19:717-729
95. Ruan S, Zhao J, He Z, Yang H, Zhang G (2018) Analysis of pathogenicity and immune efficacy of fowl adenovirus serotype 4 isolates. Poult Sci 97:2647-2653

96. Saifuddin M, Wilks CR (1990) Reproduction of inclusion body hepatitis in conventionally raised chickens inoculated with a New Zealand isolate of avian adenovirus. N Z Vet J 38:62-65
97. Saifuddin M, Wilks CR (1992) Effects of Fowl adenovirus infection on the immune system of chickens. J Comp Path 107:285-294
98. Sarfraz M, Suleman M, Tikoo SK, Wheler C, Potter AA, Gerdts V, Dar A (2017) Immune responses to *in ovo* vaccine formulations containing inactivated fowl adenovirus 8b with poly[di(sodium carboxylatoethylphenoxy)] phosphazene (PCEP) and avian beta defensin as adjuvants in chickens. Vaccine 35:981-986
99. Schachner A, De Luca C, Heidl S, Hess M (2022) Recombinantly expressed chimeric fibers demonstrate discrete type-specific neutralizing epitopes in the *Fowl aviadenovirus E* (FAdV-E) fiber, promoting the optimization of FAdV fiber subunit vaccines towards cross-protection *in vivo*. Microbiol Spectr 10:e02123-21
100. Schachner A, Gonzalez G, Endler L, Ito K, Hess M (2019) Fowl adenovirus (FAdV) recombination with intertypic crossovers in genomes of FAdV-D and FAdV-E, displaying hybrid serological phenotypes. Viruses 11:1094
101. Schachner A, Grafl B, Hess M (2021) Spotlight on fowl adenovirus (FAdV) in chickens and beyond – an unresolved host-pathogen interplay. Avian Pathol 50:2-5
102. Schachner A, Marek A, Grafl B, Hess M (2016) Detailed molecular analyses of the hexon loop-1 and fibers of fowl aviadenoviruses reveal new insights into the antigenic relationship and confirm that specific genotypes are involved in field outbreaks of inclusion body hepatitis. Vet Microbiol 186:13-20

103. Schachner A, Marek A, Jaskulska B, Bilic I, Hess M (2014) Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis–hydropericardium syndrome (HHS). Vaccine 32:1086-1092

104. Schachner A, Matos M, Grafl B, Hess M (2018) Fowl adenovirus-induced diseases and strategies for their control – a review on the current global situation. Avian Pathol 47:111-126

105. Schat K, Sellers HS (2008) Cell culture methods. In: Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR (eds) A laboratory manual for the isolation and identification of avian pathogens, 5th ed. OmniPress Inc, Madison

106. Schonewille E, Jaspers R, Paul G, Hess M (2010) Specific-pathogen-free chickens vaccinated with a live FAdV-4 vaccine are fully protected against a severe challenge even in the absence of neutralizing antibodies. Avian Dis 54:905-910

107. Schonewille E, Singh A, Göbel TW, Gerner W, Saalmüller A, Hess M (2008) Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid organs in specific pathogen-free chickens following experimental infection. Vet Immunol Immunop 121:130-139

108. Shah MS, Ashraf A, Khan MI, Rahman M, Habib M, Qureshi JA (2016) Molecular cloning, expression and characterization of 100 K gene of fowl adenovirus-4 for prevention and control of hydropericardium syndrome. Biologicals 44:19-23

109. Shah MS, Ashraf A, Rahman M, Khan MI, Qureshi JA (2012) A subunit vaccine against hydropericardium syndrome using adenovirus penton capsid protein. Vaccine 30:7153-7156

110. Shivachandra SB, Sah RL, Singh SD, Kataria JM, Manimaran K (2003) Immunosuppression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4 (FAV-4) associated with hydropericardium syndrome. Vet Res Commun 27:39-51

111. Singh A, Grewal GS, Maiti NK, Oberoi MS (2006) Effect of fowl adenovirus-1 (IBH isolate) on humoral and cellular immune competency of broiler chicks. Comp Immunol Microbiol Infect Dis 29:315-321

112. Singh A, Oberoi MS, Jand SK, Singh B (1996) Epidemiology of inclusion body hepatitis in poultry in northern India from 1990 to 1994. Revue Scientifique et Technique de l´Office International des Epizooties 15:1053-1060

