Cell-based assays to assess the immunostimulatory capacity of poultry vaccines

A rational approach to animal-free vaccine testing

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Cellulaire testen ter beoordeling van de immunostimulatoire capaciteit van pluimvee vaccines

Een rationele aanpak voor het dierproefvrij testen van vaccines

(met een samenvatting in het Nederlands)

Proefschrift

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Table of contents

Chapter 1 General introduction p 9

Chapter 2 Nitric oxide production and Fc receptor-mediated phagocytosis as functional readouts of macrophage activity upon stimuluation with inactivated poultry vaccines *in vitro* p 27

Chapter 3 Macrophage activation assays to evaluate the immunostimulatory capacity of *Avibacterium paragallinarum* in a multivalent poultry vaccine p 51

Chapter 4 *In vitro* chicken bone marrow-derived dendritic cells comprise subsets at different states of maturation p 75

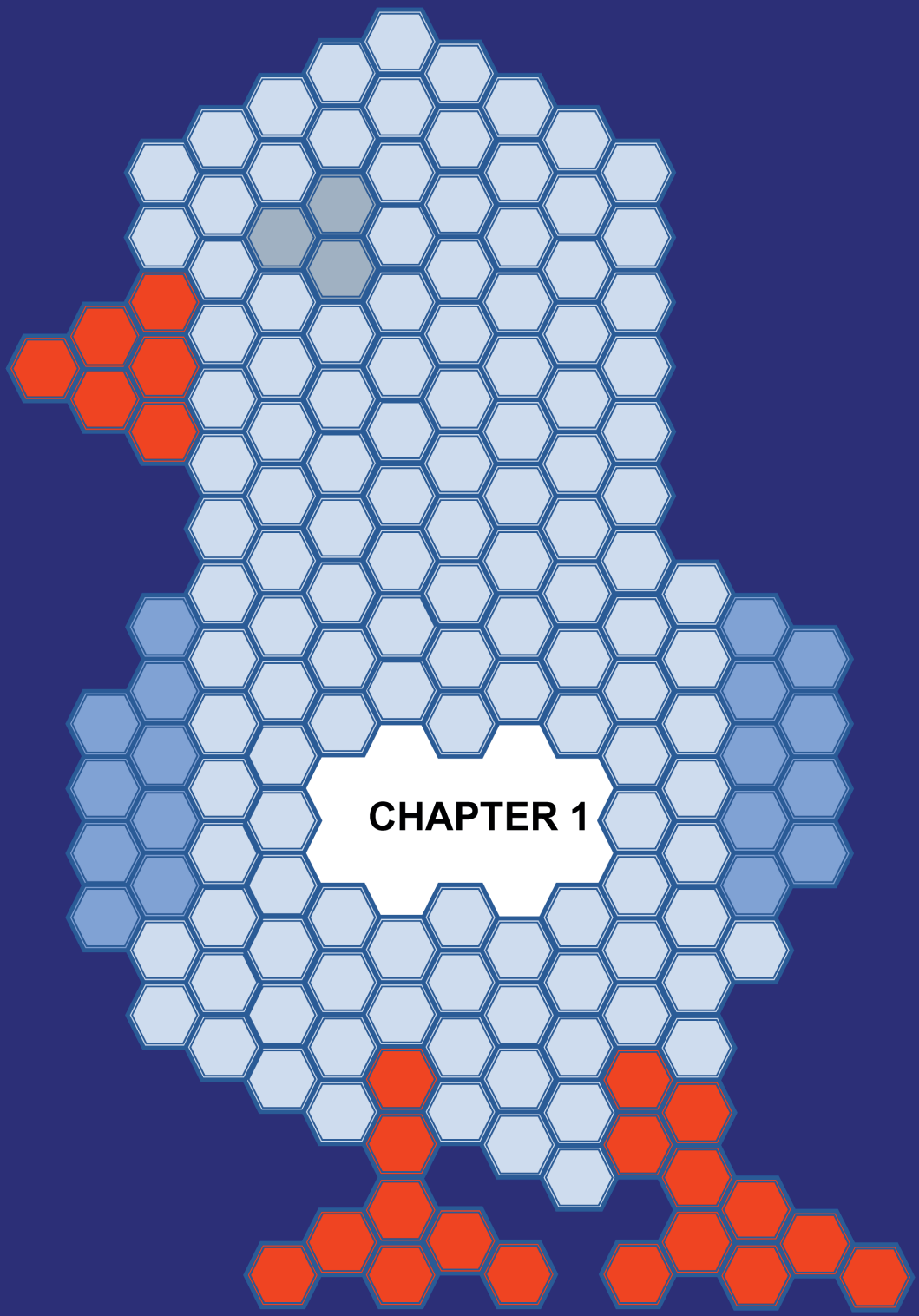
Chapter 5 Proteomic analysis of chicken bone marrow-derived dendritic cells in response to an inactivated IBV + NDV poultry vaccine p 107

Chapter 6 Assessment of antigen-specific T cell responses in chickens after vaccination with an inactivated vaccine for infectious bronchitis virus and Newcastle disease virus p 135

Chapter 7 Overcoming scientific barriers in the transition from *in vivo* to animal-free batch-release testing of human and veterinary vaccines p 147

Chapter 8 Summarizing discussion p 173

Appendix Nederlandse samenvatting p 190
Dankwoord/Acknowledgements p 194
Curriculum Vitae p 197
List of publications p 198


CHAPTER 1

General introduction

General introduction

Prevention of infectious diseases in humans and livestock relies on safe and efficacious vaccines. Vaccines are biologicals that, in many cases, comprise whole microorganisms or components thereof. Differences between vaccine batches may arise from small changes in culture conditions of these microorganisms prior to vaccine production, as well as from deviations in downstream production steps. Consequently, vaccine batches require routine quality control to test for compliance with the given specifications. For newer generation vaccines with a well-defined composition, *e.g.* recombinant subunit vaccines or DNA vaccines, quality may be assured through analytical assessment of the constituents [1–3]. In contrast, live attenuated and inactivated vaccines comprising whole organisms have a more complex composition, which complicates their assessment through analytical methods [4]. Batch quality assessment of live attenuated vaccine can be performed by culture and titration of the live pathogens present in the vaccines, but this is not possible for inactivated vaccines. The quality of inactivated vaccines is therefore often assessed through *in vivo* potency tests, in which the capacity of a vaccine batch to induce a protective immune response is evaluated.

In the poultry industry, animals are protected against infectious diseases by extensive vaccination schemes, which includes the use of many live attenuated and inactivated vaccines. Chickens receive the first live attenuated vaccines immediately after hatch, or even before hatch by *in ovo* vaccination, to provide early protection against infectious diseases like infectious bronchitis virus (IBV), Marek's disease virus (MDV) and Newcastle disease virus (NDV) [5]. Laying and breeding hens have a lifespan of more than a year during which the protective effect of vaccination may start to wane, which is why additional live attenuated or inactivated booster vaccines against *e.g.* IBV and NDV are administered before the laying period to enhance their immunity [6].

Inactivated poultry vaccines are still mostly tested for potency using *in vivo* vaccination-challenge tests or serological tests, since *in vitro* alternatives are limitedly available. However, improved knowledge on the working mechanisms of vaccines has resulted in opportunities to develop new *in vitro* test methods, which could contribute to a reduction of animal use in vaccine quality testing by replacing currently used *in vivo* tests.

Short history of vaccination

In 1798, the first vaccination was performed by Edward Jenner, who vaccinated humans with cowpox virus, which causes only mild symptoms, to prevent severe infection with smallpox virus [7]. Smallpox was an infectious disease that caused high mortality worldwide until its eradication in 1977 as a result of a global vaccination campaign [8]. The word vaccination is named after the cowpox vaccine (*Variolae vaccinae* means "smallpox of the cow") and was introduced by Louis Pasteur to use for all practices that induce protective immunity against diseases in honor of Jenner [9].

In 1879, Louis Pasteur and co-workers created the first lab-grown live attenuated vaccine by using old bacterial cultures of *Pasteurella multocida* that had lost their virulence [9]. This vaccine could be used to protect chickens against fowl cholera, caused by virulent *P. multocida* bacteria. Pasteur used similar procedures to make an anthrax vaccine for use

in sheep, cattle and horses, and a rabies vaccine, which was initially used in dogs and later in humans [10]. Many contemporary veterinary and human vaccines comprise viruses or bacteria that are attenuated by drying, heating or passage on tissue cultures, cell lines or embryonated chickens eggs [11].

In 1886, Daniel Salmon and Theobald Smith described the first inactivated vaccine, containing heat-killed *Salmonella enterica* [12]. Subsequently, heat-inactivated vaccines against typhoid, plague and cholera were successfully developed [13]. In contrast, heat-inactivation of diphtheria and tetanus did not result in effective and safe vaccines [14]. Emil von Behring and Kitasato Shibasaburo discovered that rabbits produced neutralizing antibodies upon injecting diphtheria toxin, the molecule responsible for the symptoms of diphtheria [15]. Subsequently, Smith and von Behring developed a safe and effective vaccine consisting of diphtheria toxin-antibody complexes [14,15]. The vaccine was further improved when Alexander Glenny and Henry Südmersen inactivated the toxin with formalin, resulting in a safer diphtheria toxoid vaccine [16]. In addition, they created precipitates of the toxoid vaccine by addition of acetic acid and potassium aluminium sulphate, resulting in stronger immune responses [17]. Additives that enhanced immune responsiveness were named adjuvants. Two aluminium salt adjuvants, including aluminium hydroxide and aluminium phosphate, are still used in human diphtheria and tetanus vaccines [18], as well as in many veterinary vaccines [19].

In the 1940s, water-in-oil emulsions were discovered as adjuvants, including Freund's complete adjuvant, containing mineral oil and inactivated *Mycobacterium tuberculosis* bacteria, and Freund's incomplete adjuvant, which lacks the bacteria [20–22]. Freund's adjuvants strongly stimulated antibody production, but were too reactogenic for use in humans [23]. Novartis' squalene-based MF59 emulsion adjuvant was licensed for flu vaccines in 1997 and the second adjuvant to be used in human vaccines [24]. Mineral oil adjuvants are still widely used in vaccines for veterinary use, including vaccines for poultry, cattle and fish [25].

Vaccination in poultry

Chickens are vaccinated mostly with live attenuated and inactivated vaccines, which are of low cost and suitable for mass vaccination. Live attenuated vaccines are administered *en mass* via mucosal routes through eye drops, drinking water or coarse sprays [26], and are used to prime (*i.e.*, initiate) the immune response against infectious agents. Inactivated vaccines need to be administered to individual chickens by injection and are often formulated as multivalent vaccines to reduce time and costs of vaccination. Inactivated vaccines are given to laying and breeding hens just before the laying period for protection against infectious diseases during egg production and to stimulate the transfer of maternal antibodies to offspring [27,28]. To be effective, some inactivated vaccines require a prime-boost vaccination strategy, using a live attenuated vaccine followed by an inactivated vaccine against the same pathogen. Other inactivated vaccines provide protection upon single application, including vaccines for egg-drop syndrome virus (EDSV) [6], *Salmonella* [6], colibacillosis [29] and infectious coryza [30].

Poultry vaccines protect chickens from infectious diseases, but the type of immune responses (*e.g.* neutralizing antibodies or cellular responses) that lead to protection, the so-called

correlates-of-protection, are often unknown. During the development phase, vaccines are mainly tested for their ability to induce protection. The induction serum antibody titers may be measured as well and is often presumed to correlate with protection. However, this presumption is not always based on supporting evidence from field studies [31]. There are strong indications that T cells are important for protection against several avian pathogens [32].

Despite the availability of poultry vaccines for protection against many different avian infectious diseases, there is still need to further improve vaccine effectiveness, as demonstrated by evidence that IBV [33,34] and NDV [34,35] have become endemic worldwide and in major regions of the world, respectively. The protection of poultry against viral infections like IBV has been complicated by the emergence of new virus variants, which may also emerge from live attenuated vaccines that have reversed to virulence [35] by mutations or recombination events [36–38]. Recently, recombinant DNA [39,40] and viral vector vaccines [40,41] have been developed in response to the unmet need for safer poultry vaccines and for vaccines that cross-protect against emerging pathogen variants. However, recombinant DNA, subunit and inactivated vaccines are less immunogenic compared to live attenuated vaccines and usually require adjuvants to be effective [43,44].

Overview of the immune system

Cellular and humoral (*i.e.*, part of body fluids) defense mechanisms of the immune system are divided into innate and adaptive components (**Figure 1**). The innate component is present from birth, non-specific and immediately ready to combat a pathogen at first encounter. Innate immune cells are activated by pathogen-associated molecular patterns (PAMPs), *e.g.* bacterial lipopolysaccharides or viral nucleic acids, which are recognized through pattern recognition receptors (PRRs). Innate immune cells include innate lymphoid cells, monocytes, macrophages, natural killer cells, non-conventional T cells (*e.g.* $\gamma\delta$ T cells), granulocytes and mast cells, which combat pathogens by direct killing or by secretion of pro-inflammatory cytokines. In addition innate immune cells include macrophages and dendritic cells, which also act as professional antigen-presenting cells (APCs). Professional APCs present peptides from intracellular pathogens or engulfed antigens on both major histocompatibility complexes (MHC) class I and II to T cells of the adaptive immune system (**Figure 2**). A third type of professional APC are B cells, which are part of the adaptive immune system. In contrast to professional APCs, other cells can only present antigens on MHC class I.

The adaptive immune response requires time to develop, is pathogen-specific and results in immunological memory. The latter refers to the phenomenon that upon second encounter with a pathogen, either due to previous infection or vaccination, the immune response will develop faster and stronger. The activation and differentiation of T cells requires three signals including antigen recognition, co-stimulation and cytokines (**Figure 2**). APCs express peptide-loaded MHC molecules that are recognized by T cells through T cell receptor (TCR) binding. In addition, APCs express co-stimulatory molecules in the presence of PAMPs or damage-associated molecular patterns (DAMPs) released from damaged or stressed tissues [45]. Finally, APCs, and other immune cells, release cytokines that influence the differentiation of T cells (**Figure 3**).

T cells are divided into $CD4^+$ or $CD8^+$ T cells, which have different functions during an immune response. $CD4^+$ T cells recognize peptides presented on MHC class II and differentiate into helper T subtypes that each release effector cytokines to activate specific cells of the immune system (**Table 1**). In contrast, $CD8^+$ T cells recognize peptides presented on MHC class I and differentiate into cytotoxic T cells, which kill infected and malignant host cells. Furthermore, both $CD4^+$ and $CD8^+$ T cells differentiate into memory T cells, which are reactivated upon a second encounter with the same pathogen and thus crucial for immunological memory. Some $CD4^+$ T cells will differentiate into follicular helper T cells, which assist in the differentiation of B cells that have recognized antigens through their B cell receptor (BCR) into antibody-secreting plasma cells and memory B cells. Antibodies have diverse functions, including neutralization of pathogens, killing of bacteria through activation of the complement system, facilitating the uptake of pathogens by phagocytes (opsonization), and facilitating the depletion of infected or malignant host cells through activation of innate immune cells by antibody-dependent cellular cytotoxicity. The effector

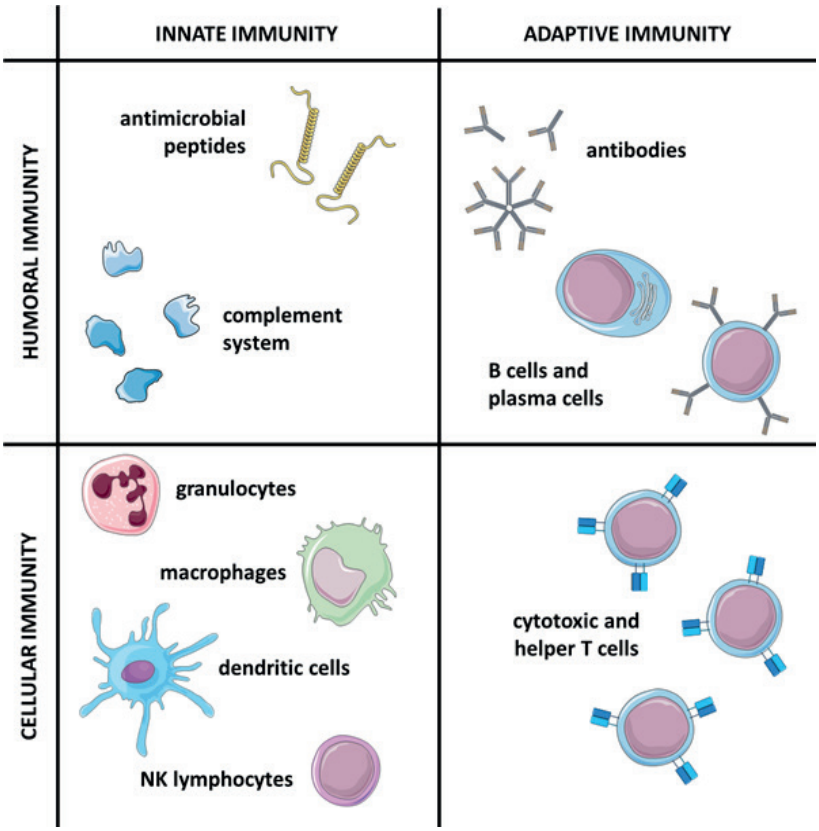


Figure 1. Components of the immune system. The immune system is divided into innate and adaptive components that each consist of cellular and humoral components. B cells and plasma cells are shown as part of the humoral instead of the cellular component of the adaptive immune system due to their effector function in antibody secretion. The icons used in this figure are adaptations from icons retrieved from the Servier Medical Art collection, which are licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

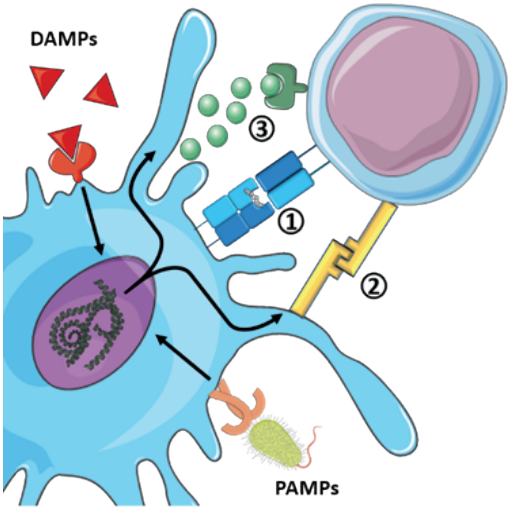


Figure 2. Three-signal model of T cell activation. APCs engulf and process antigens into peptides that can be presented on MHC molecules to T cells. T cells that recognize MHC-peptide complexes via their TCR will become activated, which is referred to as signal 1. Furthermore, T cells require two more signals for full activation including the binding of co-stimulatory receptors, providing signal 2, and cytokines, providing signal 3. APCs provide these signals upon sensing that an antigen is 'dangerous' through recognition of PAMPs or DAMPs. Without signals 2 and 3 the T cell will become anergic and unable to further respond, which is a mechanism to prevent immune responses against 'safe' antigens. The combination of cytokines that are part of signal 3 determines T cell differentiation into various T helper subsets (T_H1, T_H2, etc.). The icons used in this figure are adaptations from icons retrieved from the Servier Medical Art collection, which are licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

functions of antibodies depend on the various classes, which include immunoglobulin (Ig) A, IgD, IgE, IgG and IgM in mammals, and IgA, IgY and IgM in birds. Of these, naive B cells express IgD and IgM in mammals and IgM in birds. Antibody class switch to IgA, IgE and IgG in mammals or IgA and IgY in birds occurs when activated B cells receive differentiation signals from follicular helper T cells. The antibody most abundant in mucosal secretions is IgA in both mammals and birds. In serum, IgE and IgG antibody classes are present in mammals, whereas IgY is present in birds. Activated B cells further diversify in a process named somatic hypermutation, in which the antigen-binding regions of antibodies are enzymatically altered by gene mutations. Subsequently, follicular helper T cells are involved in a process called clonal selection, in which the B cell clones with the highest affinity for antigens are selected for. The combination of somatic hypermutation and clonal selection results in antibodies of high affinity, which is referred to as affinity maturation.

Increased knowledge about the immune system has enabled a rational approach to vaccine design. A striking example is given by conjugate vaccines (*e.g. Haemophilus influenzae* type b), comprising bacterial polysaccharides linked to a carrier protein. Initially, the vaccine only contained polysaccharides, which did not result in effective humoral immune responses due to lack of proteins required for activation of T cells and differentiation into follicular helper T cells [46]. Another example is given by acellular *pertussis* vaccines, which lead to less effective T_H2/T_H17 responses as compared to effective T_H1/T_H17 responses induced by whole cell *pertussis* vaccines in mice and humans [47,48]. The induction of a T_H1/T_H17 response could be restored by substituting the aluminum salt adjuvant of acellular *pertussis*

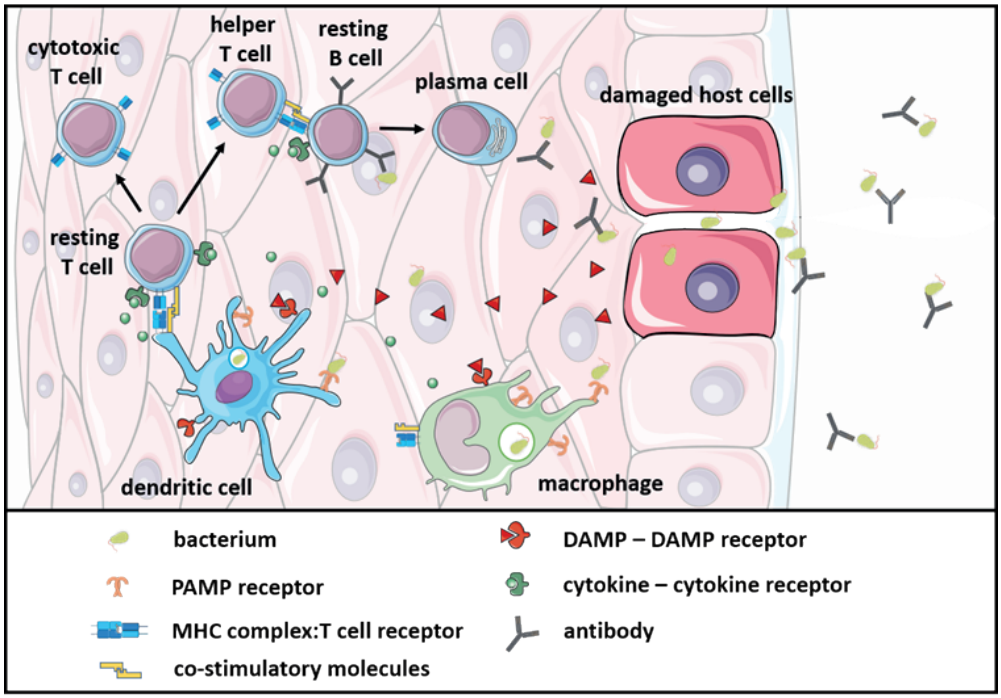


Figure 3. The immune system becomes active upon sensing pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Antigen-presenting cells (APCs), including macrophages and dendritic cells, use pattern recognition receptors (PRRs) to sense the threat of *e.g.* an infection either directly through recognition of PAMPs or indirectly through recognition of DAMPs as a result of tissue injury. Simultaneously, APCs engulf and destroy pathogens, and process pathogen-derived proteins into peptides for presentation on major histocompatibility (MHC) molecules. T cells become active when these possess T cell receptors that are able to bind the MHC-peptide complexes presented on APCs. For full activation of T cells, co-stimulatory signals and cytokines are required, which are provided by APCs upon sensing PAMPs or DAMPs. Subsequently, some T cells will differentiate in helper T cells that support the differentiation of antigen-specific B cells into antibody-producing plasma cells, providing humoral immunity, whereas others will differentiate into cytotoxic T cells, providing cellular immunity. The icons used in this figure are adaptations from icons retrieved from the Servier Medical Art collection, which are licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

vaccines for CpG oligonucleotides a T_H1/T_H17, resulting in improved efficiency of vaccination [47]. Moreover, knowledge about the interaction between vaccines and the immune system allows the development of novel *in vitro* methods to assess the quality of vaccines as will be discussed below.

Advancements in quality control of vaccines

Vaccine manufacturers and regulatory authorities are responsible for assuring the quality and safety of vaccine batches in routine quality control, also after their initial approval. This is important since vaccines are often derived from living organisms that may show inherent variability resulting in differences between batches.