113. Stadlbauer D, Rajabhathor A, Amanat F, Kaplan D, Masud A, Treanor JJ, Izikson R, Cox MM, Nachbagauer R, Krammer F (2017) Vaccination with a recombinant H7 hemagglutinin-based influenza virus vaccine induces broadly reactive antibodies in humans. MSphere 2

114. Steer PA, O´Rourke D, Ghorashi SA, Noormohammadi AH (2011) Application of high-resolution melting curve analysis for typing of fowl adenovirus in field cases of inclusion body hepatitis. Aust Vet J 89:184-192

115. Steer PA, Sandy JR, O´Rourke D, Scott PC, Browning GF, Noormohammadi AH (2015) Chronological analysis of gross and histological lesions induced by field strains of fowl adenovirus serotypes 1, 8b and 11 in one-day-old chickens. Avian Pathol 44:106-113

116. Steer PA, Sandy JR, O´Rourke D, Scott PC, Browning GF, Noormohammadi AH (2017) Chronologic analysis of gross and histologic lesions induced by field strains of FAdV-1, FAdV-8b, and FAdV-11 in six-week-old chickens. Avian Dis 61:512-519

117. Steer-Cope PA, Sandy JR, O´Rourke D, Scott PC, Browning GF, Noormohammadi AH (2019) Vaccination with FAdV-8a induces protection against inclusion body hepatitis caused by homologous and heterologous strains. Avian Pathol 48:396-405

118. Tan PK, Michou AI, Bergelson JM, Cotten M (2001) Defining CAR as a cellular receptor for the avian adenovirus CELO using a genetic analysis of the two viral fibre proteins. J Gen Virol 82:1465-1472

119. Tian K, Guo H, Li N, Zhang Y, Wang Z, Wang B, Yang X, Li Y, Zhao J (2020) Protection of chickens against hepatitis-hydropericardium syndrome and Newcastle disease with a recombinant Newcastle disease virus vaccine expressing the fowl adenovirus serotype 4 fiber-2 protein. Vaccine 38:1989-1997

120. Toro H, González C, Cerda L, Morales MA, Dooner P, Salamero M (2002) Prevention of inclusion body hepatitis/hydropericardium syndrome in progeny chickens by vaccination of breeders with fowl adenovirus and chicken anaemia virus. Avian Dis 46:547-554

121. Toro H, González O, Escobar C, Cerda L, Morales MA, Gonzalez C (2001) Vertical induction of the inclusion body hepatitis/hydropericardium syndrome with fowl adenovirus and chicken anemia virus. Avian Dis 45:215-222

122. Tufail S, Shah MA, Zafar M, Asif TA, Shehzad A, Shah MS, Habib M, Saleemi MK, Muddassar M, Mirza O, Iqbal M, Rahman M (2021) Identification of potent epitopes on hexon capsid protein and their evaluation as vaccine candidates against infections caused by members of *Adenoviridae* family. Vaccine 39:3560-3564

123. Van Den Ende M, Don PA, Kipps A (1949) The isolation in eggs of a new filtrable agent which may be the cause of bovine lumpy skin disease. J Gen Microbiol 3:174-183

124. Van den Hurk JV, Van Drunen Littel-van den Hurk S (1993) Protection of turkeys against hemorrhagic enteritis by monoclonal antibody and hexon immunization. Vaccine 11:329-335

125. Venne D (2013) Field data of the changing clinical picture over time of inclusion body hepatitis in Canada with an emphasis on diagnosis, prevention and trials on supportive treatments. In: Proceedings of the 24th Annual Australian Poultry Science Symposium, Sydney, New South Wales, Australia, February 2013. The Poultry Research Foundation and the World´s Poultry Science Association, pp 222-229.

126. Victor ME, Bengtsson A, Andersen G, Bengtsson D, Lusingu JP, Vestergaard LS, Arnot DE, Theander TG, Joergensen L, Jensen ATR (2010) Insect cells are superior to *Escherichia coli* in producing malaria proteins inducing IgG targeting PfEMP1 on infected erythrocytes. Malar J 9:325

127. Wang J, Wang S, Zou K, Zhang Y, Xu S, Yin Y (2018c) Variant serotypes of fowl adenovirus isolated from commercial poultry between 2007 and 2017 in some regions of China. Avian Dis 62:171-176

128. Wang P, Zhang J, Wang W, Li T, Liang G, Shao H, Gao W, Qin A, Ye J (2018a) A novel monoclonal antibody efficiently blocks the infection of serotype 4 fowl adenovirus by targeting fiber-2. Vet Res 49:29