Table 1. Characteristics of main helper T subsets. This table gives an overview of the most distinct T helper subtypes. Abbreviations: T_{EFF} = effector T cells; T_H1 (or 2, 17) = type 1 (or 2, 17) helper T cells; T_{FH} = follicular helper T cells and T_{REG} = regulatory T cells.

Type:		Master regulator (transcription factor)	Polarization cytokines	Signature effector cytokines	Stimulates (or inhibits)	Function
T_{EFF}	T_H1	T-bet	IL-12p70	IFN-γ	macrophages, cytotoxic T cells, NK cells	intracellular pathogens
	T_H2	GATA3	IL-4	IL-4, IL-5, IL-13	eosinophils, basophils, mast cells	extracellular parasites
	T_H17	RORγt	IL-6, IL-23, TGF-β	IL-17A, IL-17F, IL-22	neutrophils (or heterophils in birds)	extracellular bacteria, fungi
T_{FH}		Bcl-6	IL-6, IL-21	IL-21	B cells	support selection and differentiation of B cells
T_{REG}		FoxP3	IL-2, TGF-β	TGF-β, IL-10	T cells and dendritic cells	immune tolerance and resolution of immune responses

Traditionally, the quality of new batches of vaccine was assessed using *in vivo* vaccination-challenge studies. The use of large numbers of animals in experiments that cause pain and distress leads to an ethical dilemma [49–51]. Moreover, some animal models used are not reproducible or not representative for the natural pathophysiology of target pathogens [51–55]. An example of the latter is the potency test for inactivated rabies vaccines, which requires intracerebral or intraperitoneal injection of rabies virus into mice, whereas the route of transmission is through saliva [51]. Finally, the use of animal models is expensive, time consuming and may be a risk for personnel working with infected animals [51].

The 3R guidelines by William Russell and Rex Burch describe how to enable 'Replacement', 'Reduction' and 'Refinement' in animal experiments [56]. Reduction can be accomplished by statistical estimation of the minimum number of animals required to perform vaccine quality tests [56]. Refinement has been accomplished by defining humane endpoints to avoid unnecessary suffering of animals [57]. Lethal endpoints can be substituted for early clinical signs that are predictive of death or for nonclinical endpoints such as the induction of antibody titers (**Figure 4**). Ideally, *in vivo* vaccine quality tests are replaced for non-animal *in vitro* alternatives. Recently, the European Pharmacopoeia Commission has described 'Removal' as a fourth 'R' when there is scientific evidence that an animal-based test is unnecessary and can thus be removed as a legal requirement for vaccine quality testing [58]. Directive 2010/63/EU on the protection of animals used for scientific purposes forbids the use of animal experiments when non-animal alternatives are available [59]. In line with this directive, the European Pharmacopoeia is continuously being updated to remove *in vivo* tests

that are found to be unnecessary or to substitute tests for 3R alternatives [58].

Advancements in *in vitro* culturing of live pathogens has resulted in batch potency tests of live attenuated vaccines that rely on viral or bacterial titration or bacterial counts rather than animal tests [60]. However, for inactivated and toxoid vaccines this is not possible, which is why these vaccines often require *in vivo* potency tests. Many *in vivo* vaccination-challenge tests of inactivated and toxoid vaccines have been replaced by *in vivo* serological tests, which no longer require challenges with virulent pathogens [60]. Some *in vivo* potency tests of inactivated and toxoid vaccines have been replaced by *in vitro* antigen quantification methods, *e.g.* the tests for inactivated vaccines against hepatitis A virus for use in humans [61] and non-adjuvanted vaccines against Leptospira for use in dogs and cattle [62,63]. A major achievement for quality testing of poultry vaccines has been the implementation of an enzyme-lined immunosorbent assay (ELISA) based on antigen quantification as a potency test for inactivated vaccines against NDV [64].

One-to-one replacement of animal-based quality tests for established vaccines with *in vitro* alternatives can be realized when these are demonstrated to be a good correlate-of-protection. Alternatively, replacement of animal-based vaccine batch-release tests can be accomplished through the consistency approach, which is based on the principle that quality is the consequence of consistent production of subsequent batches monitored by a good manufacturing practice (GMP) quality system [65]. In addition, final batch testing is performed by analysis of a limited number of parameters that together demonstrate consistent quality of vaccine batches [66]. Since 2014, the consistency approach is promoted in the European Pharmacopoeia [67].

Box 1. Relevant definitions for vaccine evaluation.

Vaccine efficacy is the maximum effect that can be expected and more specifically refers to the proportion of vaccinated individuals for which the desired effect has been accomplished under ideal conditions [31]. The desired effect may differ between products and may include for instance protection from infection, suffering or, in the case of livestock, reduced productivity.

Vaccine potency refers to the capacity (or strength) of a vaccine to exert the desired effect [4]. This may be solely related to infectivity (live vaccines) or antigen mass (killed or subunit vaccines) but can also be strengthened by the presence of immunostimulatory constituents (*e.g.* adjuvants or PAMPs).

Immunogenicity is the capacity of a vaccine (or other substance) to provoke an immune response against the vaccine antigens. In this way immunogenicity contributes to vaccine potency, provided that a beneficial type of immune response is elicited.

Vaccine batch-release potency tests assess the ability of vaccine batches to exert the desired effect directly or indirectly by measuring immune responses (correlates-of-protection) or vaccine parameters that correlate with the desired effect.

The consistency approach for vaccine batch-release quality testing implies the use of a set of *in vitro* assays that demonstrate consistency between product profiles of a new batch and a reference batch of proven efficacy and safety [66]. Consistent batches are expected to be similar with regard to potency and safety, which would render *in vivo* batch-release testing unnecessary.

For some vaccines, antigen quantification methods are sufficient to fully replace *in vivo* potency tests. For others, a combination of *in vitro* immunochemical, physicochemical and cell-based assays may be required to assess different batches for consistency (**Figure 4**). These assays can be developed based on the current knowledge about the mechanisms by which vaccines activate the immune system. The use of cell-based assays has been proposed to mimic the most important aspects of the immune response, *e.g.* the expression of co-stimulatory molecules and cytokines [68–72].

Outline of this thesis

Currently, most inactivated poultry vaccines are tested for potency using *in vivo* vaccination-challenge or serological tests, which account for a significant proportion of laboratory animal use. The aim of this thesis is to set up *in vitro* cell-based assays to assess the immunostimulatory capacity of inactivated poultry vaccines and to determine their applicability for routine potency testing, in view of the 3Rs. Development of *in vitro* alternatives for inactivated poultry vaccines is complicated by the presence of adjuvants and the fact that most are multivalent to simultaneously induce protection against many infectious diseases. Furthermore, some pathogens like IBV are high variably and comprise different strains, which makes it more difficult to develop antigen quantification methods that cover vaccines against these different strains. Finally, most studies that have investigated the immune responses evoked by inactivated poultry vaccines have focused on serology, which is why a comprehensive understanding of the immunostimulatory properties of these vaccines is often lacking.

The first two chapters of this thesis focus on the immunostimulatory properties of inactivated poultry vaccines by investigating their effect on chicken macrophages. Macrophages have a

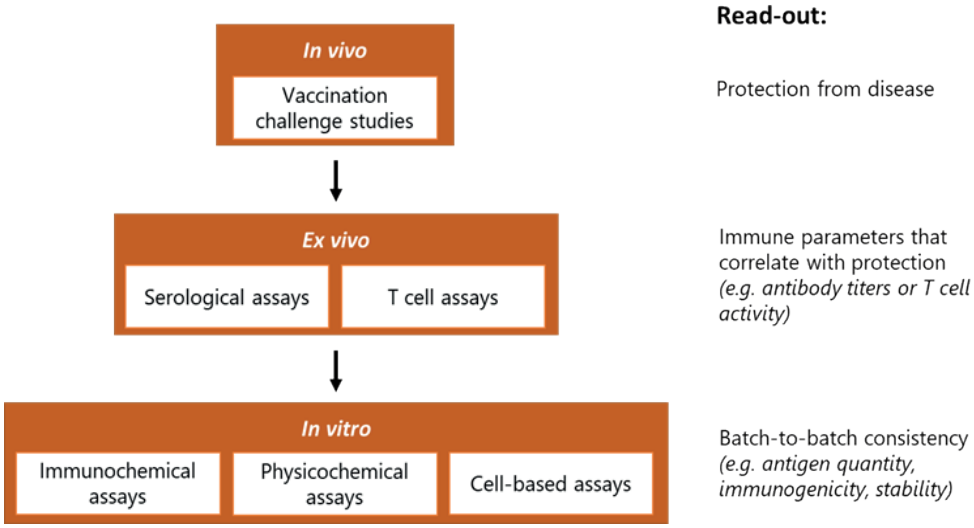


Figure 4. The transition from *in vivo* to *in vitro* vaccine batch quality testing.

clear role in the early immune response [73,74] and express a broad range of PRRs [75], and are therefore able to sense immunostimulatory properties of vaccine constituents. The cell-based assays described involve the use of the macrophage-like cell line HD11, which has been used before to investigate the capacity of pathogens to stimulate innate immune cells [76–79]. In the study presented in **Chapter 2**, a phagocytosis assay with IgY-opsonized beads is used to investigate whether inactivated poultry vaccines against IBV, NDV and EDSV affect phagocytosis by macrophages. In **Chapter 3**, the use of nitric oxide production and cytokine gene expression are described to determine the immunostimulatory properties of an inactivated octavalent vaccine against IBV, NDV, EDSV and five strains of *Avibacterium paragallinarum*, the causative agents of infectious coryza characterized by an acute respiratory disease in chickens.

Due to their long lifespan, low variability, and availability in large numbers, immortalized cell lines may be more suitable for quality control settings than primary cells. However, immortalized cell lines may have reduced sensitivity [80] or impaired immune responsiveness [81] as compared to their primary cell counterparts. Furthermore, dendritic cells, rather than macrophages, are considered to be most important for the induction of an immune response [69], and dendritic cell lines of chicken origin are not available. The study presented in **Chapter 4** describes a thorough morphological, phenotypical and functional characterization of chicken bone marrow-derived dendritic cell cultures, using similar cell culture methods as previously described by others [82,83]. In **Chapter 5**, exposure of the bone marrow-derived dendritic cells to an inactivated poultry vaccine against IBV and NDV is described. The purpose of this study was to identify biomarkers of vaccine-induced immune responses by proteomic analysis. Furthermore, the mechanisms by which inactivated poultry vaccines activate dendritic cells were explored.

As previously mentioned, the quality of inactivated poultry vaccines is often determined in vaccination-challenge or serological tests. The induction of humoral immunity is known to be an important aspect of vaccination with inactivated poultry vaccines. In contrast, knowledge about the induction of T cell-mediated immunity is still limited, although there are studies that have demonstrated a role for cellular immunity in the protection against IBV [84] and NDV [85]. The study of **Chapter 6** describes a T cell proliferation assay to evaluate cellular immunity in laying hens vaccinated with an inactivated vaccine against IBV and NDV and its potential as a quality test for this vaccine.

In **Chapter 7**, a review describes the difficulties that are being encountered during the transition from *in vivo* to *in vitro* batch testing of vaccines for both human and veterinary use, as well as possibilities to overcome these difficulties. Topics include the critical quality attributes of vaccines that require testing, the use of cell-based assays to mimic relevant aspects of *in vivo* vaccine-induced immune responses, the difficulties of adjuvanted and multivalent vaccines and how to overcome these, the use of altered batches to validate *in vitro* test methods when non-compliant vaccine batches are limitedly available, and how cooperation between different stakeholders is key to move the transition forward. Finally, the findings of this thesis, as well as the next steps required for the transition *in vitro* potency testing of inactivated poultry vaccines, are discussed in **Chapter 8**.

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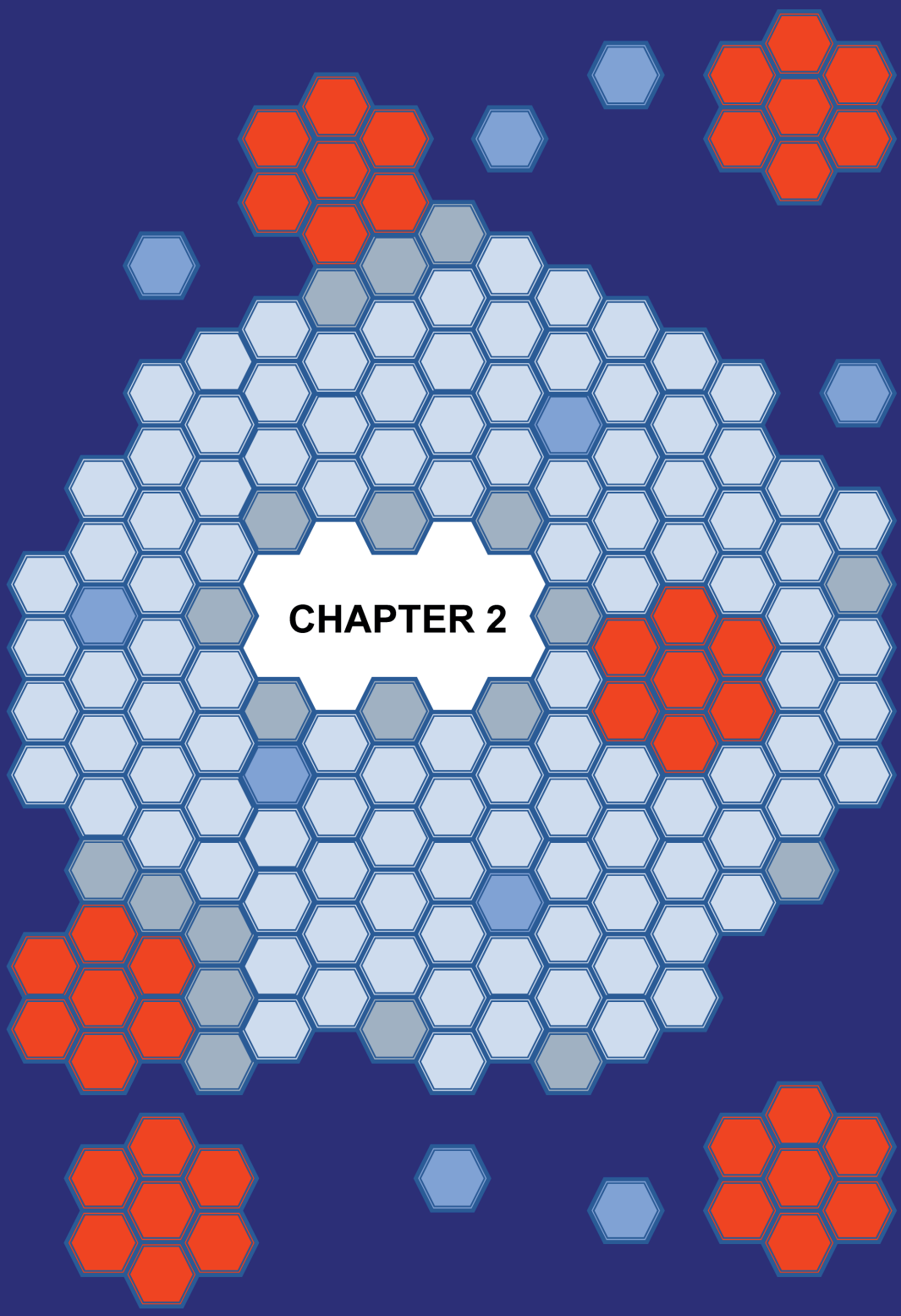
CHAPTER 2

Nitric oxide production and Fc receptor-mediated phagocytosis as functional readouts of macrophage activity upon stimulation with inactivated poultry vaccines *in vitro*

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Abstract

Vaccine batches must pass routine quality control to confirm that their ability to induce protection against disease is consistent with batches of proven efficacy from development studies. For poultry vaccines, these tests are often performed in laboratory chickens by vaccination-challenge trials or serological assays. The aim of this study was to investigate innate immune responses against inactivated poultry vaccines and identify candidate immune parameters for *in vitro* quality tests as alternatives for animal-based quality tests. For this purpose, we set up assays to measure nitric oxide production and phagocytosis by the macrophage-like cell line HD11, upon stimulation with inactivated poultry vaccines for infectious bronchitis virus (IBV), Newcastle disease virus (NDV), and egg drop syndrome virus (EDSV). In both assays, macrophages became activated after stimulation with various toll-like receptor agonists. Inactivated poultry vaccines stimulated HD11 cells to produce nitric oxide due to the presence of mineral oil adjuvant. Moreover, inactivated poultry vaccines were found to enhance Fc receptor-mediated phagocytosis due to the presence of allantoic fluid in the vaccine antigen preparations. We showed that inactivated poultry vaccines stimulated nitric oxide production and Fc receptor-mediated phagocytosis by chicken macrophages. Similar to antigen quantification methods, the cell-based assays described here can be used for future assessment of vaccine batch-to-batch consistency. The ability of the assays to determine the immunopotentiating properties of inactivated poultry vaccines provides an additional step in the replacement of current *in vivo* batch-release quality tests.

Introduction

Infectious diseases are a major problem for the poultry industry and lead to economic losses. Therefore, vaccination is essential to prevent disease outbreaks and maintain flock health. Early in life, starting before, or soon after hatch, chickens are vaccinated frequently with live attenuated vaccines to induce protective immunity. Before the first laying period, layers receive several booster vaccinations to maintain this protective immunity during their egg production phase. These may include inactivated multivalent vaccines against a combination of pathogens, such as infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome virus (EDSV), and infectious bursal disease virus (IBDV), and are often formulated as water-in-oil (w/o) emulsions using mineral oil adjuvants to potentiate the immune response [1].

Since vaccines are complex biological products, each batch requires routine quality control (QC) testing to ensure that it meets the established requirements to induce protection against disease. Quality testing for the potency of inactivated poultry vaccines used to be performed with vaccination-challenge tests, which have nowadays largely been replaced with vaccination-serology tests [2,3]. However, for many infectious diseases of poultry, the correlates of protection are still unknown and serological assays may not always be the best method to monitor vaccine potency. Moreover, there is a growing global intent to further reduce the number of laboratory animals used for routine quality control of vaccine batches [4]. As a consequence of the implementation of the 3Rs (replacement, reduction, and refinement) in European law, animal testing in the EU is only allowed when no non-animal alternatives are available [5]. The growing availability of physicochemical and cell-based alternatives has created opportunities to replace animal-based tests by methods that provide cost-effective and ethically attractive alternatives.

One strategy to ensure the quality of vaccines is the consistency approach, which implies that batch-to-batch variation of vaccines can be controlled by well-defined production processes and analysis of intermediate and final products by *in vitro* methods [6,7,8]. This approach requires a battery of methods to create a product profile that provides sufficient information to replace current animal-based tests. To assess antigen quantity, an important quality parameter of vaccines, a method like enzyme-linked immunosorbent assay (ELISA) can be used, which is in place for inactivated NDV vaccines [3,9,10] Moreover, such methods have been developed for inactivated IBV and IBDV vaccines [10,11], although they are not yet used for QC testing [3]. However, vaccine quality is usually not dependent on antigen quantity alone. Usually, additional vaccine components, like adjuvants, are required to stimulate the immune system, which is why antigen quantification methods should be complemented with other QC tests that assess the immunogenicity of these additives.

Pathogen-associated molecular patterns (PAMPs), like bacterial cell wall components or viral nucleic acids, activate innate immune cells via pattern recognition receptors (PRRs) [12,13]. Similarly, vaccine excipients, including adjuvants, can augment the immunogenicity of vaccines by activation of innate immune cells, either directly or by inflicting tissue damage, resulting in the release of endogenous danger signals, such as extracellular DNA, ATP, or heat-shock proteins, collectively called damage-associated molecular patterns (DAMPs) [14,15]. It is currently unknown whether inactivated viral poultry vaccines contain PAMPs

or other constituents leading to DAMPs that augment immune responsiveness by the activation of innate immune cells. Immunopotentiating effects of vaccine constituents on innate immune cells can be addressed *in vitro* with cell-based assays. For poultry vaccines, a good candidate for such assays is the chicken macrophage-like cell line HD11, which shows broad expression of PRRs, including toll-like receptors (TLRs) [16]. The HD11 cell line has already been employed to explore the immune-activating or immunomodulatory properties of TLR agonists [17,18,19], liposomes [20], host defense peptides [21], cytokines [22,23,24], bacteria [17,21,25], and replicating viruses [26,27,28], using nitric oxide production as a functional readout of macrophage activation. Macrophages produce nitric oxide using the enzyme-inducible nitric oxide synthase (iNOS), which is expressed upon stimulation of PRRs and downstream activation of transcription factor nuclear factor kappa B (NF-κB) [17]. In addition, the HD11 macrophage-like cell line expresses a high-affinity Fc receptor for immunoglobulin Y (IgY), the chicken Ig-like receptor AB1 (CHIR-AB1) [29], and has been used before to study phagocytosis [30,31]. *In vivo*, macrophages are strategically located at the entry sites of lymphoid tissues and are able to react to PAMPs and DAMPs in the blood lymph fluid [32,33]. Moreover, macrophages have been shown to augment immune responses after vaccination, due to their ability to produce nitric oxide and perform Fc receptor-mediated phagocytosis [34,35,36].

In this study, we stimulated the chicken macrophage-like cell line HD11 with inactivated poultry vaccines and intermediary vaccine products, including antigenic fractions and mineral oil adjuvant, to study their effects on nitric oxide production and phagocytosis and to contribute to a better understanding of their effects on the chicken innate immune system. Moreover, we explored the potential of nitric oxide production and phagocytosis as biomarkers of vaccine-induced immune activation, for future use in *in vitro* vaccine QC tests.

Materials and Methods

HD11 cell culture and stimulation

The chicken macrophage-like cell line HD11 [37], stored at -140 °C in complete Roswell Park Memorial Institute (RPMI)-1640 medium with 50% Fetal Bovine Serum (FBS) and 10% dimethyl sulfoxide (DMSO), was thawed and used after 3 to 20 passages. The cells were maintained in complete RPMI-1640 cell culture medium supplemented with GlutaMAX-I, phenol red, HEPES, 10% fetal bovine serum (FBS), 200 U/mL penicillin, and 200 U/mL streptomycin (all Gibco, Life Technologies Limited, Paisley, UK) in Corning 75-cm2 cell culture flasks (Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C, 5% CO_2, and passaged twice weekly. For experiments, HD11 cells were harvested from 75-cm2 cell culture flasks when they were at ~90% confluency using a 0.25% trypsin/EDTA solution supplemented with phenol red (Gibco, Life Technologies Limited, Paisley, UK). Subsequently, the cells were counted and resuspended at a concentration of 200,000 cells/mL. The cells were seeded at 1 mL/well complete RPMI medium in Corning Costar 24-well cell culture plates (Sigma-Aldrich, Saint Louis, MO, USA) and cultured overnight at 37 °C and 5% CO_2.

After overnight incubation, the HD11 cells were exposed to various stimuli to assess their activation using either nitric oxide production or phagocytosis as a readout. Stimuli included 100–300 ng/mL lipopolysaccharides (LPS) from *E. coli* O127:B8 (Sigma-Aldrich, Saint Louis,

MO, USA) to target TLR4, 100–500 ng/mL CpG oligonucleotides (ODNs) 2006 to target TLR21, 10 µg/mL resiquimod (R848) to target TLR7, 10 ng/mL Pam3CSK4 to target the TLR2/1 heterodimer, and 5 µg/mL zymosan from *S. cerevisiae* (all InvivoGen, San Diego, CA, USA) to target the TLR2/6 heterodimer. In addition, HD11 cells were stimulated with established inactivated poultry vaccines and/or their antigenic fractions, which were kindly provided by three pharmaceutical companies that are part of the VAC2VAC consortium (http://www.vac2vac.eu/), hereafter referred to as companies A, B, and C. The inactivated poultry vaccines used in this study contained mineral oil adjuvants in w/o formulation and included inactivated monovalent IBV (company B), inactivated bivalent IBV + NDV (companies A, B, and C), and inactivated trivalent IBV + NDV + EDSV (company A) vaccines. The inactivated poultry vaccines from companies A, B, and C were prepared in such a way that a single chicken vaccination dose corresponds to, respectively, 0.5, 0.5, and 0.3 mL. The antigenic fractions, hereafter referred to as antigens, comprised whole inactivated IBV (companies A, B, and C) and NDV (company B), which were propagated on embryonated chicken eggs, harvested from the allantoic cavity, and inactivated using either formaldehyde or β-propiolactone. Allantoic fluid without virus (company A), mineral oil (company A), and an "empty vaccine" consisting of allantoic fluid without virus formulated with mineral oil (company B) were included as controls.

Griess assay to measure nitric oxide production by HD11 cells

Nitric oxide production by HD11 cells was measured by the Griess assay [38] 48 h after stimulation. First, 50 µL of supernatants were harvested from triplicate wells and transferred to a 96-well flat-bottom plate (Corning B.V. Life Sciences, Amsterdam, The Netherlands) to measure the nitrite concentration. A 3.13–200 µM $NaNO_2$ nitrite standard dilution series (Sigma-Aldrich, Merck, St. Louis, MO, USA) was included to generate a standard curve. Griess assay reagents were made by dissolving N-(1-naphtyl)ethylenediamine at 3 g/L and sulfanilamide at 10 g/L (both from Sigma-Aldrich, Merck, St. Louis, MO, USA) in 2.5% phosphoric acid (Supelco, Merck, St. Louis, MO, USA). The Griess reagents were mixed 1:1 and 50 µL was added to the wells with cell culture supernatants and standards. The Griess reagents mixture turned purple upon reaction with nitrite ions in the cell culture supernatant. The optical density (OD) at 540 nm of each well was measured using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) to determine the nitrite concentration of each sample according to the nitrite standard curve.

Phagocytosis of IgY-Opsonized Beads by HD11 Cells

IgY-opsonization of fluorescent beads

The phagocytosis assay was performed with chicken IgY-opsonized fluorescent beads, which were prepared by mixing 1 µm crimson carboxylate-modified FluoSpheres (Invitrogen, Life Technologies Europe BV, Bleiswijk, The Netherlands) at a final concentration of 7.2×10^9/mL with an egg yolk IgY fraction (Agrisera AB, Vännäs, Sweden) at a final concentration of 14.4 mg/mL in a glass tube, followed by overnight mixing in an orbital rotator at 4 °C. The next day, the beads were washed twice by adding 10 mL Dulbecco's phosphate-buffered saline without calcium and magnesium ($DPBS^{-/-}$; Lonza, Basel, Switzerland) and centrifugated at 3000x *g* for 20 min between washes. Finally, the beads were resuspended in $DPBS^{-/-}$ at a concentration of 3.5×10^9 beads/mL. Coupling of IgY was confirmed by staining the beads with 0.5 µg/mL R-phycoerythrin (PE)-labeled mouse anti-chicken monoclonal antibodies (SouthernBiotech, Birmingham, AL, USA) in fluorescence-activated cell sorting (FACS)

buffer containing $DPBS^{-/-}$ + 0.5% bovine serum albumin and 0.005% sodium azide (both from Sigma-Aldrich, Saint Louis, MO, USA) and analysis using a CytoFLEX LX flow cytometer and 375-, 561-, and 638-nm lasers (Beckman Coulter Inc., Brea, CA, USA) (**Supplementary Materials Figure S1**).

Phagocytosis by HD11 cells

Phagocytosis of IgY-opsonized beads was measured 24 h after stimulation of the HD11 cells. First, three wells of a 24-well plate with HD11 cells were harvested using $DPBS^{-/-}$ supplemented with 5 mM UltraPure EDTA (Invitrogen, Life Technologies Europe BV, Bleiswijk, The Netherlands) to determine the cell counts per well, which ranged between 0.5 and 1.0 x 10^6 cells. IgY-opsonized beads were added at a 1:1 bead-to-cell ratio to the HD11 cells of the remaining wells followed by a 4-h incubation at 37 °C, 5% CO_2 to allow the cells to phagocytose the beads. Next, the cells were harvested using $DPBS^{-/-}$ supplemented with 5 mM UltraPure EDTA and centrifuged at 400x *g* for 3 min. The cells were transferred to 96-well V-bottom plates (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands), washed in $DPBS^{-/-}$, and stained for cell viability in 50 µL $DPBS^{-/-}$ with 1:400 Zombie Aqua Fixable Viability Dye (BioLegend Inc., San Diego, CA, USA) for 20 min at 4 °C. Subsequently, the cells were washed twice and fixed in 200 µL $DPBS^{-/-}$ with 2% paraformaldehyde (Alfa Aesar, Haverhill, MA, USA) for 10 min at room temperature (RT). Finally, the cells were washed once more in FACS buffer and resuspended in 200 µL of FACS buffer. Up to 50,000 cells were analyzed using the CytoFLEX LX flow cytometer.

Data analysis was performed using FlowJo Software v. 10.6 (FlowJo LCC, Ashland, OR, USA) and Prism 8.4 (Graphpad Software Inc., San Diego, CA, USA). The viability of the cells was expressed as the percentage of HD11 cells negative for Zombie Aqua Fixable Viability Dye. Only samples with ≥100 viable cells in the live gate (**Figure 2b**) were included in the analysis of changes in bead uptake. The fluorescent content of HD11 cells measured using a 660/10-nm bandpass filter after excitation with the 638-nm laser was directly proportional to the number of beads engulfed. Furthermore, the HD11 containing a single bead were visible as the first positive peak in a histogram showing the fluorescent intensity at 660/10 nm. From this, the average bead uptake by each sample was calculated by:

$$(1) \qquad beads/cell = \frac{MFI_{total}}{MFI_{1\ bead/cell}}$$

with MFI_{total} for the mean fluorescent intensity (MFI) at 660/10 nm of each sample $MFI_{1\ bead/cell}$ for the MFI at 660/10 nm of cells containing 1 bead/cell (see also **Figure 1a**). Next, the fold change in bead uptake by HD11 cells after stimulation was calculated by:

$$(2) \qquad foldchange = \frac{beads/cell_{stimulated}}{beads/cell_{unstimulated}}$$

Flow cytometric side scatter to determine vaccine decomposition

An accumulation of vacuoles was observed in HD11 cells exposed to inactivated poultry vaccines or the empty vaccine control (without viral antigens), which was captured by light microscopy using an an EVOS FL microscope (AMG, Mill Creek, Washington, DC, USA).

The vacuoles were considered to be vaccine-derived lipid droplets, since they were only observed in the presence of inactivated poultry vaccines containing emulsified mineral oil adjuvant. The accumulation of lipid droplets was considered as a surrogate marker for vaccine decomposition and quantified by measuring the average side scatter (SSC) of HD11 cells by flow cytometry, in accordance with a previous study [39].

Involvement of the Fc receptor CHIR-AB1 in IgY-opsonized bead uptake by HD11 cells

HD11 cells stimulated for 24 h with different concentrations of LPS or inactivated IBV antigen (company B) were assessed for expression of CHIR-AB1 by subsequently staining the cells in 50 µL of FACS buffer with 1:20-diluted hybridoma supernatant containing mouse-anti-chicken CHIR-AB1 (clone 8D12, mouse IgG2b, gift from Thomas W. Göbel, LMU Munich, Munich, Germany) and 0.1 µg/mL allophycocyanin (APC)-labeled goat-anti-mouse IgG2a (SouthernBiotech, Birmingham, AL, USA) for 20 min at 4 °C, with two washing steps in FACS buffer in between. Next, the cells were washed once in FACS buffer and once in $DPBS^{-/-}$ followed by staining in 50 µL of $DPBS^{-/-}$ with 1:400 Zombie Aqua Fixable Viability Dye (BioLegend Inc., San Diego, CA, USA) for 20 min at 4 °C. Finally, the cells were washed once more in FACS buffer and resuspended in 200 µL of FACS buffer for analysis using the CytoFLEX LX flow cytometer.

To determine the involvement of CHIR-AB1 in the uptake of IgY-opsonized beads, different concentrations of mouse-anti-chicken CHIR-AB1 were administered to HD11 cells 10 min before addition of the beads to block interactions between CHIR-AB1 and IgY-opsonized beads. Subsequent steps were according to the phagocytosis assay as described.

Confocal microscopy to assess internalization of IgY-opsonized beads by HD11 cells

HD11 cells were prepared for confocal microscopy to confirm the internalization of IgY-opsonized beads. Ethanol-cleaned 12-mm glass coverslips (Waldemar Knittel Glasbearbeitungs GmbH, Brunswick, Germany) were added to the 24-well cell culture plates before HD11 cells were seeded and subjected to the phagocytosis assay as described. After 4 h of incubation with IgY-beads, the cells were washed twice with cold DPBS with calcium and magnesium ($DPBS^{+/+}$; Lonza, Basel, Switzerland) before staining in $DPBS^{+/+}$ with 2 µg/mL wheat germ agglutinin (WGA)-Alexa Fluor 488 (Invitrogen, Life Technologies Europe BV, Bleiswijk, The Netherlands) for 20 min at 4 °C. Subsequently, the cells were washed thrice with cold $DPBS^{+/+}$ and fixed in $DPBS^{+/+}$ with 4% paraformaldehyde at RT for 30 min. Next, the cells were washed three times with $DPBS^{-/-}$ + 10 mM glycine (Merck Millipore, Burlington, MA, USA) to quench the remaining paraformaldehyde. The cells were washed once more in distilled water before the coverslips with HD11 cells were mounted on Polysine microscope slides (Menzel Glazer GmbH & Co KG, Braunschweig, Germany) using Fluorsave Reagent (Calbiochem, Merck Millipore, Burlington, MA, USA). The cells were captured, and bead internalization was analyzed using a TCS-SPE-II confocal microscope (Leica Microsystems B.V., Amsterdam, The Netherlands) and 488- and 635-nm diode lasers. Microscopic images were further processed using Fiji software [40].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.4 software. When the assumptions of normally distributed data and residuals were met, a one-way ANOVA with Holm–Sidak's

multiple comparisons test was used to test for statistically significant differences between stimulated and unstimulated control samples. When the assumptions of normality were not met, a non-parametric Kruskal–Wallis test with Dunn's multiple comparisons test was used instead. A *p*-value of <0.05 was considered statistically significant.

Results

High concentrations of inactivated poultry vaccines induce nitric oxide production by HD11 Cells

Production of nitric oxide by HD11 cells stimulated with TLR agonists, used as positive controls, was determined by the Griess assay. Stimulation with LPS resulted in 106.1 ±2.2 µM, CpG in 115.2 ±4.2 µM, and R848 in 84.4 ±1.4 µM nitric oxide in the culture supernatant (**Figure 1a**). Stimulation of HD11 cells with dose ranges of inactivated IBV and NDV antigens provided by different companies did not result in nitric oxide production (**Figure 1b**). In contrast, nitric oxide was produced at low quantities when HD11 cells were stimulated with an inactivated monovalent IBV vaccine from company B (4.8 ±0.5 µM) and inactivated bivalent IBV + NDV vaccines from companies A (4.2 ±0.3 µM) and B (4.9 ±0.5 µM) (**Figure 1c**). An "empty vaccine" without inactivated viral antigens also induced the production of low quantities of nitric oxide (4.9 ±0.2 µM). All vaccines contained a mineral oil adjuvant and were formulated as water-in-oil emulsions. Nitric oxide production was not significantly different from unstimulated HD11 cells when non-emulsified mineral oil was added to the HD11 cells (respictively 2.0 ±0.3 µM and 2.6 ±0.1 µM). Taken together, small amounts of nitric oxide were produced by HD11 cells upon exposure to the inactivated poultry vaccines, which may be induced by the presence of emulsified mineral oil.

Phagocytosis of IgY-opsonized beads by HD11 Cells is enhanced upon stimulation with TLR agonists

The ability of HD11 cells to phagocytose IgY-opsonized beads after 24 h of stimulation with TLR agonists was assessed by a 4-h co-incubation (**Figure 2a** and **Supplementary Materials Video S1**). HD11 cells showed increased uptake of IgY-opsonized beads upon stimulation with LPS (2.18 ±0.05-fold), CpG (1.99 ±0.12-fold), R848 (1.66 ±0.04-fold), Pam3CSK4 (1.87 ±0.08-fold), and zymosan (1.59 ±0.07-fold) compared to unstimulated cells (**Figure 2b**,**c**). The viability of HD11 remained unaffected by stimulation with zymosan and was only slightly affected by LPS, CpG, R848, or Pam3CSK4 (**Supplementary Materials Figure S2**).

Allantoic fluid-containing inactivated IBV and NDV antigens enhance phagocytosis by HD11 Cells

Next, the effects of inactivated IBV and NDV antigens on phagocytosis by HD11 cells were determined. IBV antigen from company B (maximum fold change 3.54 ±0.19 at 10 µL/mL) led to a higher induction of bead uptake, at a lower dose, than IBV antigens from companies A (maximum fold change 2.69 ±0.35 at 18 µL/mL) or C (maximum fold change 2.81 ±0.36 at 30 µL/mL) (**Figure 3a**). Inactivated NDV antigens from company B (maximum fold change 3.81 ±0.29 at 10 µL/mL) also enhanced phagocytosis by HD11 cells. Beyond the doses inducing a maximum bead uptake, increasing doses of IBV and NDV antigen led to a decrease in phagocytosis (**Figure 3a**), concurrent with decreased cell viability (**Figure 3b**).

IBV and NDV antigens both contained viruses that were whole inactivated after propagation in embryonated chicken eggs and harvest from the allantoic cavity. For this reason, allantoic fluid from non-inoculated eggs was tested for its ability to stimulate phagocytosis and found to enhance phagocytosis by 3.06 ±0.21-fold (**Figure 3c**). Similar to the IBV and NDV antigens, increasing doses of allantoic fluid also led to a decrease in cell viability (**Figure 3d**).

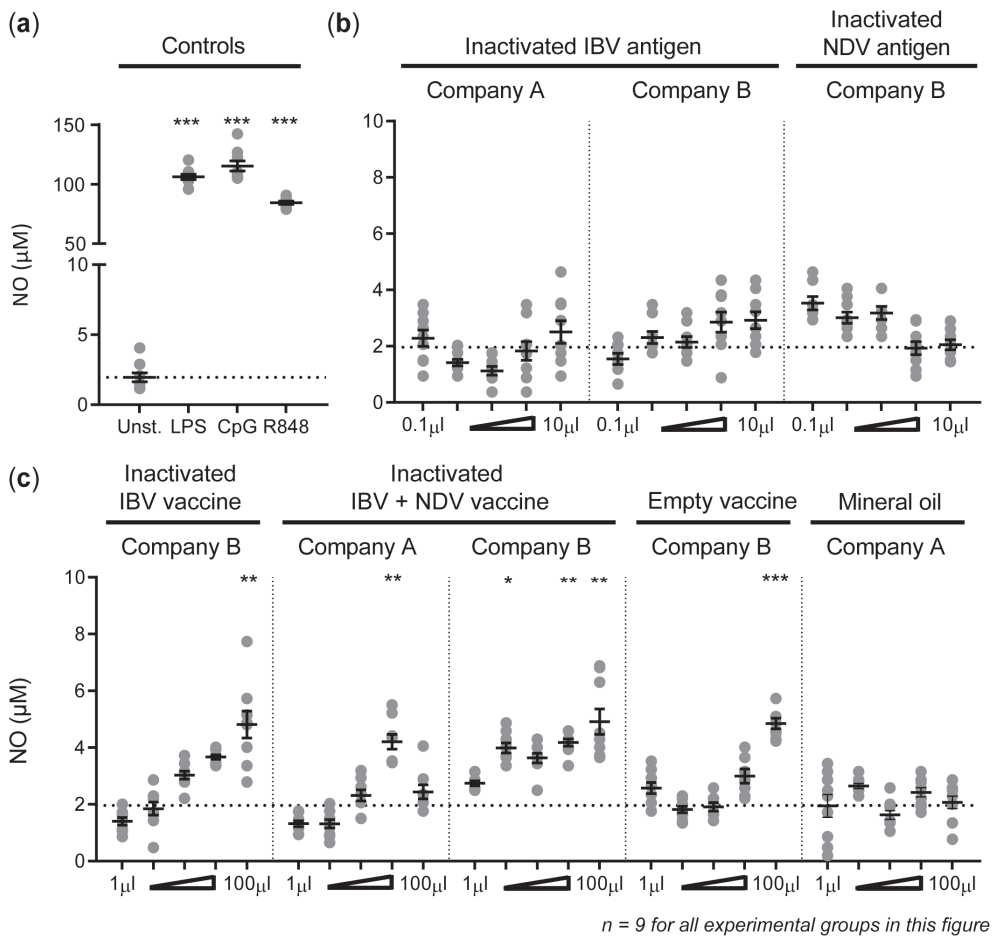


Figure 1. Inactivated poultry vaccines induced nitric oxide production by HD11 cells, whereas inactivated IBV and NDV antigens did not. (**a**) Nitric oxide production by HD11 cells was assessed upon stimulation with TLR agonists, *i.e.*, 100 ng/mL LPS, 100 ng/mL CpG, and 10 μg/mL R848. (**b**) In addition, HD11 cells were exposed to inactivated IBV and NDV antigens (companies A and B) in doses ranging from 0.1–10 μL/mL. (**c**) Finally, HD11 cells were exposed to vaccines, an "empty vaccine" containing allantoic fluid without inactivated viruses, and mineral oil in doses ranging from 1–100 μL/mL. Three independent experiments were performed, and the experimental conditions of each independent experiment were tested in triplicate. Error bars represent the standard error of the mean (SEM). The experimental groups were tested for statistically significant increases in nitric oxide production as compared to unstimulated HD11 cells using a Kruskal–Wallis test and Dunn's multiple comparisons test. Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Inactivated IBV and NDV vaccines can enhance phagocytosis by HD11 cells

Five inactivated vaccines, all containing a mineral oil adjuvant in a w/o formulation, were used to stimulate HD11 cells and included a monovalent IBV vaccine (company B), three bivalent IBV + NDV vaccines (companies A, B, and C), and a trivalent IBV + NDV + EDSV vaccine (company A). Both bi- (2.60 ±0.35-fold) and trivalent (1.95 ±0.10-fold) vaccines from company A led to a clear increase in the uptake of IgY-opsonized beads (**Figure 4a**). The bivalent vaccine from company C resulted in maximal uptake at 10µL/mL (1.49 ±0.06). The vaccines from company B did not enhance phagocytosis. Vaccine doses beyond those inducing a maximum increase of phagocytosis resulted in decreased bead uptake, concurrent with decreased cell viability (**Figure 4a** and **Supplementary Materials Figure S3**). An increasing dose of bivalent vaccine from company B also led to a decrease in cell viability without any enhancement of phagocytosis.

The mineral oil adjuvant of the vaccines acts as a slowly decomposing depot [41], resulting in a gradual release of vaccine components, such as antigen, allantoic fluid, and mineral oil. We hypothesized that the decomposition rate of an emulsified vaccine affects its release of allantoic fluid and thus its effect on phagocytosis. Light microscopy showed the intracellular accumulation of vacuoles in HD11 cells exposed to the vaccines (**Figure 4b**), suggesting that HD11 cells engulfed emulsified mineral oil released by the decomposing vaccines and stored this into lipid droplets. Because of this finding, we aimed to use flow cytometric SSC to quantify the accumulation of lipid droplets as a readout for vaccine decomposition. SSC can be used as a readout for engulfed particle content, as demonstrated by the high correlation between the SSC and the number of fluorescent IgY beads after the phagocytosis assay in unstimulated HD11 cells (r^2 = 0.996) (**Figure 4c**). Next, SSC was found to become higher with increasing doses of the empty vaccine control from company B until saturation was reached (r^2 = 0.972) (**Figure 4d**), demonstrating that SSC could indeed be used to quantify vaccine decomposition. The SSC of HD11 cells upon exposure to the vaccines was analyzed to determine whether there was a relationship between the vaccine decomposition rate and bead uptake. For the bi- and trivalent vaccines from company A, bead uptake increased simultaneously with SSC, suggesting that the increase in bead uptake correlated with vaccine decomposition (**Figure 4a**,**e**). HD11 cells were saturated with lipid droplets after exposure to 30 µL/mL bivalent vaccine from company A, which is the dose at which phagocytosis was found to be maximally enhanced (**Figure 4a**,**e**). The SSC also increased with increasing concentrations of the mono- and bivalent vaccines from companies B and C, but this did not result in similar changes in bead uptake. This indicates that differences between the vaccines in their capacity to induce phagocytosis cannot solely be explained by different decomposition rates.

Fc receptor CHIR-AB1 is responsible for the enhancement of IgY-opsonized bead uptake by HD11 cells upon exposure to inactivated IBV antigen

Since we chose to use IgY-opsonized beads, we investigated whether the increase in phagocytosis was dependent on the high-affinity IgY Fc receptor CHIR-AB1. Its expression increased upon 24 h of stimulation with LPS (maximum 2.79 ±0.09-fold change in gMFI) or inactivated IBV antigen (maximum 2.23 ±0.19-fold change in gMFI) (**Figure 5a**,**b**). For IBV antigen, CHIR-AB1 expression reached its peak after stimulation with a dose of 10 µL/mL

and decreased at higher doses, concurrent with the previously described cytotoxicity of the antigen (**Figure 3b**). Next, HD11 cells were incubated with a CHIR-AB1 blocking antibody before performing the phagocytosis assay. The increased bead uptake upon exposure to an IBV antigen diminished with increasing concentrations of the blocking antibody (from 1.83 ±0.23 beads/cell to 0.32 ±0.04 beads/cell) (**Figure 5d**). In contrast, bead uptake in unstimulated or LPS-stimulated HD11 cells was less affected by CHIR-AB1 blocking (Unst: from 0.67 ±0.05 beads/cell to 0.50 ±0.04 beads/cell; LPS: from 1.35 ±0.15 beads/cell to 0.89 ±0.05 beads/cell).

Discussion

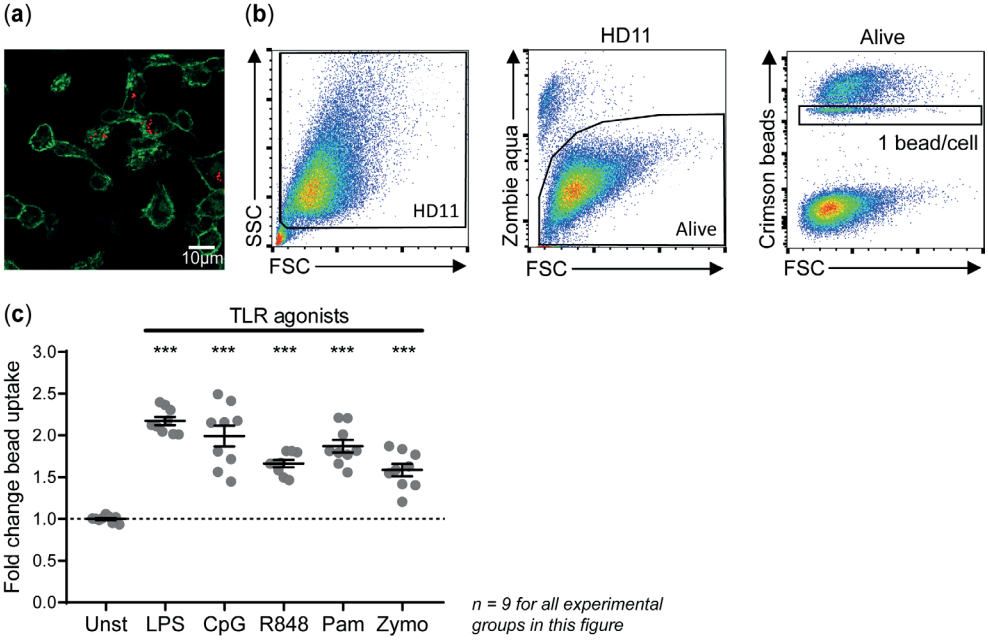


Figure 2. TLR agonists stimulated the uptake of IgY-opsonized beads by HD11 cells. (**a**) Confocal microscopy confirmed the uptake of IgY-opsonized beads by HD11 cells. The surface of unstimulated HD11 cells was made visible by WGA-Alexa Fluor 488 shown in green and IgY-opsonized beads are shown in red. A corresponding video showing the 3-D model of this composition can be found in **Supplementary Materials Video S1**. (**b**) Bead uptake by HD11 cells was quantified by flow cytometry. HD11 cells were gated for their scatter profile (FSC/SSC) and viability (zombie aqua live/dead staining). Moreover, HD11 cells with 1 bead/cell were gated to determine the fluorescence of a single bead, from which the average beads/cell for all HD11 cells could be calculated. (**c**) HD11 cells were stimulated with 300 ng/mL LPS, 500 ng/mL CpG, 10 µg/mL R848, 10 ng/mL Pam3CSK4 (Pam), 5 µg/mL zymosan (Zymo), or left unstimulated (Unst). The results are expressed as fold changes in bead uptake after stimulation in comparison to unstimulated controls. Three independent experiments were performed, and the experimental conditions of each independent experiment were tested in triplicate. Error bars represent the standard error of the mean (SEM). The experimental groups were tested for statistically significant differences in bead uptake between stimulated and unstimulated groups using a one-way ANOVA and Holm–Sidak's multiple comparisons test. Statistical significance is indicated by *** $p < 0.001$.