129. Wang X, Tang Q, Chu Z, Wang P, Luo C, Zhang Y, Fang X, Qiu L, Dang R, Yang Z (2018b) Immune protection efficacy of FAdV-4 surface proteins fiber-1, fiber-2, hexon and penton base. Virus Res 245:1-6

130. Wang X, Tang Q, Qiu L, Yang Z (2019) Penton-dodecahedron of fowl adenovirus serotype 4 as a vaccine candidate for the control of related diseases. Vaccine 37:839-847

131. Wohlfart C (1988) Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms. J Virol 62:2321-2328

132. Xia J, Yao K, Liu Y, You G, Li S, Liu P, Zhao Q, Wen Y, Wu R, Huang X, Cao S, Han X, Huang Y (2017) Isolation and molecular characterization of prevalent Fowl adenovirus strains in southwestern China during 2015-2016 for the development of a control strategy. Emerg Microbes & Infect 6:e103

133. Xiang S, Huang R, He Q, Xu L, Wang C, Wang Q (2022) Arginine regulates inflammation response-induced by Fowl adenovirus serotype 4 via JAK2/STAT3 pathway. BMC Vet Res 18:189

134. Xie Q, Cao S, Zhang W, Wang W, Li L, Kan Q, Fu H, Geng T, Li T, Wan Z, Gao W, Shao H, Qin A, Ye J (2021a) A novel fiber-2-edited live attenuated vaccine candidate against the highly pathogenic serotype 4 fowl adenovirus. Vet Res 52:35

135. Xie Q, Wang W, Kan Q, Mu Y, Zhang W, Chen J, Li L, Fu H, Li T, Wan Z, Gao W, Shao H, Qin A, Ye J (2022) FAdV-4 without Fiber-2 is a highly attenuated and protective vaccine candidate. Microbiol Spectr 10:e01436-21

136. Xie Q, Wang W, Li L, Kan Q, Fu H, Geng T, Li T, Wan Z, Gao W, Shao H, Qin A, Ye J (2021b) Domain in Fiber-2 interacted with KPNA3/4 significantly affects the replication and pathogenicity of the highly pathogenic FAdV-4. Virulence 12:754-765

137. Yates VJ, Fry DE (1957) Observations on a chicken embryo lethal orphan (CELO) virus. Am J Vet Res 18:657-660

138. Yin D, He L, Zhu E, Fang T, Yue J, Wen M, Wang K, Cheng Z (2021) A fowl adenovirus serotype 4 (FAdV-4) Fiber2 subunit vaccine candidate provides complete protection against challenge with virulent FAdV-4 strain in chickens. Vet Microbiol 263:109250

139. Yin D, Yin L, Wang J, Shen X, Dai Y, Zhao R, Hu X, Hou H, Zhang D, Wang G, Qi K, Pan X (2022) Antiviral and virucidal activities of Camptothecin on Fowl adenovirus serotype 4 by blocking virus replication. Front Cell Infect Microbiol 12:823820

140. Zhang Y, Liu A, Cui H, Qi X, Liu C, Zhang Y, Li K, Gao L, Wang X, Pan Q, Gao Y (2022b) An inactivated vaccine based on artificial non-pathogenic fowl adenovirus 4 protects chickens against hepatitis-hydropericardium syndrome. Vet Microbiol 264:109285

141. Zhang Y, Liu A, Jiang N, Qi X, Gao Y, Cui H, Liu C, Zhang Y, Li K, Gao L, Wang X, Pan Q (2022a) A novel inactivated bivalent vaccine for chickens against emerging hepatitis-hydropericardium syndrome and infectious bursal disease. Vet Microbiol 266:109375

142. Zhang Y, Pan Q, Guo R, Liu A, Xu Z, Gao Y, Cui H, Liu C, Qi X, Zhang Y, Li K, Gao L, Wang X (2021) Immunogenicity of novel live vaccine based on an artificial rHN20 strain against emerging Fowl adenovirus 4. Viruses 13:2153

143. Zhao Z, Song Y, Huang Z, Liu L, Liao H, Sun W, Tao M, Li L, Li X (2022) Immunity analysis against Fowl adenovirus serotype 4 (FAdV-4) based on Fiber-2 trimer protein with the different virulence. Virus Res 308:198652

144. Zia MR, Rahman SU, Siddique M, Sandhu MA (2001) Comparative efficiency of two oil emulsion hydropericardium/hepatitis syndrome vaccines. Int J Agric Biol 3:436-438