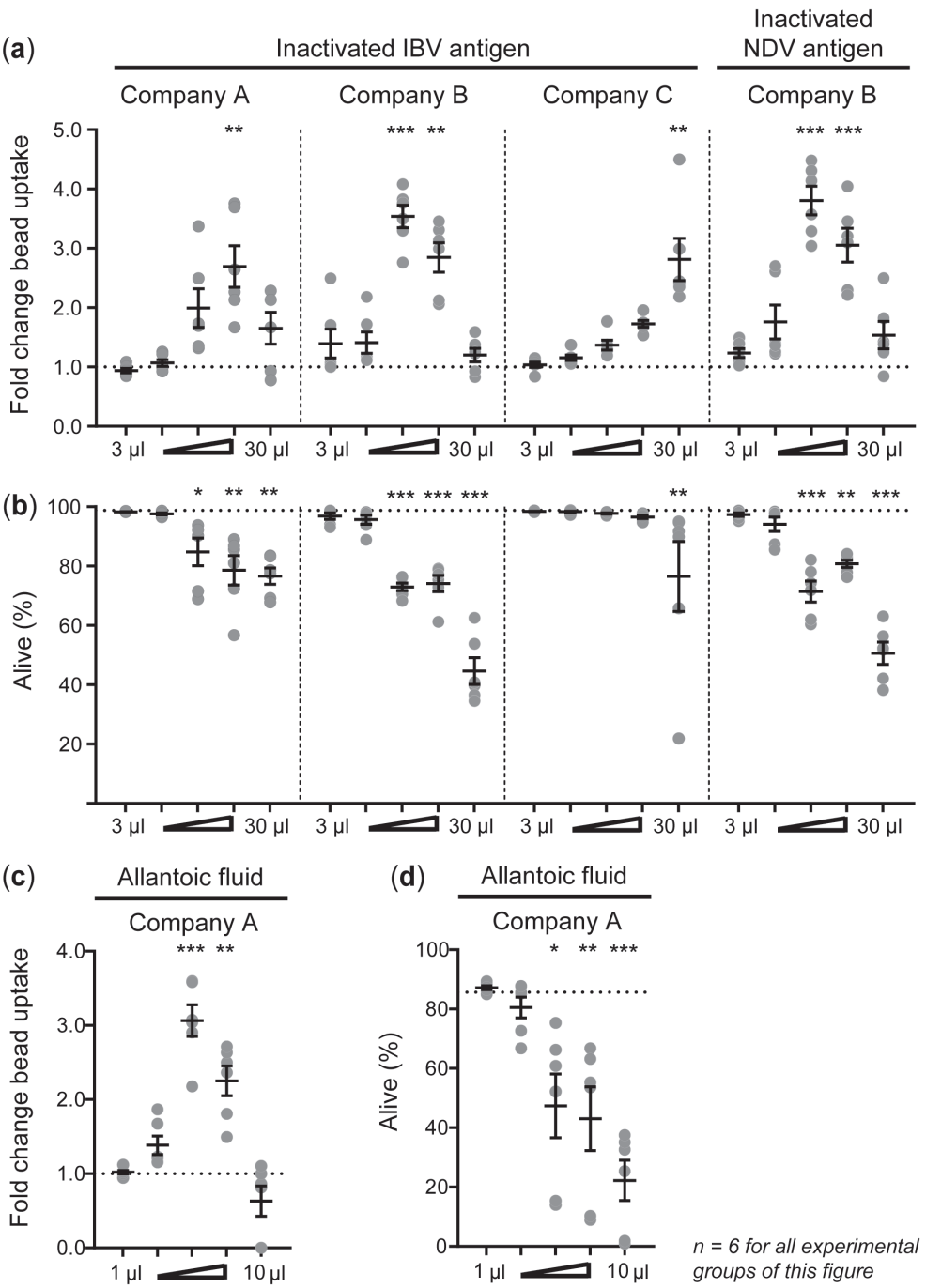


Figure 3. Phagocytosis of IgY-opsonized beads by HD11 cells is increased upon exposure to inactivated IBV and NDV antigens. (**a**) Differences in bead uptake upon exposure to IBV and NDV antigens are expressed as fold changes compared to unstimulated controls. (**b**) The effects of IBV and NDV antigens on HD11 cell viability, as determined by Zombie Aqua Fixable Viability Dye, is expressed

as the percentage of living cells (% alive). Inactivated IBV antigens were provided by three different companies (A–C) and inactivated NDV antigen was provided by one company (B). In addition, the effects of allantoic fluid without virus (provided by company A) on HD11 cell phagocytosis capacity (**c**) and cell viability (**d**) were determined. The x-axis shows the titrated doses at which IBV antigens, NDV antigens, or allantoic fluid without antigens were added, expressed as µL dose, added to 1 mL of cell culture medium. Three independent experiments were performed, and the experimental conditions of each independent experiment were tested in duplicate. Error bars represent the SEM. The experimental groups were tested for statistically significant differences in bead uptake and viability between stimulated and unstimulated groups using Kruskal–Wallis tests and Dunn's multiple comparisons tests. Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

In the present study, putative activating properties of inactivated poultry vaccines and their constituents on innate immune cells were assessed through measurement of nitric oxide production and phagocytosis by the chicken macrophage-like cell line HD11, as potential biomarkers for *in vitro* assessment of batch-to-batch consistency of vaccines. Small amounts of nitric oxide were produced in the presence of inactivated IBV and NDV vaccines or an empty vaccine without viral antigens. In contrast, nitric oxide was not produced in the presence of inactivated IBV or NDV antigens or non-emulsified mineral oil. These findings indicate that nitric oxide was produced by HD11 cells due to stimulation with the mineral oil adjuvant in its emulsified form.

The absence of nitric oxide production in the presence of viral antigens was unexpected, since HD11 cells do express TLR7 [42] and TLR21 [43], PRRs for viral single-stranded RNA and double-stranded DNA, respectively. Moreover, TLR7 agonist R848 and TLR21 agonist CpG were able to induce high amounts of nitric oxide production. One hypothesis is that the nucleic acids from the inactivated viral antigens did not end up in the endosomes where these TLRs are present [42]. Alternatively, the recognition of the viral nucleic acids could be impaired by chemical modification, as a result of viral inactivation, or degradation. It has been shown for H5N1 influenza vaccines that TLR7 is involved in the generation of an effective adaptive immune response and that TLR7 activation is severely reduced after inactivation of the antigen with formalin or β-propiolactone [44,45].

The phagocytosis assays showed that both inactivated poultry vaccines and antigens enhanced the phagocytosis of IgY-opsonized beads by HD11 cells. Moreover, allantoic fluid without antigens stimulated HD11 cells to enhance phagocytosis, similar to allantoic fluid containing inactivated IBV and NDV antigens. These results indicate that allantoic fluid, present in both the vaccines and antigen preparations, was responsible for the enhancement of phagocytosis. Allantois fluid contains a high concentration of uric acid, which may form monosodium urate crystals [46,47]. Previous studies have shown that uric acid is released from cells damaged by aluminum salt adjuvants and may form monosodium urate crystals that act as immunostimulatory DAMPs [47]. The stimulatory effects of allantoic fluid on phagocytosis may therefore be caused by the presence of uric acid precipitates. Vaccination studies in mice have shown that DAMPs stimulate antigen uptake by macrophages *in vitro* and enhance antibody titers *in vivo* [48,49,50]. Whether allantoic fluid stimulates phagocytosis and enhances antibody titers in chickens *in vivo* will be of interest for further investigation.

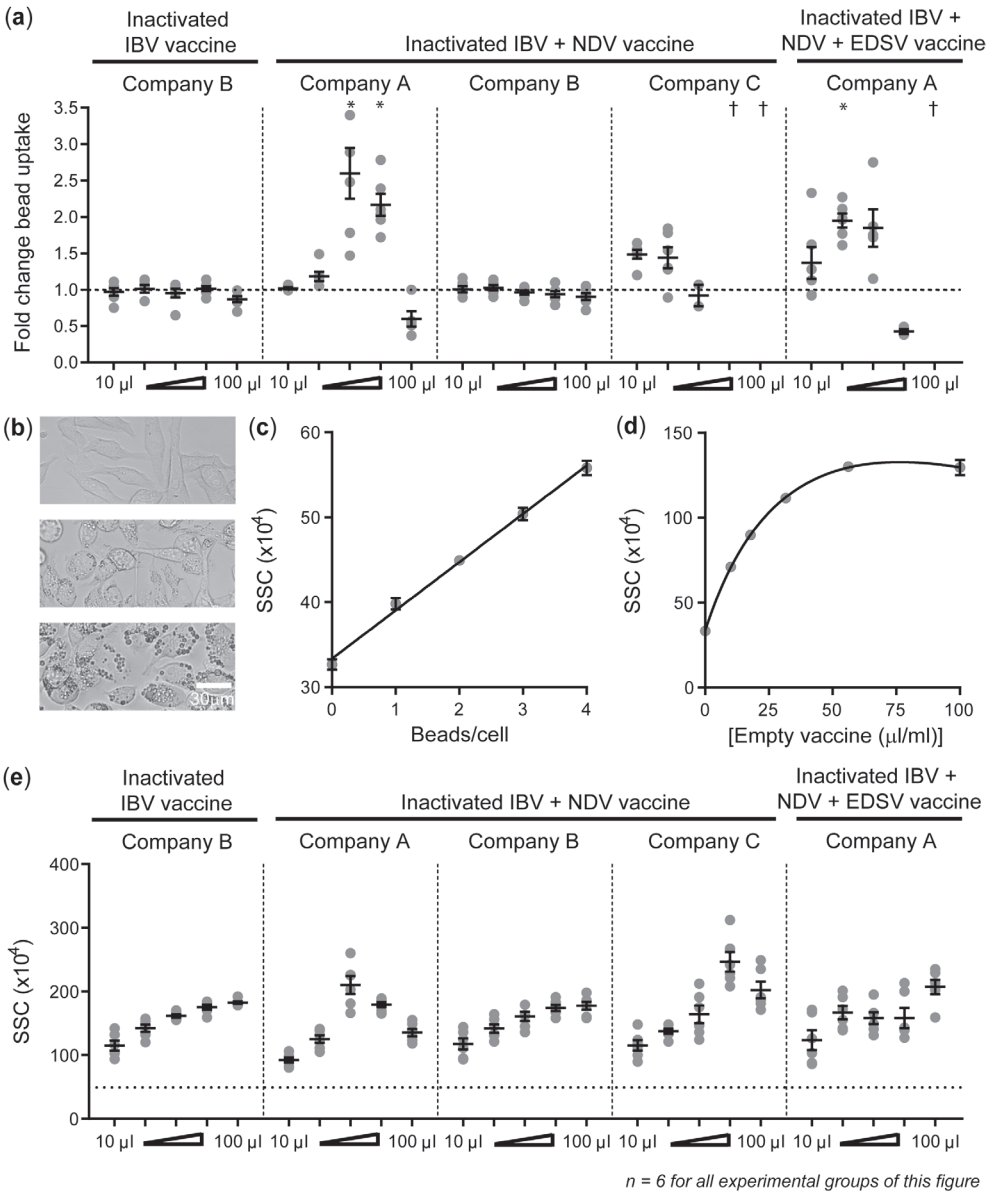


Figure 4. Phagocytosis capacity of HD11 cells can be increased upon exposure to inactivated viral w/o vaccines. (**a**) The fold change in bead uptake by HD11 cells upon stimulation with vaccines is compared to unstimulated controls. The x-axis shows the graded doses at which the vaccines have been added, expressed as µL dose, added to 1 mL of cell culture medium. † indicates that datapoints were missing because the threshold of ≥100 viable cells was not reached. (**b**) Light microscopy photos show unstimulated HD11 cells (top), HD11 cells exposed to 10 µL/mL inactivated bivalent vaccine B (middle), and 100 µL/mL inactivated bivalent vaccine B (top). (**c**) A linear correlation curve shows the relationship between the average flow cytometric SSC and number of IgY-opsonized beads/cell for HD11 cells containing 0–4 beads/cell. (**d**) A non-linear saturation curve shows the relationship between the average SSC and different doses of empty vaccine (without viral antigens) from

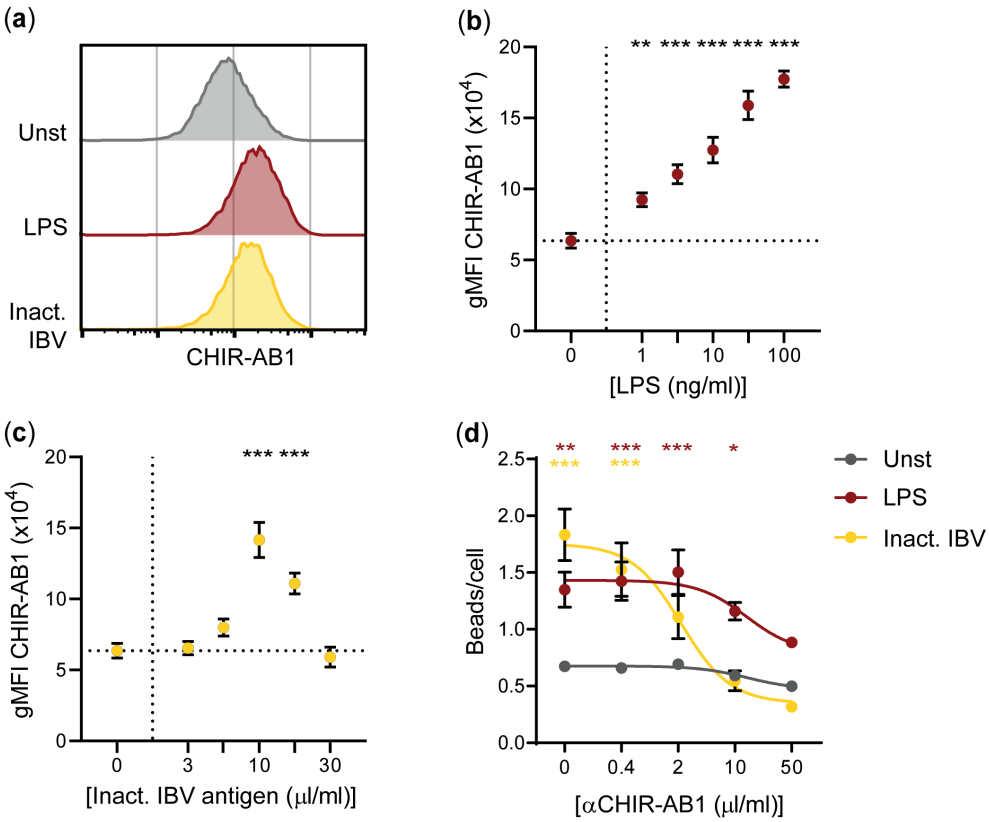
company B. (**e**) The flow cytometric SSC of HD11 cells is shown for graded doses of the different vaccines. Three independent experiments were performed, and the experimental conditions of each independent experiment were tested in duplicate. Error bars represent the SEM. The experimental groups were tested for statistically significant differences in bead uptake and SSC between stimulated and unstimulated groups using Kruskal–Wallis tests and Dunn's multiple comparisons tests. Statistical significance is indicated by * $p < 0.05$. For (**e**), all data was found to be statistically different from the unstimulated sample with $p < 0.001$.

Stimulation with inactivated IBV antigens from different sources induced different outcomes of the phagocytosis assay, both in terms of the dose at which the highest bead uptake was observed and the maximal bead uptake, whereas no differences were observed between IBV and NDV antigens from the same source. Similarly, vaccines from different courses resulted in different outcomes. However, this does not affect the feasibility of assessing vaccine batch quality under the consistency approach, which aims to select a number of parameters for individual vaccines or intermediate products to prove batch-to-batch consistency.

Besides antigens, the inactivated poultry vaccines in this study contained mineral oil adjuvants in w/o formulation, which has been shown to form a depot with a low decomposition rate *in vivo*, leading to slow release kinetics of the antigen [51,52]. In our study, some vaccines enhanced bead uptake by HD11 cells, whereas other vaccines did not, depending on the manufacturer of the vaccines. These results may be explained by differences in the antigen dose or release kinetics of the different products, which depends on the product ingredients and the manufacturing process. Light microscopy showed the accumulation of lipid droplets in HD11 cells upon stimulation with emulsified mineral oil or vaccines containing emulsified mineral oil. We used flow cytometric SSC, which measured cell contents and granularity [39], to assess quantitative differences in the accumulation of lipid droplets as a read-out for decomposition of the vaccines. Although, SSC increased upon incubation with higher concentrations of the individual vaccines, this did not always result in increased bead uptake. This suggests that the variation in phagocytosis between the vaccines cannot solely be explained by differences in decomposition rates. Therefore, phagocytosis may be affected by other determinants of the vaccine formulation, including differences in antigen content, the immunostimulatory capacity of the allantoic fluid, or cytotoxicity.

Since CHIR-AB1 is known as a high-affinity chicken IgY Fc receptor that signals through Fc ε receptor I gamma chain (FcεRIγ) upon interaction with heat-aggregated IgY [29], we investigated its involvement in the uptake of IgY-opsonized beads by HD11 cells upon stimulation. We observed increased CHIR-AB1 expression on HD11 cells upon stimulation with LPS and inactivated IBV antigen. In addition, blocking CHIR-AB1 diminished the increase in phagocytosis as a result of stimulation. Hence, it must be concluded that inactivated IBV antigen led to an increase in phagocytosis due to an increase in the expression of CHIR-AB1. The increase in phagocytosis was therefore Fc receptor dependent. In a previous study, LPS was not found to affect CHIR-AB1 expression by primary macrophages, which contrasts to our findings in the HD11 cell line [29]. The difference may also be explained by the use of another type of LPS.

The nitric oxide production assay and the phagocytosis assay presented in this study showed different levels of macrophage activity upon stimulation with individual inactivated poultry vaccines. Based on our results, nitric oxide production by HD11 cells seemed to be



n = 6 for all experimental groups of this figure

Figure 5. The induction of phagocytosis after exposure to inactivated IBV antigen is dependent on the IgY Fc receptor CHIR-AB1. (**a**) Representative histograms show CHIR-AB1 expression by HD11 cells after 24 h without stimulation, stimulation with 100 ng/mL LPS, or stimulation with 10 µL/mL IBV antigen. (**b**,**c**) CHIR-AB1 surface expression by HD11 cells was quantified and expressed as the geometric mean fluorescent intensity (gMFI) after 24 h stimulation with different concentrations of LPS (**b**) and IBV antigen (**c**). (**d**) Unstimulated HD11 cells and HD11 cells stimulated with LPS or inactivated IBV antigen for 24 h received the blocking antibody 8D12 specific for chicken CHIR-AB1 10 min before the addition of IgY-opsonized beads. The average number of phagocytosed beads per HD11 cell is shown for different concentrations of blocking antibody. Three independent experiments were performed, and the experimental conditions of each independent experiment were tested in duplicate. Error bars represent the SEM. The experimental groups were tested for statistically significant differences in CHIR-AB1 expression or bead uptake between stimulated and unstimulated groups using one-way ANOVA tests and Holm–Sidak's multiple comparisons tests. Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

induced by the mineral oil adjuvant, when present in a water-in-oil formulation. Fc receptor-mediated phagocytosis by HD11 cells was found to be induced by allantoic fluid, which is present in inactivated IBV and NDV antigens of poultry vaccines. Nitric oxide production was not induced by allantoic fluid, as demonstrated by the absence of nitric oxide after exposure to IBV and NDV antigens containing allantoic fluid. In this exploratory study, these

two assays were able to show different immunopotentiating properties of the vaccines and hence may be used in the future as complementary assays to test for immunostimulatory properties of vaccines. Allantoic fluid might function as an inherent adjuvant and facilitate the immune response, although this remains to be confirmed with *in vivo* experiments. Finally, the assays used in this study may be applied in the future as quality control tests for inactivated poultry vaccines. Future studies will be needed to address the capacity of these cell-based assays to evaluate vaccine batch-to-batch consistency and detect non-conforming batches, as compared to the animal-based quality control tests that are currently in place as gold standards. Obviously, the capacity of inactivated poultry vaccines to induce antigen-specific immunity requires additional assessments, like antigen quantification or stability, to ensure vaccine quality. The nitric oxide production and phagocytosis assays with HD11 cells might contribute to future efforts to replace current *in vivo* vaccine batch-release quality tests for *in vitro* alternatives.

Conclusions

In this exploratory study we demonstrated that inactivated poultry vaccines are able to activate chicken innate immune cells *in vitro*. Stimulation of the chicken macrophage-like cell line HD11 with the inactivated poultry vaccines resulted in the production of nitric oxide due the presence of mineral oil adjuvant. Furthermore, inactivated poultry vaccines enhanced Fc receptor-mediated phagocytosis due to the presence of allantoic fluid in the vaccine antigen preparations. Taken together, these two *in vitro* assays were able to show different immunopotentiating properties of the vaccines and hence may be used in the future as complementary assays to test for immunostimulatory properties of vaccines.

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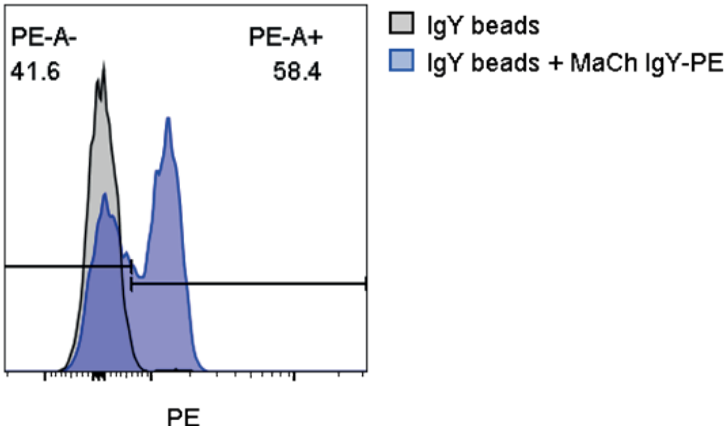
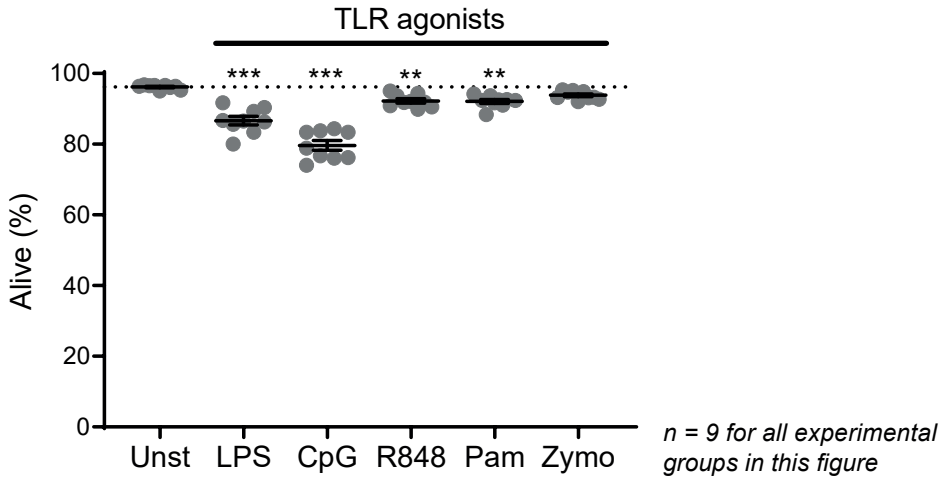
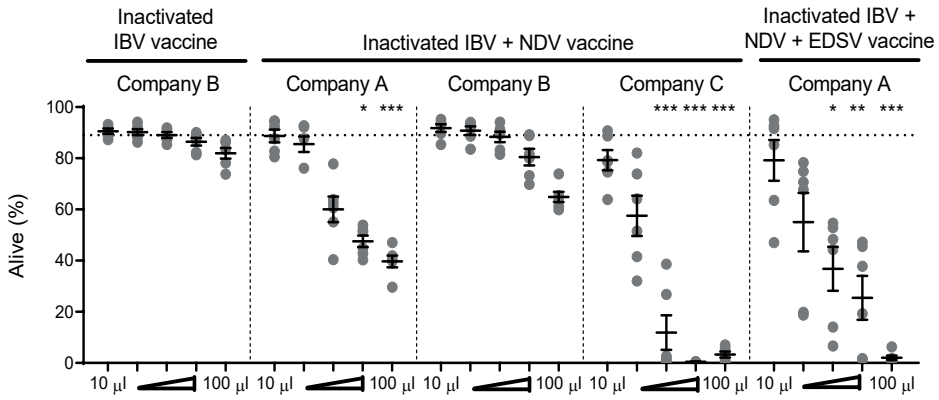
Supplementary Materials



Supplementary Figure S1. A major part of the beads used for the phagocytosis assay were coupled to IgY. An overlay of histograms shows IgY-coated beads without staining in grey and IgY-coated beads stained with a PE-conjugated mouse anti-chicken IgY in blue.



Supplementary Figure S2. Stimulation with TLR agonists showed little effects on HD11 cell viability. The effect of TLR agonists on HD11 cell viability, as determined by zombie aqua fixable/live dead staining, is expressed as the percentage of living cells (%alive). HD11 cells were stimulated with 300 ng/ml LPS, 500 ng/ml CpG, 10 µg/ml R848, 10 ng/ml Pam3CSK4 (Pam), 5 µg/ml zymosan (Zymo), or left unstimulated (Unst). The data comprise three independent replicates performed *in triplo*. Error bars represent the SEM. The experimental groups were tested for statistically significant differences in viability between stimulated and unstimulated groups using a Kruskal-Wallis test and Dunn's multiple comparisons test. Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.



n = 6 for all experimental groups in this figure

Supplementary Figure S3. The viability of HD11 cells was affected by various inactivating poultry vaccines to different extents. The effect of inactivated w/o vaccines on HD11 cell viability, as determined by zombie aqua fixable/live dead staining, is expressed as the percentage of living cells (%alive). HD11 cells were stimulated with a monovalent vaccine (IBV) from company B, bivalent vaccines (IBV + NDV) from companies A, B, and C, and a trivalent vaccine (IBV + NDV + EDSV) from company A. The data comprise three independent replicates performed *in duplo*. Error bars represent the SEM. The experimental groups were tested for statistically significant differences in viability between stimulated and unstimulated groups using a Kruskal-Wallis test and Dunn's multiple comparisons test. Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Supplementary Video S1. A 3D model of IgY-opsonized beads engulfed by HD11 cells. This movie is available online at https://www.mdpi.com/2076-393X/8/2/332/s1.

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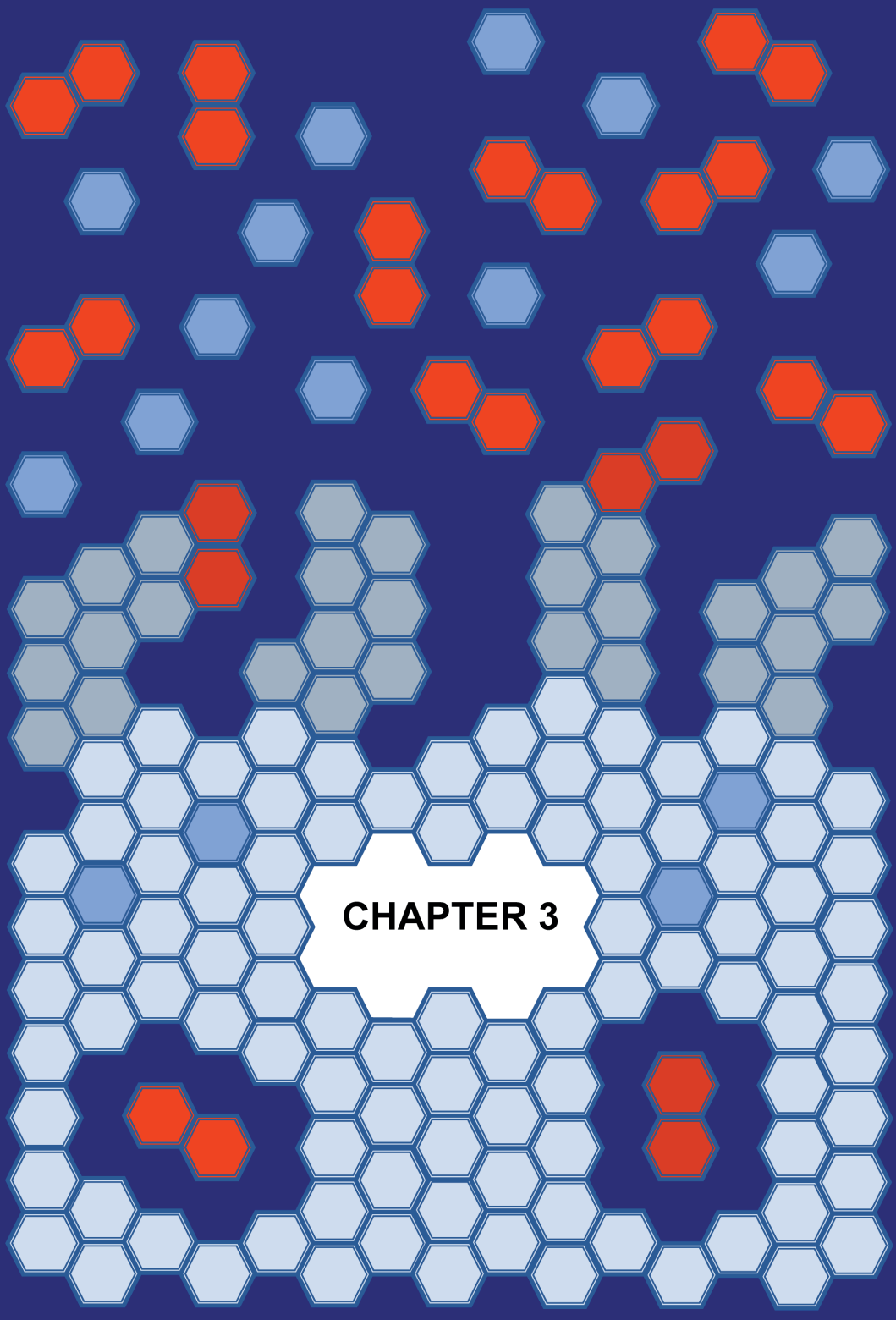
CHAPTER 3

3

Macrophage activation assays to evaluate the immunostimulatory capacity of *Avibacterium paragallinarum* in a multivalent poultry vaccine

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Abstract

High-quality vaccines are crucial to prevent infectious disease outbreaks in the poultry industry. *In vivo* vaccination tests are routinely used to test poultry vaccines for their potency, *i.e.*, their capacity to induce protection against the targeted diseases. A better understanding of how poultry vaccines activate immune cells will facilitate the replacement of *in vivo* potency tests for *in vitro* assays. Using the chicken macrophage-like HD11 cell line as a model to evaluate innate immune responses, the current explorative study addresses the immunostimulatory capacity of an inactivated multivalent vaccine for infectious bronchitis, Newcastle disease, egg-drop syndrome, and infectious coryza. The vaccine stimulated HD11 cells to produce nitric oxide and to express pro-inflammatory cytokines *IL-1*β, *TNF*, and *IL-12p40*, chemokines *CXCLi1* and *CXCLi2*, and the anti-inflammatory cytokine *IL-10*, but only when inactivated *Avibacterium paragallinarum*, the causative agent of infectious coryza, was present. Lipopolysaccharides from *Avibacterium paragallinarum* were crucial for the production of nitric oxide and expression of *IL-1*β and *CXCLi1*. The described immune parameters demonstrate the capacity of this multivalent vaccine to activate innate immune cells and may in the future, combined with antigen quantification methods, contribute to vaccine quality testing *in vitro*, hence the replacement of current *in vivo* vaccination tests.

Introduction

In the poultry industry, chickens are routinely vaccinated against infectious diseases to maintain flock health. Vaccine batches are subjected to routine quality control (QC) to ensure their potency, *i.e.*, their capacity to induce protection against the targeted infectious diseases. For many poultry vaccines, current QC test approaches include *in vivo* vaccination-challenge or vaccination-serology tests, which require large numbers of chickens. Alternatively, poultry vaccines could be tested in accordance with the consistency approach [1], which implies that quality can be proven by *in vitro* methods that demonstrate the similarity between the product profiles of a new batch and reference batches of proven clinical efficacy and safety. The characteristics that determine the potency of a vaccine have to be predetermined and may include properties like antigen quantity and the capacity to stimulate innate immune responses [1,2,3].

Infectious coryza is an upper respiratory tract infection caused by the Gram-negative bacterium *Avibacterium* (*Av.*) *paragallinarum*, which is responsible for worldwide economic losses due to mortality and reduced egg production in chickens [4]. Breeding and laying hens can be protected against infectious coryza by inactivated vaccines comprising multiple *Av. paragallinarum* isolates of serovars A, B, and C [5]. Like the vaccine used in this study, *Av. paragallinarum* can be incorporated in inactivated multivalent vaccines that also comprise infectious bronchitis virus (IBV), egg-drop syndrome '76 virus (EDSV) and Newcastle disease virus (NDV), to reduce the time and costs of vaccination [6]. Inactivated NDV vaccines can be assessed for potency using an *in vitro* enzyme-linked immunosorbent assay (ELISA) [7,8,9] and inactivated IBV and EDSV vaccines can be assessed for potency by *in vivo* vaccination-serology tests [10,11], which have a lower impact on animal welfare than traditional vaccination-challenge tests. In contrast, inactivated infectious coryza vaccines still rely on vaccination-challenge tests to prove their potency and are hence in need of reliable *in vitro* alternatives [12,13]. The capacity of a vaccine to induce protective immunity depends in part on its capacity to stimulate innate immune cells through recognition of immunostimulatory constituents including adjuvants and intrinsic pathogen-associated molecular patterns (PAMPs) like bacterial cell wall components [14,15]. The importance of PAMPs for effective vaccination has already been demonstrated for human vaccines against bacterial pathogens [16] like *Bordetella pertussis* [17,18] and Mycobacterium tuberculosis [14,19]. However, these immunostimulatory constituents have not been described for most poultry vaccines. Here, we investigated whether infectious coryza vaccines contain PAMPs that activate innate immune cells.

In general, bacterial PAMPs are recognized by innate immune cells through pattern recognition receptors (PRRs), which are broadly expressed by phagocytes like macrophages. Furthermore, the importance of macrophages in detecting bacterial PAMPs during vaccination has been demonstrated in macrophage-depletion studies in mice [20], which justifies the use of this cell type in *in vitro* vaccine quality assessment. A prime candidate to characterize innate immune responses evoked by poultry vaccines is the chicken macrophage-like cell line HD11. The HD11 cell line was originally described as LSCC-HD (MC/MA1) [21] but later renamed to HD11 [22]. This cell line recognizes bacteria using a broad repertoire of PRRs, including most chicken toll-like receptors (TLRs) [23] and mannose receptors. Activation of HD11 cells can be observed by increased phagocytosis [24,25,26], cytokine

expression [27,28], and nitric oxide production by the inducible nitric oxide synthase (iNOS) [27,28,29,30,31]. As vaccines for *Av. paragallinarum* are commonly multivalent and also used to protect chickens against other pathogens, we used two multivalent vaccines in the current study. To identify which responses were directed against *Av. paragallinarum*, an octavalent vaccine comprising inactivated IBV, NDV, EDSV, and 5 strains of *Av. paragallinarum* was used next to a trivalent vaccine that only contained inactivated IBV, NDV, and EDSV. In this explorative study, we aimed to identify candidate immune parameters for an *in vitro* QC test for inactivated poultry vaccines against *Av. paragallinarum* and contribute to the replacement of current *in vivo* vaccination-challenge tests.

Materials and Methods

HD11 cell culture and stimulation

Batches of chicken macrophage-like HD11 cells [21] were suspended in complete Roswell Park Memorial Institute (RPMI)-1640 medium with 50% FBS (both from Gibco, Life Technologies Limited, Paisley, UK) and 10% DMSO (Honeywell, Bucharest, Romania) and stored at −140 °C. After thawing, they were maintained and propagated in a complete RPMI-1640 cell culture medium supplemented with GlutaMAX-I, phenol red, HEPES, 10% FBS, 200 U/mL penicillin, and 200 U/mL streptomycin (all from Gibco) at 37 °C, 5% CO_2 in Corning 75 cm2 cell culture flasks (Sigma-Aldrich, Saint Louis, MO, USA). The cells were passaged twice weekly by washing the cells in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS; Lonza, Basel, Switzerland) and detaching adherent cells using a 0.25% trypsin/EDTA solution supplemented with phenol red (Gibco). For the experiments, HD11 cells were harvested after 3 to 20 passages using the trypsin/EDTA solution, counted, and resuspended at a concentration of 200,000 cells/mL in complete RPMI-1640 medium. The cells were seeded at 1 mL/well in Corning Costar 24-well cell culture plates (Sigma-Aldrich) and cultured overnight at 37 °C and 5% CO_2.

HD11 cells were exposed to various stimuli to assess their activation using nitric oxide production as a readout. First of all, TLR agonists were given at eleven different concentrations with increments of 100.25 between consecutive concentrations to create dose-response curves and to test the ability of HD11 cells to recognize different PAMPs. Stimuli included 1.0–300 ng/mL lipopolysaccharides (LPS) from *Escherichia* (*E.*) coli O127:B8 (Sigma-Aldrich) to target TLR4, 0.2–60 ng/mL Pam3CSK4 to target the TLR2/1 heterodimer, 0.2–60 µg/mL zymosan from *Saccharomyces* (*S.*) *cerevisiae* to target the TLR2/6 heterodimer, 0.1–30 µg/mL high molecular weight (1.5-8kb) polyinosinic-polycytidylic acid (poly[I:C]) to target TLR3, 0.1–30 µg/mL resiquimod (R848) to target TLR7, and 3–1000 ng/mL CpG oligonucleotides (ODN) 2006 to target TLR21 (all InvivoGen, San Diego, CA, USA). In addition, HD11 cells were exposed to established tri- an octavalent inactivated poultry vaccines kindly provided by Boehringer Ingelheim (Ingelheim am Rhein, Germany) as a partner of the VAC2VAC consortium (http://www.vac2vac.eu/). The octavalent vaccine comprised of whole-inactivated IBV, NDV, EDSV, and inactivated isolates of five serovars of *Av. paragallinarum*, including one isolate of serovar A, three of serovar B, and one of serovar C. The trivalent vaccine used in this study comprised whole-inactivated IBV, NDV, and EDSV only. Both vaccines were adjuvanted as mineral oil-based water-in-oil emulsions. The vaccines were prepared such that a single chicken vaccination dose corresponded to 0.5 mL vaccine. HD11 cells were exposed to the vaccines at a fixed concentration of 1 µL/mL or

to different concentrations ranging from 0.2 to 30 µL/mL to create dose-response curves.

In accordance with a previous publication [7], total antigenic fractions were extracted from the vaccines by adding vaccine to isopropyl myristate (Sigma-Aldrich) in a 1:5 ratio, vigorous mixing on a vortex mixer for 1 min, and centrifugation at 1000x *g*. The lower water phase contained the antigenic fraction, which was gently resuspended and collected. HD11 cells were exposed to extracted antigens at a fixed concentration of 0.5 µL/mL or different concentrations ranging from 0.2–30 µL/mL to create dose-response curves. Furthermore, *Av. paragallinarum* bacteria were purified from 200 µL extracted antigens by centrifuging three times at 15,000x *g* and washing the bacterial pellet in PBS. Finally, the bacterial pellet was resuspended in 200 µL PBS. A pellet was absent when the trivalent vaccine comprising only viral antigens was exposed to the same procedure. HD11 cells were exposed to the purified bacteria at different concentrations ranging from 0.1–10 µL/mL to create dose-response curves.

The contribution of LPS, present within the antigens extracted from the octavalent vaccine, to the activation of HD11 cells was determined using the LPS-binding antibiotic polymyxin B (Sigma-Aldrich) [32]. For these experiments, 300 ng/mL *E. coli* LPS, 300 ng/mL CpG ODN2006, 0.5 µL/mL octavalent vaccine and 1.0 µL/mL extracted antigens were resuspended in complete RPMI-1640 and pre-incubated with 1, 10, or 100 µg/mL polymyxin B for 1 h at 37 °C. Subsequently, these mixtures were added to the HD11 cells and incubated for 48 h to evaluate the production of nitric oxide or for different periods of time between 0–48 h to evaluate the gene expression of candidate biomarkers over time.

Griess test to evaluate nitric oxide production by stimulated HD11 cells

Nitric oxide production by HD11 cells was measured after 48 h of stimulation by the Griess test as previously described [33]. Griess test reagent was made by dissolving N-(1-naphtyl)ethylenediamine at 3 g/L and sulfanilamide at 10 g/L (both from Sigma-Aldrich) in 2.5% phosphoric acid (Supelco, Merck, St. Louis, MO, USA) and mixing the two solutions 1:1. From the stimulated HD11 cell cultures, 50 µL supernatant was harvested, transferred to a 96-well flat-bottom plate (Corning B.V. Life Sciences, Amsterdam, the Netherlands), and mixed with 50 µL/well Griess test reagent. The mixture turns purple upon reaction with nitrite ions in the cell culture supernatant. The median optical density at 540 nm was determined for each well using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The corresponding nitrite concentrations were calculated according to a nitrite standard curve made from a dilution series between 3.13–200 µM sodium nitrite (Sigma-Aldrich). For HD11 stimulated with TLR agonists or purified inactivated *Av. paragallinarum* bacteria, the results of the Griess test were plotted using the four-parameter logistic regression model from GraphPad Prism 8 software (GraphPad Software)

$$E(C) = E_{max} + \frac{E_{max} - E_{min}}{1 + (c / EC_{50})^{n}} \quad (1)$$

where E(C) = effect at a given concentration (C), E_{max} = maximum observed effect, E_{min} = minimum observed effect, EC_{50} = half-maximum effective concentration, and n = Hill

coefficient. The model was used to determine the maximum nitric oxide production (E_{max}) and the concentration that gives half-maximal nitric oxide production (EC_{50}) as readouts for the capacity of the stimuli to induce nitric oxide production.

Relative expression of iNOS and cytokines using real-time quantitative PCR

To determine iNOS and cytokine gene expression, HD11 cells were harvested, either 8 h after stimulation or at different time points between 0–48 h (as indicated in **Figure 3**), using 200 µL PBS + 5 mM UltraPure EDTA (Invitrogen™, Life Technologies Europe BV, Bleiswijk, the Netherlands) and centrifugated at 400x *g*. The supernatant was discarded, and the pelleted cells were lysed in RLT buffer (Qiagen GmbH, Hilden, Deutschland) and stored at –20 °C until further processing. After thawing, RNA isolation was performed with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Deutschland) including a DNase treatment using the RNase-Free DNase Set (Qiagen GmbH, Hilden, Deutschland). Next, cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) according to the manufacturer's instructions. RT-qPCR reactions were performed with 100 nM FAM-TAMRA-labelled TaqMan probes (listed in **Table 1**) together with 600 nM primers and TaqMan Universal PCR Master Mix or without probes using 400 nM primers and SYBR Green Master Mix (all from Applied Biosystems, Life Technologies Europe BV, Bleiswijk, the Netherlands). RT-qPCR reactions were performed using a CFX Connect qPCR detection system (Bio-Rad), using a program of 40 cycles with melting temperatures of 58 (SYBR Green probes) or 59 °C (TaqMan probes), and analyzed with CFX Maestro software (Bio-Rad). All RT-qPCR reactions were evaluated for proper amplification efficiency (95–105%) using serial dilutions of reference cDNA or plasmids before testing the samples (*data not shown*). RT-qPCR reactions were performed in triplicate for every sample. Changes in gene expression over time upon stimulation were assessed using t = 0 h as a reference time point and expressed as $2^{-\Delta\Delta Ct}$-values, according to the Livak method [34] with Ct being the number of cycles before a signal above the threshold (background) level was reached. The results were normalized to gene expression levels of the housekeeping genes 28S and GAPDH. An exception is the IL-10 gene expression data of **Supplementary Figure S2**, which were expressed as 40-Ct values according to a previous publication [35] as this method was more suitable when one or more data points did not reach a signal above the threshold within 40 cycles.

Flow cytometric assessment of HD11 cell viability after stimulation

HD11 cells were harvested 48 h after stimulation using 200 µL PBS + 5 mM UltraPure EDTA and centrifuged at 400 x *g*. The cells were transferred to a 96-well V-bottom plate, washed in PBS with 0.5% bovine serum albumin, 0.005% sodium azide (FACS buffer; both from Sigma-Aldrich), and stained for viability in 200 µL FACS buffer with 1.25 µg/mL 7-aminoactinomycin D (7-AAD; BD Biosciences, Pharmingen, San Diego, CA, USA). The staining of the cells was analyzed using the 488 nm laser of a CytoFLEX LX flow cytometer (Beckman Coulter Inc., Brea, CA, USA). The fraction of viable 7-AAD-negative HD11 cells was determined using FlowJo Software v. 10.5 (FlowJo LCC, Ashland, OR, USA). The cytotoxicity of the extracted antigens and purified bacteria from the octavalent vaccine was expressed as the concentration at which 50% of HD11 cells had died, *i.e.*, the 50% lethal concentration (LC_{50}).

Table 1. Primer and probe sequences. All probes contain the 5′ reporter dye FAM and the 3′ fluorescent quencher TAMRA.

Gene	NCBI Reference	Type	Sequences (5′-3′)
iNOS	NM_204961.1	Forward	TGGGTGGAAGCCGAAATA
		Reverse	GTACCAGCCGTTGAAAGGAC
TNF	MF000729.1	Forward	CGCTCAGAACGACGTCAA
		Reverse	GTCGTCCACACCAACGAG
CXCLi1	NM_205018.1	Forward	CCAGTGCATAGAGACTCATTCCAAA
		Reverse	TGCCATCTTTCAGAGTAGCTATGACT
GAPDH	NM_204305.1	Forward	GTGGTGCTAAGCGTGTTATC
		Reverse	GCATGGACAGTGGTCATAAG
IL-1β	NM_204524.1	Forward	GCTCTACATGTCGTGTGTGATGAG
		Reverse	TGTCGATGTCCCGCATGA
		Probe	CCACACTGCAGCTGGAGGAAGCC
IL-4	NM_001007079.1	Forward	AACATGCGTCAGCTCCTGAAT
		Reverse	TCTGCTAGGAACTTCTCCATTGAA
		Probe	AGCAGCACCTCCCTCAAGGCACC
IL-6	NM_204628.1	Forward	GCTCGCCGGCTTCGA
		Reverse	GGTAGGTCTGAAAGGCGAACAG
		Probe	AGGAGAAATGCCTGACGAAGCTCTCCA
CXCLi2	NM_205498.1	Forward	GCCCTCCTCCTGGTTTCA
		Reverse	TGGCACCGCAGCTCATT
		Probe	TCTTTACCAGCGTCCTACCTTGCGACA
IL-10	NM_001004414.2	Forward	CATGCTGCTGGGCCTGAA
		Reverse	CGTCTCCTTGATCTGCTTGATG
		Probe	CGACGATGCGGCGCTGTCA
IL-12p35	NM_213588.1	Forward	TGGCCGCTGCAAACG
		Reverse	ACCTCTTCAAGGGTGCACTCA
		Probe	CCAGCGTCCTCTGCTTCTGCACCTT
IL-12p40	NM_213571.1	Forward	TGGGCAAATGATACGGTCAA
		Reverse	CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT
		Probe	CAGAGTAGTTCTTTGCCTCACATTTT
IFN-α	XM_015277440.2	Forward	GACAGCCAACGCCAAAGC
		Reverse	GTCGCTGCTGTCCAAGCATT
		Probe	CTCAACCGGATCCACCGCTACACC
IFN-γ	NM_205149.1	Forward	GTGAAGAAGGTGAAAGATATCATGGA
		Reverse	GCTTTGCGCTGGATTCTCA
		Probe	TGGCCAAGCTCCCGATGAACGA
28S	XR_003078040.1	Forward	GGCGAAGCCAGAGGAAACT
		Reverse	GACGACCGATTTGCACGTC
		Probe	AGGACCGCTACGGACCTCCACCA

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). When the assumptions of normally distributed data and residuals were met, a one-way ANOVA with Holm–Sidak's multiple comparisons test was used to test

for statistically significant differences between stimulated and unstimulated control samples. Relative gene expression data were log-transformed to create normally distributed data. When the assumptions of normality were not met, a non-parametric Kruskal–Wallis test with Dunn's multiple comparisons test was used instead. A *p*-value of <0.05 was considered statistically significant.

Results

The inactivated octavalent poultry vaccine induces HD11 cells to produce nitric oxide

HD11 cells were stimulated for 48 h by different TLR agonists derived from or mimicking components from bacteria (Pam3CSK4, LPS, CpG), viruses (R848, CpG, poly[I:C]), and fungi (zymosan). Nitric oxide production was analyzed using the Griess test (**Figure 1**). The most potent inducer of nitric oxide production was Pam3CSK4 (EC_{50} = 23.0 ng/mL; E_{max} = 101 µM nitrite), which stimulates the chicken TLR2/1 heterodimer. Other potent stimuli were the TLR4 agonist *E. coli* LPS (EC_{50} = 122 ng/mL; E_{max} = 93.5 µM nitrite) and the TLR21

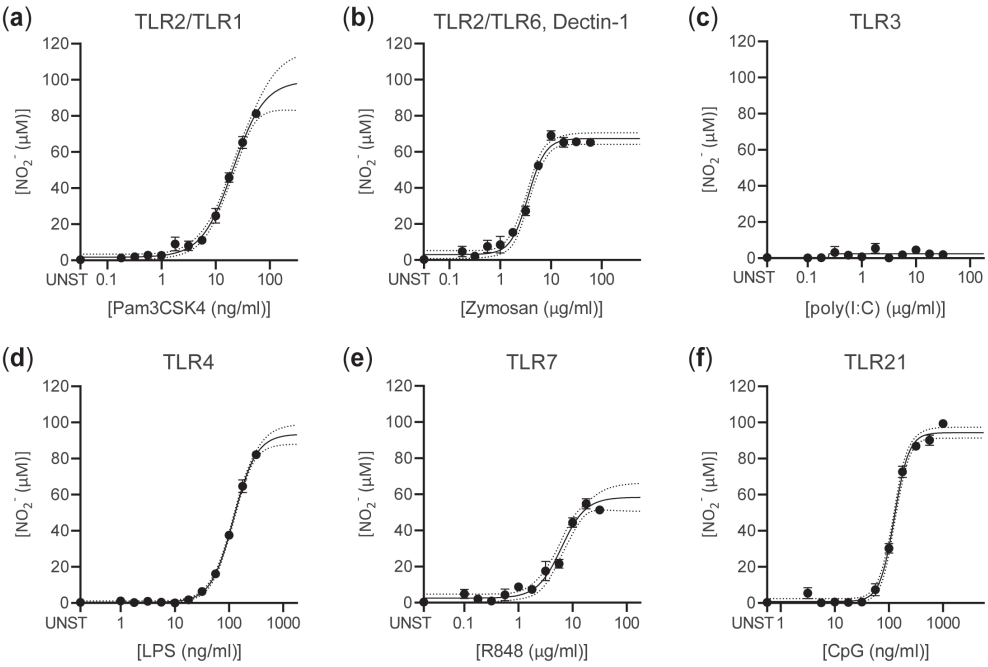

Figure 1. The macrophage-like HD11 cell line produces nitric oxide upon stimulation with a broad range of TLR agonists for 48 h. HD11 cells were stimulated with titrated concentrations of the TLR agonists Pam3CSK4 (**a**), zymosan (**b**), poly(I:C) (**c**), LPS (**d**), R848 (**e**), and CpG (**f**). The expected receptors for each agonist are shown above each panel. Nitric oxide production is expressed as the concentration of nitrite ions (NO_2^-) in the cell culture supernatant, as measured by the Griess test. Four parameter logistic curves were plotted together with their confidence intervals (dotted lines). Unstimulated HD11 cells (UNST) were used as a negative control and HD11 cells stimulated with 300 ng/ml LPS (**d**) were used as a positive control in this and all subsequent experiments. The experiment was performed in triplicate. The error bars represent the SEM.

agonist CpG ODN2006 (EC_{50} = 127 ng/mL; E_{max} = 94.7 µM nitrite). High concentrations of R848 (EC_{50} = 6.42 µg/mL; E_{max} = 57.1 µM nitrite), stimulating TLR7, and *S. cerevisiae* zymosan (EC_{50} = 3.68 µg/mL; E_{max} = 66.3 µM nitrite), stimulating both the TLR2/6 heterodimer and Dectin-1, were required to induce nitric oxide production. Nitric oxide was not detected upon exposure to high molecular weight poly(I:C) oligonucleotides. Overall, these results demonstrate that nitric oxide production can be used as a readout to determine the capacity of various compounds to stimulate innate immune cells like macrophages.

Next, we investigated whether an inactivated octavalent poultry vaccine for IBV, NDV, EDSV, and five serovars of *Av. paragallinarum* contains any immunostimulatory constituents that may stimulate nitric oxide production by HD11 cells. Stimulation with this vaccine for 48 h at concentrations ranging from 0.56 to 3.2 µL/mL induced significantly higher nitric oxide production compared to unstimulated cells (E_{max} = 34.7 µM nitrite at 1.0 µL/mL) (**Figure 2a**). At doses > 1.0 µL/mL, nitric oxide production gradually decreased, which can be explained by the observed cytotoxicity of the vaccine at high concentrations, as assessed by 7-AAD viability staining (**Supplementary Figure S1**).

Gene expression of iNOS was measured to investigate the activation of the nitric oxide production pathway over time as the accumulation of nitric oxide cannot be detected at early time points (*not shown*). Expression of iNOS was strongly upregulated between 4 and 6 h after exposure to 1.0 µL/mL octavalent vaccine, reaching a 59-fold increase at t = 6 h in comparison to t = 0 h (**Figure 2b**). The maximum increase in iNOS expression was observed at t = 24 h (311-fold) and expression remained high at least up to 48 h (289-fold), which was the last time point assessed.

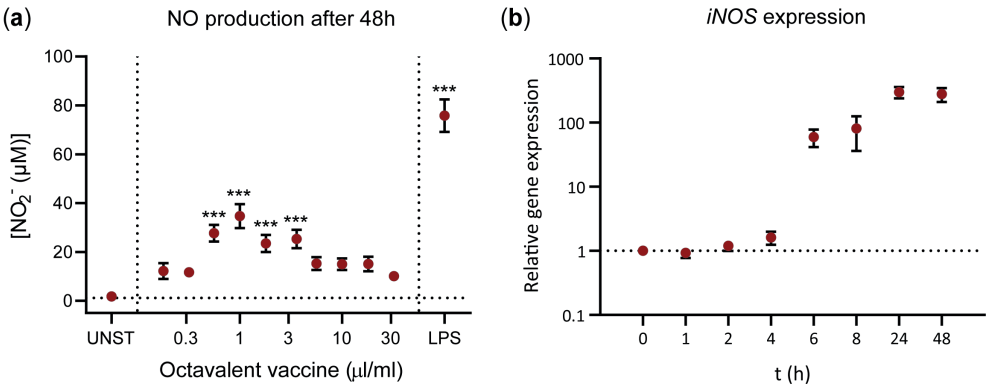

Figure 2. Nitric oxide was produced upon exposure to octavalent vaccine (IBV + NDV + EDSV + 5x *Av. paragallinarum*) for 48 h. (**a**) HD11 cells were exposed to titrated concentrations of the octavalent vaccine. Moreover, unstimulated HD11 cells (UNST) and HD11 cells stimulated with 300 ng/ml LPS are shown as negative and positive controls. The data comprises three independent technical replicates performed in triplicate. The error bars represent the SEM. A Kruskal–Wallis test combined with Dunn's multiple comparisons test was used to test for significant induction of nitric oxide production upon stimulation. *** $p < 0.001$. (**b**) RT-qPCR was performed on HD11 cell samples stimulated with 1 µL/mL octavalent vaccine and harvested at the given time points between 0 and 48 h. iNOS expression is shown relative to t = 0 h and expressed as $2^{-\Delta\Delta Ct}$ values as calculated using the Livak method and GAPDH and 28S as a reference gene. The experiment was performed in triplicate. The error bars represent the SEM.

Stimulation with the octavalent vaccine results in enhanced gene expression of cytokines and chemokines

Cytokine gene expression by vaccine-stimulated HD11 cells was followed over time. As early as 1 h after exposure to the vaccine, HD11 cells upregulated gene expression levels of the pro-inflammatory cytokine IL-1β (4.3-fold) and IL-8-like chemokines CXCLi1 (2.9-fold) and CXCLi2 (3.3-fold) (**Figure 3a–c**), which increased up to 8 h after exposure to respectively 584-, 99.9-, and 225-fold. Expression of IL-1β subsequently decreased to 30.0-fold at 24 h and 9.3-fold at 48 h as compared to t = 0 h (**Figure 3a**), whereas expression of CXCLi1 and CXCLi2 remained high up to 48 h (**Figure 3b**,**c**). HD11 cells showed a modest upregulation of the pro-inflammatory cytokine TNF after exposure to the octavalent vaccine, reaching its peak at t = 8 h with a 4.0-fold increase in expression (**Figure 3d**). In contrast, stimulation with the vaccine did not induce gene expression of IL-6 and IFN-α at any of the time points studied (**Figure 3e**,**f**). Expression of the anti-inflammatory cytokine IL-10 was elevated after

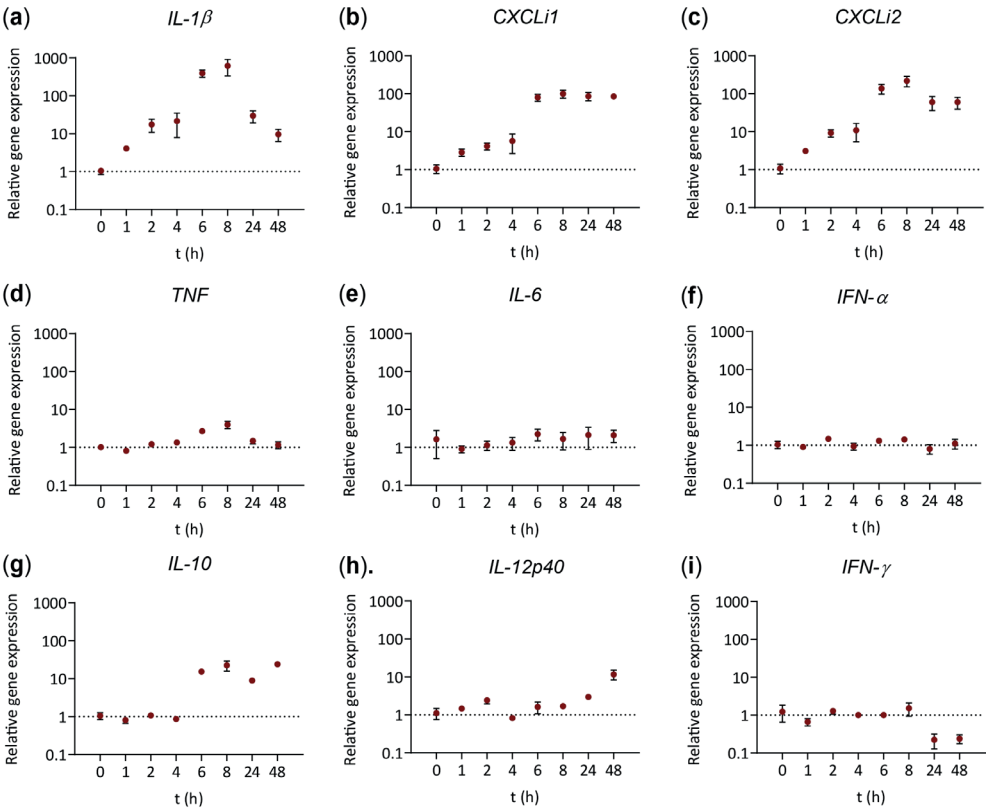

Figure 3. The octavalent vaccine induced the expression of pro-inflammatory cytokines at an early time point, followed by expression of the anti-inflammatory cytokine IL-10 and finally IL-12p40. HD11 cells were stimulated with 1 µL/mL octavalent vaccine and harvested at the indicated time points between 0 and 48 h. RT-qPCR was performed for the cytokines IL-1β (**a**), CXCLi1 (**b**), CXCLi2 (**c**), TNF (**d**), IL-6 (**e**), IFN-α (**f**), IL-10 (**g**), IL-12p40 (**h**) and IFN-γ (**i**). The relative gene expression levels were normalized against t = 0 h and expressed as $2^{-\Delta\Delta Ct}$ values as calculated using the Livak method and both GAPDH and 28S as reference genes. The experiment was performed in triplicate. The error bars represent the SEM.

6 h to 19.5-fold and remained at that level up to 48 h (**Figure 3g**). Expression of the T_H1 or 17-inducing cytokine IL-12p40, depending on its heterodimerization with respectively IL-12p35 [36] or IL-23p19 [37], was increased maximally 11.7-fold at t = 48 h (**Figure 3h**). Expression of T_H1-inducing cytokine IFN-γ was found slightly decreased at 24 and 48 h (4.50- and 4.14-fold decrease respectively) compared to t = 0 h (**Figure 3i**). The T_H2-inducing cytokine IL-4 and T_H1-inducing cytokine IL-12p35 were not detected (*not shown*). Taken together, HD11 cells showed increased expression of pro-inflammatory cytokines IL-1β and TNF, and chemokines CXCLi1 and CXCLi2 within 8 h after exposure to the octavalent vaccine, which was followed by the induction of the anti-inflammatory cytokine IL-10 and the T_H1/T_H17-inducing cytokine IL-12p40.

Av. paragallinarum antigens contribute to the stimulatory capacity of the octavalent vaccine

To investigate whether *Av. paragallinarum* antigens were able to stimulate HD11 cells, the responses induced by the octavalent vaccine were compared to responses induced by a trivalent vaccine containing the same viral antigens but without bacterial *Av. paragallinarum* antigens. In contrast to the octavalent vaccine, the trivalent vaccine did not lead to nitric oxide production by HD11 cells (**Figure 4a**). These results strongly suggested that the *Av. paragallinarum* antigens were responsible for the nitric oxide production by HD11 cells. Next, antigens were extracted from the emulsion vaccines using isopropyl myristate. As shown in **Figure 4b**, the antigens extracted from the octavalent vaccine induced more nitric oxide production than the octavalent vaccine itself (50.2 µM vs. 6.7 µM nitrite at 0.3 µL/mL, respectively). In contrast, the antigens extracted from the trivalent vaccine did not induce nitric oxide production. Compared to the complete octavalent vaccine with an LC_{50} of 18 µL/mL, the extracted antigens were more cytotoxic for HD11 cells with a LC_{50} of 0.26 µL/mL (**Supplementary Figure S1**). To determine whether these cytotoxic effects were due to the bacterial antigens or other vaccine constituents, the bacteria were recovered from the antigenic fraction of the octavalent vaccine by centrifugation at high speed, followed by extensive washing in PBS, and resuspended in PBS according to the original volume of antigenic fraction (**Supplementary Figure S2**). The bacteria stimulated HD11 cells to produce high concentrations of nitric oxide (E_{max} = 57.1 µM nitrite at 1.0 µL/mL) over a wide range of concentrations (**Figure 4c**), whereas the cytotoxicity of the purified bacteria (LC_{50} = 4.2 µL/mL) was reduced compared to the antigenic fraction (LC_{50} = 0.26 µL/mL) (**Supplementary Figure S1**). The potency of the purified bacteria to induce nitric oxide production, as expressed by an EC_{50}-value, was found to be = 0.276 µL/mL.

Next, the gene expression levels of iNOS and cytokines were determined 8 h after stimulation with either the tri- or octavalent vaccine. Exposure to the octavalent vaccine led to significantly increased expression of iNOS (36.4-fold), IL-1β (71.4-fold), TNF (1.7-fold), CXCLi1 (46.9-fold), CXCLi2 (55.7-fold) (**Figure 4e–i**), and IL-10 (40-Ct: 4.0 for octavalent vaccine vs. 1.1 for unstimulated; **Supplementary Figure S3**). In contrast, the trivalent vaccine did not induce the expression of iNOS or any of the cytokines. Expression IFN-α was slightly decreased after stimulation with either the tri- (2.1-fold decrease) or octavalent (2.0-fold decrease) vaccine (**Figure 4j**).

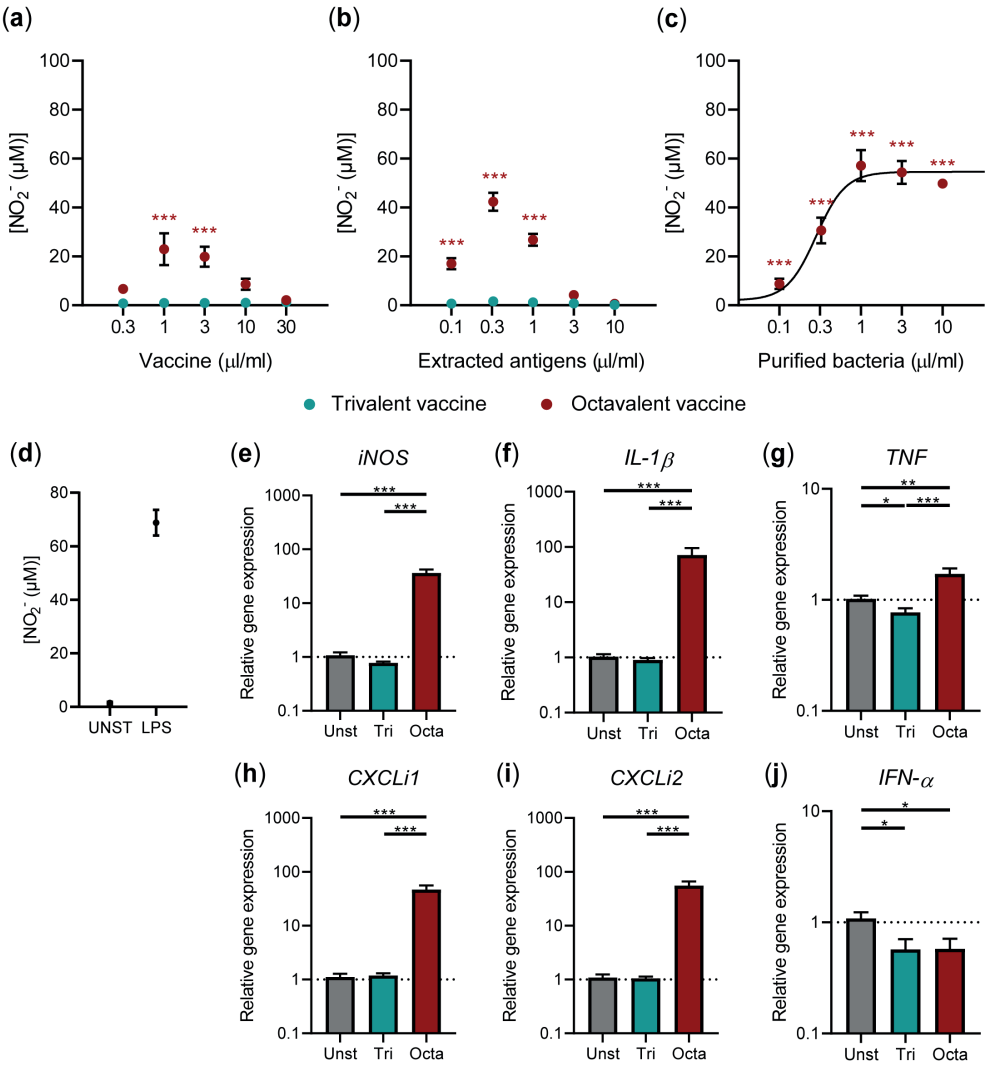

Figure 4. A trivalent vaccine without *Av. paragallinarum* antigens did not induce nitric oxide production or expression of pro-inflammatory cytokines. (**a**) Nitric oxide production was determined for HD11 cells exposed to titrated doses of trivalent (IBV + NDV + EDSV) and octavalent vaccine (IBV + NDV + EDSV + 5x *Av. paragallinarum*) for 48 h. (**b**) Antigenic fractions were extracted from the tri- and octavalent vaccines using isopropyl myristate and given to HD11 cells to determine nitric oxide production after 48 h. (**c**) The bacterial pellet was purified from the antigenic fraction of the octavalent vaccine and given to HD11 cells to determine nitric oxide production after 48 h. A four-parameter logistic curve could be calculated and was plotted. (**d**) The controls of the nitric oxide production assay included unstimulated HD11 cells (UNST) and HD11 cells stimulated with 300 ng/ml LPS. (**e-j**) Expression levels of iNOS (**e**), IL-1β (**f**), TNF (**g**), CXCLi1 (**h**), CXCLi2 (**i**), and IFN-α (**j**) by HD11 cells were determined 8 h after stimulation with 1.0 µL/mL tri- or octavalent vaccine. The values are expressed as $2^{-\Delta\Delta Ct}$ values as calculated using the Livak method and both GAPDH and 28S as reference genes. All figures show three independent technical replicates. The error bars represent the SEM. A Kruskal-Wallis test combined with Dunn's multiple comparisons test was used to test for statistical significance of the data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The relative gene expression data were log-transformed prior to the statistical analysis to generate normally distributed data.

Activation of HD11 cells by the octavalent vaccine largely depends on *Av. Paragallinarum* antigen-associated LPS

Finally, the antigenic fraction of the octavalent vaccine was pre-incubated with the LPS-binding antibiotic polymyxin B to determine the contribution of LPS, embedded in the cell wall of *Av. paragallinarum* bacteria, to HD11 cell activation. First, *E. coli* LPS (**Figure 5a**) and CpG ODN2006 (**Figure 5b**), acting as positive and negative controls, were both preincubated with polymyxin B for 1 h and subsequently administered to HD11 cell cultures. Polymyxin B significantly inhibited LPS-induced nitric oxide production (36.5% reduction), whereas CpG-induced nitric oxide production remained unchanged. Next, the antigenic fraction of the octavalent vaccine was preincubated with polymyxin B and administered to the cells (**Figure 5c**), which resulted in a significant reduction in nitric oxide production of 65.7% as compared to exposure to the antigenic fraction alone. Moreover, the treatment of the antigenic fraction of the octavalent vaccine with polymyxin B resulted in a significant reduction in the expression of iNOS (67.6% reduction; **Figure 5d**), IL-1β (80.0% reduction; **Figure 5e**), and CXCLi1 (58.9% reduction; **Figure 5f**). The expression levels of CXCLi2 (35.1% reduction; **Figure 5g**) and IL-10 (43.5% reduction; **Figure 5h**) tended to be inhibited by pre-incubation with polymyxin B, but this was not statistically significant. Taken together, these experiments suggest that LPS present in *Av. paragallinarum* antigens significantly contributed to the activation of HD11 cells upon exposure to the octavalent vaccine.

Discussion

In this explorative study, we aimed to investigate the capacity of an octavalent vaccine containing inactivated IBV, NDV, EDSV, and five serovars of *Av. paragallinarum* to activate innate immune cells, in view of identifying potential immune parameters that could be used in the future for *in vitro* vaccine QC testing of inactivated *Av. paragallinarum*. The vaccine was found to activate the chicken macrophage-like cell line HD11 shortly after stimulation, resulting in the expression of the nitric oxide-producing enzyme iNOS and the pro-inflammatory cytokines IL-1β, TNF, CXCLi1, and CXCLi2. From 6 h after stimulation, HD11 cells expressed the anti-inflammatory cytokine IL-10, which inhibits TLR signaling [38] and may be responsible for the subsequent return of IL-1β and TNF expression to baseline levels. In contrast, HD11 cells were not activated by a trivalent vaccine containing the same inactivated viral antigens but without inactivated *Av. paragallinarum* antigens, indicating that bacterial PAMPs were important for the evoked immune response. Furthermore, pre-treating the extracted antigens with the LPS-neutralizing antibiotic polymyxin B [32] largely averted nitric oxide production and cytokine expression by HD11 cells, showing that bacterial LPS was an important immunostimulatory factor of the octavalent vaccine. Hence, stimulation of HD11 cells by the octavalent vaccine must at least partly be dependent on TLR4, the designated PRR for LPS [39]. This is supported by an *in vivo* study, showing upregulation of the TLR4 signaling pathway in the nasal tissues of chickens infected with *Av. paragallinarum* [40].

The differences in macrophage activation by the octa- and trivalent vaccine were striking and may be explained by the presence or absence of bacterial PAMPs from *Av. paragallinarum* acting as an endogenous adjuvant of the octavalent vaccine. This is in agreement with *in vivo* data demonstrating that chickens developed strong granulomatous reactions involving macrophages at injection sites of inactivated bacterial *Av. paragallinarum* or Mycoplasma

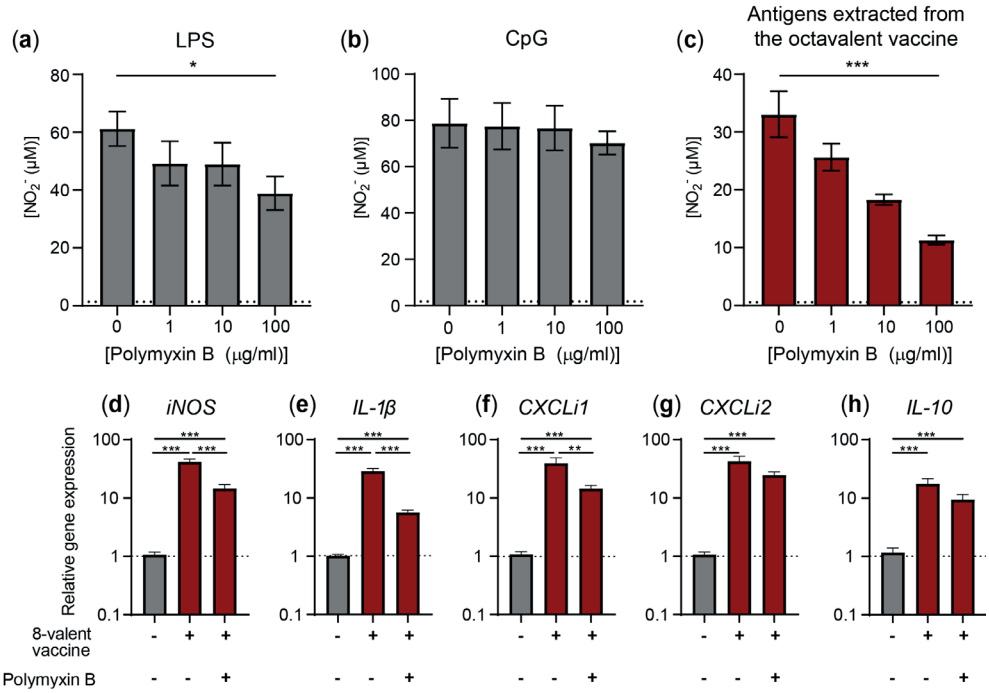

Figure 5. Nitric oxide production upon exposure to the antigenic fraction of the octavalent vaccine could be inhibited by the LPS-binding antibiotic polymyxin B. (**a**–**c**) HD11 cells were pre-incubated in cell culture media without or with 1, 10, or 100 µg/ml polymyxin B for 1 h at 37 °C and subsequently exposed to cell culture media containing 300 ng/ml LPS (**a**), 300 ng/ml CpG (**b**) or 0.5 µL/mL antigens extracted from the octavalent vaccine (**c**) for 48 h. Unstimulated controls are represented by the dotted lines. The first bar of panel a is the result of HD11 stimulated with 300 ng/ml without polymyxin B and thus represents the positive control. (**d**–**h**) Expression levels of iNOS (**d**), IL-1β (**e**), CXCLi1 (**f**), CXCLi2 (**g**), and IL-10 (**h**) by HD11 cells were determined 8 h after stimulation with 1 µL/mL octavalent vaccine and, when indicated, 1 h pre-incubation with 100 µg/ml polymyxin B. The values are expressed as $2^{-\Delta\Delta Ct}$ values as calculated using the Livak method and both GAPDH and 28S as reference genes. The data comprises three independent technical replicates performed in triplicate. Unstimulated controls are represented by the dotted lines. The error bars represent the SEM. A Kruskal–Wallis test combined with Dunn's multiple comparisons test was used to test for statistical significance of the data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The relative gene expression data were log-transformed prior to the statistical analysis to create normally distributed data.

gallisepticum vaccines, but not at injection sites of inactivated viral NDV, IBV, avian reovirus, or infectious bursal disease virus vaccines [41,42,43]. The presence of bacterial antigens containing PAMPs in the multivalent vaccine most likely also boosts the immune responses against the antigens of viral origin, as demonstrated for an experimental vaccine against both inactivated viral influenza A and Streptococcus pneumonia in mice [15].

The role of nitric oxide production and cytokines to the induction of vaccine-mediated protection against *Av. paragallinarum* in chickens is still unknown. A genome-wide association study in chickens has shown that there is an association between serological responses and small nucleotide polymorphism within and surrounding the gene encoding for iNOS, suggesting that iNOS may also be important in chickens during vaccination [44]. Studies

with iNOS, IL-17, IL-4, and IFN-γ-deficient mice have demonstrated the importance of nitric oxide [45] and cytokine production [17] for effective vaccination against *Bordetella pertussis*, also being a Gram-negative bacterium, and Trypanosoma cruzi [46]. Studies in humans mice and humans have more specifically demonstrated the importance of the cytokine-mediated induction of a mixed T_H1/T_H17 response for effective vaccination against *Bordetella pertussis* [17,18]. Similarly, a study in chickens has demonstrated that a nasal challenge with Pasteurella multocida, a member of the same bacterial family as *Av. paragallinarum*, results in a mixed T_H1/T_H17 response [47]. Similar to mice, a strong pro-inflammatory cytokine response is likely to be important for effective vaccination in chickens, as demonstrated for a flagellin-adjuvanted vaccine for Pasteurella multocida [48]. A nasal challenge with *Av. paragallinarum* in chickens was previously shown to result in the increased local gene expression of IL-6, which may contribute to a T_H17 adaptive immune response in mammals [49], whereas no changes were observed for the T_H1-inducing cytokine IL-12p35 [50]. In this *in vitro* study, we found increased gene expression of the T_H1/T_H17-inducing cytokine IL-12p40. We did unexpectedly not observe increased IL-6 expression by HD11 cells after stimulation with the octavalent vaccine, despite previous studies showing that bacterial stimulation induces IL-6 expression in HD11 cells [24]. The T_H1-incuding cytokine IL-12p35 and the T_H2-incuding cytokine IL-4 remained undetectable in HD11 cells at any of the time points after stimulation with the octavalent vaccine. The type of adaptive immune response that is triggered by *Av. paragallinarum* during natural infection or vaccination remains interesting for further investigation.

Since the potency of a vaccine does not depend solely on antigen quantity but also on its immunostimulatory capacity, the preservation and consistency of LPS and other PAMPs in inactivated *Av. paragallinarum* vaccines is likely to be important for vaccination [51,52,53,54]. The immunostimulatory capacity of *Av. paragallinarum* bacteria incorporated in the octavalent vaccine could be evaluated using the nitric oxide production assay and was expressed as the half-maximum effective concentration (EC_{50})-value, which here is the vaccine concentration that gives half-maximal nitric oxide production. This opens up the possibility to use the nitric production assay, in addition to antigen quantification methods, to test vaccines containing inactivated *Av. paragallinarum* (and potentially other Gram-negative bacteria) for potency without the use of animals. The bacterial antigens of the octavalent vaccine were extracted from the w/o emulsion with isopropyl myristate, followed by centrifugation steps, to enable the quantification of the immunostimulatory capacity of the vaccine. Testing extracted antigens, rather than the native vaccine formulation, is a strategy that is also used to test inactivated NDV vaccines for potency by an ELISA and has been implemented in the European Pharmacopoeia monograph 0870 [7,9]. Future studies should address the suitability of the nitric oxide assay to discriminate between vaccine batches of different potency, including non-conforming batches, in accordance with the consistency approach [1]. Furthermore, in our study, we observed that high cell passage numbers of the HD11 cell line may slightly affect the sensitivity of the nitric oxide production assay (*not shown*), which needs to be addressed in assay validation studies. The inclusion of reference standards for normalization might be required to improve precision. Finally, the performance of the assay as a QC test should be compared to the animal-based vaccination-challenge test that is currently in place as the gold standard for infectious coryza vaccines [13].

Conclusions

This explorative study aimed to investigate the immunostimulatory capacity of an inactivated octavalent vaccine for IBV, NDV, EDSV, and *Av. paragallinarum* and to identify immune parameters that could potentially influence vaccine potency. We have found that this inactivated octavalent poultry vaccine activates the chicken macrophage-like cell line HD11 due to the presence of LPS associated with *Av. paragallinarum* antigens, which resulted in the production of nitric oxide and expression of the pro-inflammatory cytokine IL-1β and chicken IL-8-like chemokine CXCLi1. In contrast, a trivalent vaccine containing inactivated IBV, NDV, and EDSV viral antigens, which are also present in the octavalent vaccine, did not induce nitric oxide production or cytokine expression by HD11 cells, further demonstrating that the responses measured in the assays were specific for the *Av. paragallinarum* antigens of the octavalent vaccine. Furthermore, the nitric oxide production assay was shown to be potentially useful as an *in vitro* potency test for inactivated poultry vaccines against *Av. paragallinarum* using the EC_{50} of the purified bacteria as a readout for potency. Therefore, this study may contribute to the replacement of current animal-based vaccine QC tests and improve animal welfare.

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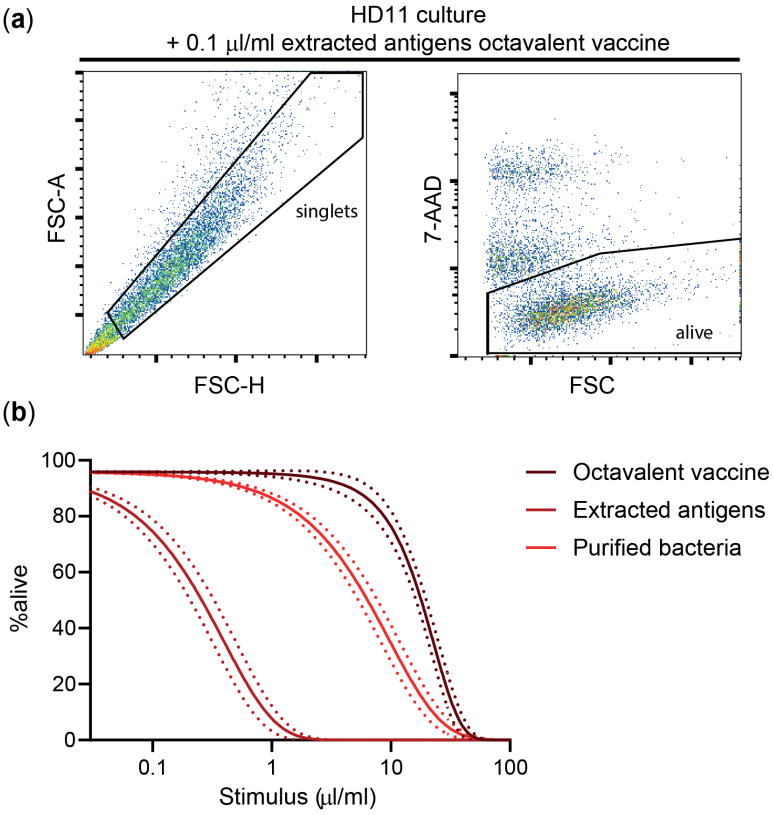
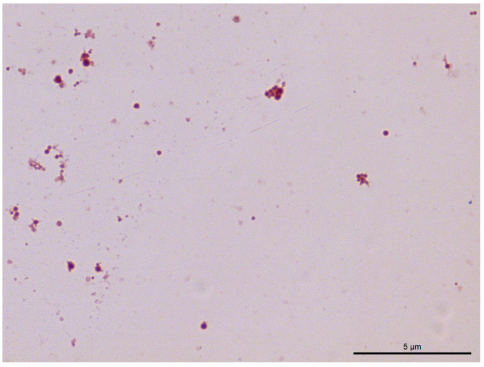
Supplementary Materials

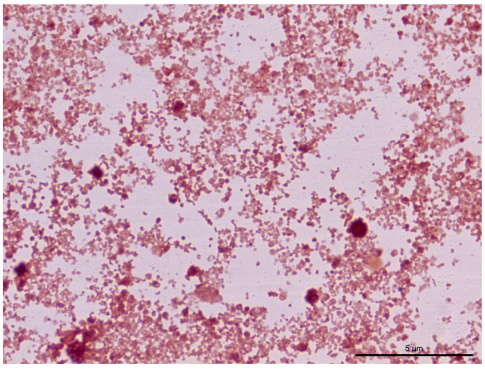


Supplementary Figure S1. Cytotoxicity of the octavalent vaccine, extracted antigens and purified *Av. paragallinarum* bacteria. (**a**) The HD11 cells were stained with 7-aminoactinomycin D to determine the percentage of viable cells by flow cytometry. The cells were first gated for single cells, also excluding debris. Next, cells negative for 7-AAD were considered to be alive. (**b**) HD11 cells were stimulated with graded doses of octavalent vaccine (0.3 – 30 µl/ml), the antigens extracted from the octavalent vaccine (0.1 – 10 µl/ml) and purified *Av. paragallinarum* bacteria (0.1 – 10 µl/ml) for 48 h. The graph was created by plotting survival curves with confidence intervals.

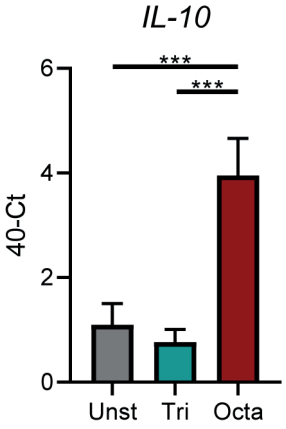
(**a**) Extracted antigens from trivalent vaccine



(**b**) Extracted antigens from octavalent vaccine



Supplementary Figure S2. Gram staining of extracted antigens from the tri- (**a**) and octavalent (**b**) vaccines. *Av. paragallinarum* bacteria were purified from the octavalent vaccine as described in the Materials and Methods. As a control, the antigens from the trivalent vaccines were purified using the same procedure. The images were taken at 1000x magnification using an Olympus BX60 microscope and immersion oil. The scale bars in black represent 5 µm.



Supplementary Figure S3. The octavalent vaccine induced gene expression of IL-10, whereas a trivalent vaccine without *Av. paragallinarum* antigens did not. Gene expression levels of IL-10 by HD11 cells were determined 8 h after stimulation with 1.0 µl/ml tri- or octavalent vaccine and are expressed as 40-Ct values, as described by Eldaghayes et al. [34]. The figure shows three independent technical replicates. The error bars represent the SEM. A Kruskal-Wallis test combined with Dunn's multiple comparisons test was used to test for statistical significance of the data. *** $p < 0.001$.

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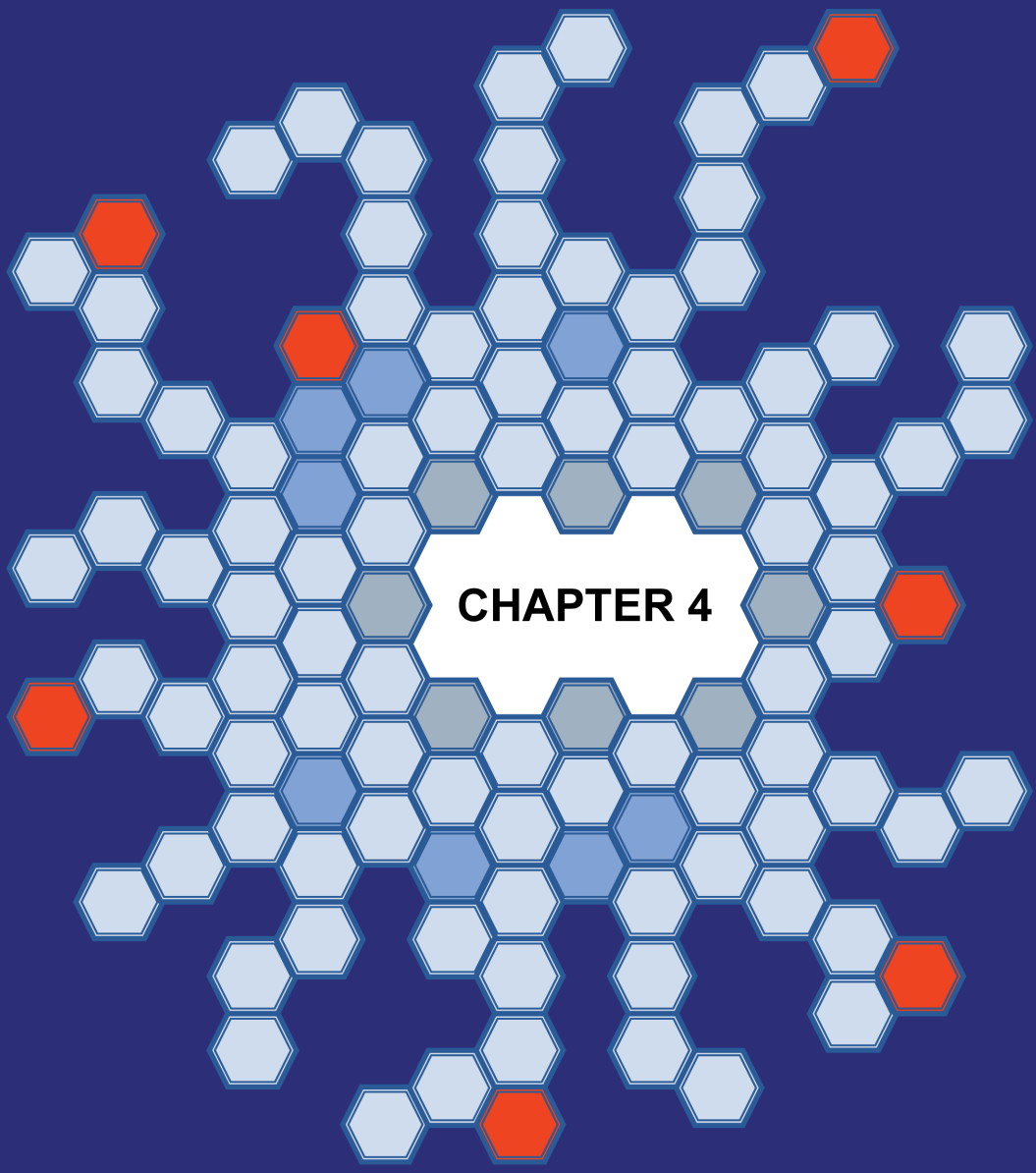
CHAPTER 4

In vitro chicken bone marrow-derived dendritic cells comprise subsets at different states of maturation

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Abstract

Research in chickens has been fundamental for the discovery of basic aspects of the immune system and has led to an interest in the in-depth characterization of avian immune cell types including dendritic cells (DCs). The *in vitro* generation and expansion of chicken bone marrow-derived DCs (chBMDCs) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) has provided a way to study chicken DCs, which are only present at limited cell numbers *in vivo*. This method has been employed to study the interactions between chicken DCs and pathogens or vaccines. However, a detailed characterization of the chBMDC culture is still lacking. In the present study, we performed an elaborate phenotypical and functional analysis of the chBMDC culture and addressed its heterogeneity. After 8 days of culture, chBMDCs comprised major histocompatibility complex class II (MHC-II)^{low} and MHC-II^{high} subsets with different morphologies. Compared with MHC-II^{low} chBMDCs, the MHC-II^{high} subset showed a more mature phenotype, with higher expressions of CD1.1, CD40, CD80, CCR7, and CD83, and a relatively low opsonophagocytic capacity. Nevertheless, MHC-II^{high} chBMDCs did not show an increased capacity to induce T-cell proliferation. Therefore, MHC-II^{high} chBMDCs were found to be semi-mature. Interestingly, the presence of the semi-mature MHC-II^{high} chBMDC subset reduced when cells were cultured in the presence of IL-4. Finally, prolonged cell culture after fluorescence-activated cell sorting (FACS) converted the semi-mature MHC-II^{high} subset back into the immature phenotype of the MHC-II^{low} subset, demonstrating plasticity of their maturation state. This detailed characterization explained the heterogeneity of the chBMDC culture by the simultaneous presence of immature and semi-mature chBMDC subsets, in addition to cells without features of antigen-presenting cells. Our findings are instrumental for the interpretation of experiments using the chBMDC culture in past and future research by providing insights into its phenotypically and functionally distinct cell types.

Introduction

Dendritic cells (DCs) are important innate immune cells that capture and process antigens to present them to cells of the adaptive immune system [1]. Adaptive immune responses result in pathogen-specific and long-lasting immunological memory, which enables the immune system to act more rapidly upon a second encounter with the pathogen. Vaccination against pathogens critically depends on DCs, which, respectively, support and fine-tune antigen presentation by co-stimulatory molecules and cytokines. The expression pattern of co-stimulatory molecules and cytokines by DCs depends on signals from their environment, including pathogen-associated molecular patterns (PAMPs) [1, 2] damage-associated molecular patterns (DAMPs) [3], and cytokines [1, 2, 4].

DCs are rare cells in all tissues and hard to isolate, which makes the use of primary DCs in functional assays challenging. To overcome this problem, granulocyte-macrophage colony-stimulating factor (GM-CSF)-differentiated bone marrow-derived DC (BMDC) culture methods have been developed to generate DCs in large numbers [5]. The availability of cultured BMDCs facilitates their use in *in vitro* screening methods, including immunogenicity tests for vaccines and toxicity tests for allergens [6–8]. In addition to well-characterized murine BMDC culture methods, such methods have been developed for veterinary species, including dogs [9], cats [10], cattle [11], sheep [12], pigs [13], and chickens [14].

Chicken BMDCs (chBMDCs) have been cultured in the presence of both recombinant chicken GM-CSF and interleukin-4 (IL-4) and were defined as DCs because of their typical stellate morphology and high expression of both major histocompatibility complex class II (MHC-II) and CD11b/c [14]. This chBMDC culture method has led to several studies into the role of chicken DCs in infection and vaccination. Maturation of chBMDCs has been observed after stimulation with lipopolysaccharide (LPS) or CD40L, as demonstrated by increased surface expression of co-stimulatory molecules CD40, CD83, and CD86; reduced phagocytosis and endocytosis; and an increased ability to induce a mixed lymphocyte reaction [14]. Similarly, chBMDCs have been found to mature upon exposure to avian influenza virus [15, 16], infectious bursal disease virus [17], or Salmonella enteritidis and Salmonella gallinarum vaccine candidates [18, 19].

Despite the widespread use of BMDCs originating from chickens and other species, a recent transcriptome study showed that murine GM-CSF-differentiated BMDCs differ phenotypically from murine DC populations *in vivo* [20]. Moreover, this study revealed that murine BMDC cultures comprise both CD11b^{high} MHC-II^{low} macrophage-like and CD11b^{low} MHC-II^{high} DC-like subsets that are closely related, but still phenotypically and functionally different. These findings had implications for conclusions drawn using *in vitro* murine BMDC cultures as a model for DC biology *in vivo* and are part of the ongoing discussion on how to distinguish DCs and macrophages [20–25]. In addition, these findings stressed the importance of thorough characterization of the cellular subsets present in *in vitro* BMDC cultures and triggered us to explore in depth the nature of chBMDCs raised *in vitro* with GM-CSF and to determine whether these indeed represent DC-like cells.

The initial results of the present study showed that the chBMDC culture was heterogeneous and comprised MHC-II^{low} and MHC-II^{high} subsets, similar to observations in murine

BMDC cultures. Therefore, we hypothesized that chBMDC culture comprised MHC-II^{low} macrophage-like and MHC-II^{high} DC-like subsets. However, in contrast to murine BMDC cultures, the MHC-II^{low} and MHC-II^{high} subsets of the chBMDC culture were found to reflect different maturation states rather than distinct cell types. MHC-II^{high} chBMDCs were found to exhibit increased expression of costimulatory molecules, also in the absence of stimuli. These findings on chBMDCs may have important consequences for conclusions drawn in past and future studies that make use of the chBMDC culture as a model for *in vivo* DC biology in chickens, in particular studies that assess chBMDC maturation.

Materials and Methods

Bone marrow isolation

Eighteen-day-old embryonated NOVOgen Brown eggs were obtained from a commercial breeder (Verbeek Broederij, Zeewolde, the Netherlands). Chicken embryos were removed from the eggs and euthanized by decapitation. Next, the tibiae and femurs were collected, bone heads were removed, and bone marrow was harvested by flushing the bones with RPMI-1640 cell culture medium supplemented with GlutaMAX™-I, phenol red, and HEPES (Gibco™, Life Technologies Limited, Paisley, UK) under sterile conditions using a Plastipak™ 10-ml syringe with a Microlance™ 3 21-G needle (both from BD Biosciences, Pharmingen, San Diego, CA, USA). Bones and bone marrow cells were kept on ice during the whole procedure. Bone marrow cells from 200 embryos were pooled, gently squeezed through a Falcon® 70-µm cell strainer (Corning®, Corning B.V. Life Sciences, Amsterdam, the Netherlands), and stored at −140°C in RPMI, 50% chicken serum (Gibco™, Life Technologies Limited, Paisley, UK), and 10% DMSO (Honeywell, Bucharest, Romania). This procedure resulted in batches comprising 1.3–2.3 x 10^9 bone marrow cells, which were frozen at a concentration of 2.5–5 x 10^7 cells per cryotube.

chBMDC culture

As previously described by others [26], chBMDCs were cultured from isolated bone marrow cells in RPMI-1640 cell culture medium supplemented with 5% chicken serum and 50 U/ml of penicillin–streptomycin (all from Gibco™, Life Technologies Limited, Paisley, UK) in the presence of recombinant GM-CSF (and IL-4) at 41°C, 5% CO_2. Recombinant GM-CSF and IL-4 were produced using COS-7 cells transfected with pCI-neo (Promega Corporation, Madison, Wisconsin, USA) expressing the relevant cytokine, which were a kind gift from P. Kaiser and L. Rothwell (Roslin Institute, Edinburgh, UK). The concentrations of the recombinant cytokines are given as a dilution of supernatant from transfected COS-7 cultures in accordance with a previous study [27]. GM-CSF was used at the titrated concentration (2 µl/ml) that resulted in the highest percentage of MHC-II$^+$ CD40$^+$ CD80$^+$ cells. In one experiment, the chBMDC culture was supplemented with GM-CSF and titrated concentrations of IL-4. Bone marrow cells were seeded at 2.5 x 10^6 cells per milliliter in 75-cm2 cell culture flasks in 15 ml of RPMI-1640 medium per flask, in 25-cm2 cell culture flasks in 5 ml of RPMI-1640 medium per flask, in Costar® six-well plates in 2 ml of RPMI-1640 medium per well, or in Costar® 24-well plates in 0.5 ml of RPMI-1640 medium per well (all from Corning®, Corning B.V. Life Sciences, Amsterdam, the Netherlands) depending on the required sample size. Early in the morning at day 3, culture medium with non-adherent cells was removed, and fresh RPMI-1640 medium with GM-CSF (and IL-4) was added. Late in the afternoon at day 4, the cultures received another volume of RPMI-1640 medium with

GM-CSF (and IL-4). The morphology of chBMDCs was examined by light microscopy using an EVOS FL microscope (AMG, Mill Creek, Washington, USA). In selected experiments, chBMDC cultures were matured by 100 ng/ml of LPS O127:B8 (Sigma-Aldrich, Saint Louis, MO, USA) stimulation for 24 h at day 7. To harvest the cultures at day 8, the medium with non-adherent cells was first collected. Subsequently, loosely adherent cells were washed and collected with Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium ($DPBS^{-/-}$; Lonza, Basel, Switzerland). Finally, the remaining adherent cells were incubated in $DPBS^{-/-}$ supplemented with 5 mM UltraPure EDTA (Invitrogen™, Life Technologies Europe BV, Bleiswijk, the Netherlands) for 10 min at room temperature (RT) before being collected as well. All cell-containing fluids (cell culture medium, $DPBS^{-/-}$, and $DPBS^{-/-}$ 5 mM EDTA) obtained during the harvest procedure were pooled for subsequent experiments.

Flow cytometry analysis and antibodies

Antibodies and streptavidin conjugates used in this study are listed in **Table 1**. All were diluted in fluorescence-activated cell sorting (FACS) buffer, containing $DPBS^{-/-}$ + 0.5% BSA and 0.005% NaN3 (both from Sigma-Aldrich, Saint Louis, MO, USA), which was used for staining and washing steps. Antibodies and streptavidin conjugates were used at titrated concentrations to stain 0.5–1.0 x 10^6 freshly harvested chBMDCs per 50 µl for 20 min at 4°C. Between staining steps, chBMDCs were washed twice with FACS buffer. To assess viability, the cells were first washed in $DPBS^{-/-}$ and then stained with Zombie Aqua Fixable Viability Dye (BioLegend Inc., San Diego, CA, USA) diluted in $DPBS^{-/-}$ for 20 min at 4°C. From each sample, 50,000–100,000 chBMDCs were analyzed using a CytoFLEX LX flow cytometer, equipped with 375-, 405-, 488-, 561-, 638-, and 808-nm lasers (Beckman Coulter Inc., Brea, CA, USA), FlowJo Software v. 10.5 (FlowJo LCC, Ashland, OR, USA), and Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

Immunofluorescence microscopy analysis

Ethanol-cleaned 12-mm glass coverslips (Waldemar Knittel Glasbearbeitungs GmbH, Brunswick, Germany) were placed into the wells of a 24-well cell culture plate. Next, bone marrow cells were seeded and cultured in complete RPMI culture medium in the presence of recombinant GM-CSF at 41°C, 5% CO_2. At day 8, differentiated chBMDCs on glass coverslips were either washed three times with $DPBS^{-/-}$ or first stained with fluorescently labeled lectin wheat germ agglutinin (WGA)-Alexa Fluor 488 (Invitrogen™, Life Technologies Europe BV, Bleiswijk, the Netherlands). Staining with WGA-Alexa Fluor 488 was performed by washing the cells twice with cold DPBS with calcium and magnesium, followed by staining with WGA-Alexa Fluor 488 diluted in DPBS with calcium and magnesium for 20 min at 4°C. Fixation was performed in $DPBS^{-/-}$ with 4% paraformaldehyde (Alfa Aesar, Haverhill, MA, USA) for 30 min at RT. Subsequently, the fixatives were quenched by washing the fixed samples three times with $DPBS^{-/-}$ and 10 mM glycine (Merck Millipore, Burlington, MA, USA) and blocked in blocking buffer, containing $DPBS^{-/-}$, 0.05% Tween-20 (Sigma-Aldrich, Saint Louis, MO, USA), and 2% bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA), overnight at 4°C. The coverslips were stained with the cells faced-down on Parafilm in 25 µl of blocking buffer with primary antibodies for 2 h and secondary antibodies for 1 h at RT (antibodies are listed in **Table 1**). In addition, a nuclear staining was performed in 25 µl of blocking buffer at 10 µg/ml with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Saint Louis, MO, USA) for 5 min at RT. Between staining steps, the samples were washed three

Table 1. Antibodies used in this study

Antibody name	Isotype	Clone	Target	Figure	Source
MaCh-MHC-II Alexa Fluor 488	IgG1	2G11	MHC-II	1	Southern Biotech
MaCh-Ia PE	IgM	Cia	MHC-II	2, 6, 7, 9	
MaCh-Ia UNLB	IgM	Cia	MHC-II	3	
MaCh-b2-microglobulin UNLB	IgG1	F21-21	MHC-I	2, 6	
MaCh-monocyte/macrophage UNLB	IgG1	KUL01	MRC1L-B	2, 6	
MaCh-c-kit BIOT	IgG2a	Kit2c75	c-kit, CD117	2, 6	
MaCh-CD1.1 UNLB	IgG1	CB3	CD1.1	2, 6	
M IgM, k isotype Ctrl PE	IgM	MM-30	-	2, 6	BioLegend
MaCh-CD40 UNLB	IgG2a	AV79	CD40	2, 6	Bio-Rad
MaCh-CD80 UNLB	IgG2a	IAH:F864DC7	CD80, B7-1	2, 3, 6, 7, 9	
MaCh-CSF1R UNLB	IgG1	ROS-AV170	CSF1R, CD115	2, 3, 6, 7, 9	
MaCh-5C7 UNLB	IgG1	5C7	Putative CD11b/c*	2, 6	Produced in house†
RaM-IgG1 BV421	IgG	RMG1-1	Mouse IgG1	7, 9	BioLegend
Streptavidin APC	-	-	Biotin	2, 6	BD Pharmingen
GaM-IgG1 APC	IgG	-	Mouse IgG1	2, 6	Southern Biotech
GaM-IgG2a APC	IgG	-	Mouse IgG2a	2, 6, 7, 9	
GaM-IgM Alexa Fluor 488	IgG	-	Mouse IgM	3	
GaM-IgG2a Alexa Fluor 488	IgG	-	Mouse IgG2a	3	Invitrogen
GaM-IgG1 Alexa Fluor 647	IgG	-	Mouse IgG1	3	

M, mouse; Ch, chicken; G, goat; R, rat; MHC-II, major histocompatibilitycomplex class II; MRC1L-B, mannose receptor C-type 1-like B; CSF1R, colony-stimulating factor 1 receptor;APC, allophycocyanin; PE, R-phycoerythrin; BV421, Brilliant Violet 421.*It is yet uncertain whether MαCh-5C7 recognizes CD11b or CD11c.†Hybridoma was a gift from T.W. Göbel(Ludwig Maximilians Universität, Munich, Germany). Sources: SouthernBiotech, Birmingham, AL, USA; Bio-Rad Laboratories B.V., Veenendaal, the Netherlands; BioLegend Inc., SanDiego, CA, USA; Invitrogen†, Life Technologies Europe BV, Bleiswijk, the Netherlands; BD Biosciences, Pharmingen, San Diego, CA, USA.

times with DPBS$^{-/-}$ with 0.05% Tween-20. The last wash step was performed in distilled water, before mounting the samples on Polysine® microscope slides (Menzel Glaser GmbH & Co KG, Braunschweig, Germany) in a FluorSave reagent (Calbiochem®, Merck Millipore, Burlington, MA, USA). The samples were captured using a TCS-SPE-II confocal microscope (Leica Microsystems B.V., Amsterdam, the Netherlands) equipped with 405-, 488-, 561-, and 635-nm diode lasers and processed using Fiji software [28].

Phagocytosis of IgY-opsonized beads by chBMDC subsets

Chicken serum IgY fraction (Agrisera AB, Vännäs, Sweden) was added at 14.4 mg/ml to 1.44 x 10^{10} beads per milliliter of 1-µm crimson carboxylate-modified FluoSpheres (Invitrogen™, Life Technologies Europe BV, Bleiswijk, the Netherlands) and incubated overnight on an orbital shaker at 4°C to create IgY-opsonized beads. The next day, the beads were washed twice and resuspended in DPBS$^{-/-}$ with centrifugation steps at 3,000 x *g* for 20 min at 4°C in between. To confirm IgY coupling, the beads were stained in FACS buffer with MαCh IgY-PE (SouthernBiotech, Birmingham, AL, USA) and analyzed on the CytoFLEX LX flow cytometer (*data not shown*). Next, the IgY-coupled beads were used in a phagocytosis assay to assess bead uptake by chBMDCs. After 8 days of culture in a 24-well plate, chBMDCs from one well were harvested and counted to determine the number of IgY-opsonized beads needed to obtain a 1:1 bead-to-cell ratio. Next, crimson beads were added to the remaining wells followed by 4-h incubation at 41°C, 5% CO_2, to allow phagocytosis by chBMDCs. Subsequently, chBMDCs were harvested and stained for flow cytometry or confocal microscopy according to the methods described above. For flow cytometry, the cells were stained for MHC-II expression and viability, using Zombie Aqua Fixable Viability Dye, and analyzed using the CytoFLEX LX flow cytometer. For confocal microscopy, the cells were stained with WGA-Alexa Fluor 488, MαCh-Ia BIOT, and streptavidin Alexa Fluor 405 and analyzed using the TCS-SPE-II confocal microscope.

IL-4 bioactivity assessment by ^{3}H-thymidine incorporation by PBMCs

The ^{3}H-thymidine incorporation assay to measure IL-4 bioactivity was modified from a published method to assess peripheral blood mononuclear cell (PBMC) proliferation [27]. Heparinized blood was collected from healthy chickens (under registration number AVD108002016642-1 from the Dutch Central Authority for Scientific Procedures on Animals). The chickens were daily monitored by animal caretakers for signs and symptoms of disease, which were absent for the chickens used in this study. PBMCs were isolated from heparinized blood by density gradient separation using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA) according to standard procedure. Collected PBMCs were resuspended in 2 ml of Iscove's modified Dulbecco's medium (IMDM) culture medium supplemented with GlutaMAX™-I, phenol red, and HEPES, with 8% fetal bovine serum (FBS), 2% chicken serum, and 50 U/ml of penicillin–streptomycin (all from Gibco™, Life Technologies Limited, Paisley, UK). The cells were counted and seeded in a 96-well flat-bottom culture plate with 100 µl of IMDM medium per well containing 2 x 10^{5} cells. The cells received different concentrations (ranging 1:25–1:250) of cell culture supernatant from COS-7 cells transfected with an IL-4 or empty pCI vector and were incubated for 4 days at 41°C, 5% CO_2. Subsequently, 0.4 µCi of ^{3}H-thymidine per well was added to the culture for 18 h, and the cells were harvested using a Harvester 96 (TOMTEC Imaging Systems GmbH, Unterschleißheim, Germany). ^{3}H-Thymidine incorporation by the cells was determined in a 1,450 MicroBeta Plus liquid scintillation counter (Wallac, PerkinElmer Life Sciences, Zaventem, Belgium).

Separation of chBMDC subsets by FACS

For sorting, chBMDCs were stained with antibodies specific for MHC-II, colony-stimulating factor 1 receptor (CSF1R), and CD80 as before. In addition, the dye 7-aminoactinomycin D (7-AAD; BD Biosciences, Pharmingen, San Diego, CA, USA) was added to the cells for viability assessment. Next, the cells were resuspended in $DPBS^{-/-}$, 1% FBS, 2 mM EDTA, and 0.005% NaN3 and flushed through a 70-µm cell strainer to create single-cell suspensions. MHC-II^{high} $CSF1R^{low}$ and MHC-II^{low} $CSF1R^{high}$ chBMDC subsets were sorted by FACS with a BD Influx cell sorter, equipped with 405-, 488-, 561-, and 635-nm lasers (BD Biosciences, Pharmingen, San Diego, CA, USA). Each chBMDC subset constituted close to 25% of the original sample. Approximately 2 x 10^6 cells were sorted for both subsets to perform quantitative real-time PCR (RT-qPCR). In parallel, the cells were analyzed before and after FACS using the CytoFLEX LX flow cytometer. Moreover, some cells were sorted onto a Polysine® microscope slide, fixed with 4% paraformaldehyde, and analyzed by confocal microscopy using the TCS-SPE-II microscope to confirm the expression patterns of MHC-II, CD80, and CSF1R by the chBMDC subsets. In addition, chBMDCs were sorted to evaluate the phenotypic stability of the cells by prolonged cell culture. Sorted chBMDCs were seeded into 24-well plates at 350,000 cells per well in 1 ml of RPMI culture medium with GM-CSF and incubation for 1 or 3 days of prolonged cell culture at 41°C, 5% CO_2, before repeated flow cytometric analysis.

Gene expression analysis of separated chBMDC subsets using RT-qPCR

Sorted chBMDC subsets were lysed in RLT buffer (Qiagen GmbH, Hilden, Deutschland) and stored at –20°C until RNA isolation. RNA isolation was performed with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Deutschland) according to the manufacturer's instructions, including a DNase treatment step using the RNase-Free DNase Set (Qiagen GmbH, Hilden, Deutschland). Next, cDNA was prepared using the reverse transcriptase from the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) according to the manufacturer's instructions. RT-qPCRs were performed with primers (listed in **Table 2**) and either FAM-TAMRA-labeled TaqMan probes combined with TaqMan Universal PCR Master Mix or SYBR Green Master Mix without probes (all from Life Technologies Europe BV, Bleiswijk, the Netherlands). RT-qPCRs were performed with a CFX Connect and analyzed with the CFX Maestro software (both from Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). All RT-qPCRs were evaluated for proper amplification efficiency (95–105%) using serial dilutions of reference cDNA from splenocytes that were stimulated with concanavalin A for 24 h or from HD11 cells that were stimulated with LPS for 3 h. RT-qPCRs were performed in triplo for every sample, and the average gene expression levels were expressed as 40-Ct values, as described by Eldaghayes et al. [29]. The results were normalized toward gene expression levels of the housekeeping genes 28S and GAPDH.

Mixed lymphocyte reaction between chBMDC subsets and PBMCs

PBMCs were isolated from heparinized blood by density gradient separation using Ficoll-Paque PLUS according to standard procedure and resuspended in X-VIVO 15 cell culture medium. The cells were seeded in a 96-well flat-bottom culture plate with 100,000 cells per well. Unsorted and sorted MHC-II^{low} and MHC-II^{high} chBMDC subsets were resuspended in X-VIVO 15 medium and added to the PBMCs at different effector-to-target (E:T) ratios, ranging 1:2–1:8. A positive control was created by addition of 1 µg/ml of anti-CD3, 1 µg/ml of anti-CD28, and 1:50 supernatant from a COS-7 cells transfected with a pCI-neo

Table 2. All primer and probe sequences are given from the 5' to 3' ends.

Gene	NCBI Reference	Type	Sequences (5'-3')
MERTK	NM_204988.1	Forward	TGTGGAAGGATGGCAGGGAG
		Reverse	GCACGGATGCTGAATGTAGAGG
ZBTB46	XM_015296613.2	Forward	CTGGACCTGTGGAAGAGGAAAC
		Reverse	CGGTAGTGGGAGGCAATCTC
iNOS	NM_204961.1	Forward	TGGGTGGAAGCCGAAATA
		Reverse	GTACCAGCCGTTGAAAGGAC
TLR4	NM_001030693.1	Forward	GTCCCTGCTGGCAGGAT
		Reverse	TGTCCTGTGCATCTGAAAGCT
GAPDH	NM_204305.1	Forward	GTGGTGCTAAGCGTGTTATC
		Reverse	GCATGGACAGTGGTCATAAG
CD14	NM_001139478.1	Forward	GGACGACTCCACCATTGACAT
		Reverse	GGAGGACCTCAGGAACCAGAA
		Probe	AATGATCTTCCTGATTTGCAGACTGCCAA
CCR6	XM_015284122.2	Forward	GCCAGCCGCAGAAGAATGTA
		Reverse	TGTGGAGAAGAGTTTCAGAATGCT
		Probe	CAGAGTCGTGCAACATCGTCTGACCTACA
CCR7	NM_001198752.1	Forward	CATGGACGGCGGTAAACAG
		Reverse	TCATAGTCGTCGGTGACGTTGT
		Probe	TGAGGGTCACCATCGCTTTCAGCC
DEC205	NM_001037836.1	Forward	AACACGATGCCAGCTCTCAA
		Reverse	TTGACATGAAACGTAAGCTTCCTT
		Probe	CTACCAGTTCAACACCCAGTCTGCTCTTTCTTG
DC-SIGN	NM_205484.1	Forward	TCTCGCTGAGCAGAATGAGTTG
		Reverse	GATGAGGTGGGAGTGCATCTC
		Probe	CACAAAGCGAAGGCGGAGTGCG
CD83	XM_418929.6	Forward	TTGGCGACAGAATAGCATGG
		Reverse	CAGGGAGCCTCCAAGTCCTT
		Probe	AAGTCCTTGATGTGGAATCTCGTCATCCA
28S	XR_003078040.1	Forward	GGCGAAGCCAGAGGAAACT
		Reverse	GACGACCGATTTGCACGTC
		Probe	AGGACCGCTACGGACCTCCACCA

In the absence of probe sequences, quantitative real-time PCR (RT-qPCR)was performed with SYBR Green Master Mix

construct expressing chicken IL-2. A negative control was created by adding an additional X-VIVO 15 medium. The cells were cultured for 3 days at 41°C, 5% CO_2. Subsequently, 0.4 µCi of ^{3}H-thymidine per well was added to the culture for 18 h, and the cells were harvested using a Harvester. ^{3}H-Thymidine incorporation by the cells was determined in a 1,450 MicroBeta Plus liquid scintillation counter.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). The data were tested for the assumptions of normally distributed data. Flow cytometry expression data showing the geometric mean fluorescent intensity (gMFI) was log-transformed in order to generate normally distributed data. Paired t-tests were used to test for statistically significant differences between MHC-II^{low} and MHC-II^{high} chBMDC subsets. A *p*-value of <0.05 was considered statistically significant.

Results

chBMDC cultures are heterogeneous and comprise MHC-II^{low} and MHC-II^{high} subsets cells that differ in morphology and phenotype

Bone marrow cells, derived from bones of 18-day embryonated chicken embryos, were cultured in the presence of GM-CSF for 7 days to generate chBMDCs. The bone marrow isolates comprised mainly myeloid immune cells, including monocytes, thrombocytes, thromboblasts, granulocytes, and erythrocytes (**Supplementary Figure S1a**). After 7 days of culturing, nearly all cells were found to be CD45$^+$ and thus hematopoietic (**Supplementary Figure S1b**). In agreement with previous studies, chBMDCs formed clusters holding veiled cells (**Figures 1a**,**c**, arrows), typical for DC morphology [14], and highly expressing MHC-II (**Figure 1b**). MHC-II-expressing cell clusters did not appear in the absence of GM-CSF (**Supplementary Figure S2**). Beyond the clusters, cells with different morphologies were visible. These were large, round, without protrusions, and highly granular (**Figure 1a**, stars). Upon LPS stimulation, cell clusters became less dense, and many individual small cells with elongated protrusions became visible, a feature typical for mature DCs (**Figure 1d**, arrows).

Next, a phenotypic analysis of the chBMDC culture by flow cytometry identified three subsets, distinguished by forward scatter (FSC) vs. MHC-II expression: FSC^{low} with no or low expression of MHC-II (FSC^{low}), FSC^{int} with high expression of MHC-II (MHC-II^{high}), and FSC^{high} with low expression of MHC-II (MHC-II^{low}) (**Figure 2a**). These chBMDC subsets were evaluated for the expression of myeloid markers including integrin CD11b/c, costimulatory receptors CD40 and CD80, CSF1R, stem cell growth factor receptor c-kit, mannose receptor C-type 1-like B (MRC1L-B), non-classical MHC molecule CD1.1, and MHC class I component β2-microglobulin (β2m). FSC^{low} cells showed high expression of CD11b/c, but no expression of CD40 and CD1.1 (**Figure 2b**). CD80, c-kit, MRC1L-B, and β2m were expressed at moderate levels. CSF1R and c-kit were expressed by some FSC^{low} cells, but not by others, showing further heterogeneity within this subset. Since FSC^{low} cells were largely positive for MRC1L-B but showed no or low expression of MHC molecules and costimulatory molecules, these were likely to represent undifferentiated monocytes. Both MHC-II^{high} and MHC-II^{low} cells showed expression of MHC molecules and

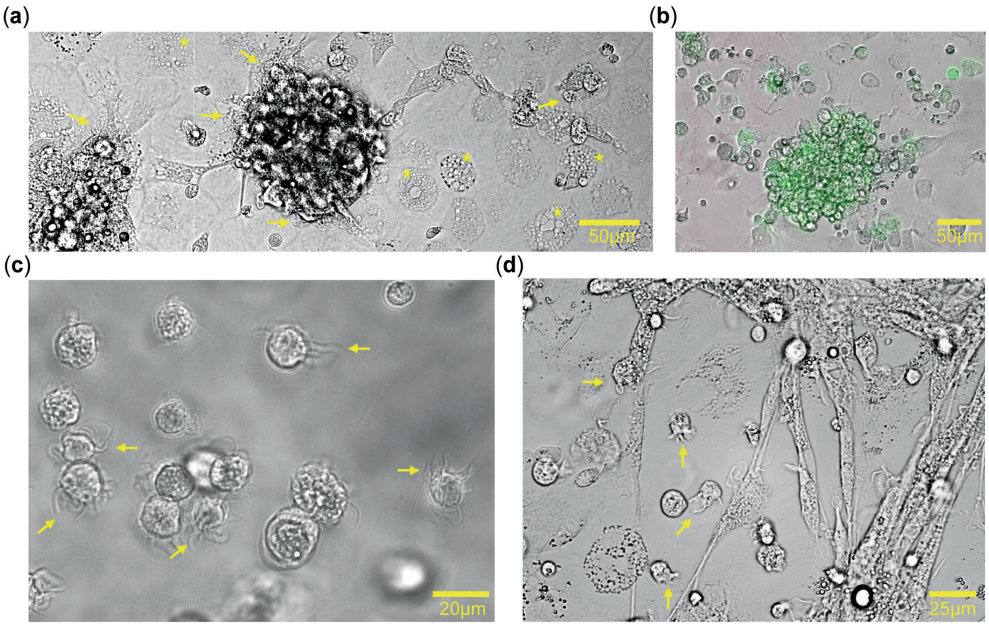

Figure 1. Chicken bone marrow-derived dendritic cell (chBMDC) cultures contained clusters of major histocompatibility complex class II (MHC-II)$^{+}$ cells. (**a**) Bright-field light microscopy shows unstimulated chBMDCs after 7 days of culture. A cluster with chBMDCs is visible at the center. Yellow arrows indicate dendritic cells, visible as veiled cells that show protrusions. Yellow stars indicate highly granular cells without protrusions. (**b**) An overlay of light and fluorescent microscopy shows a cluster of unstimulated chBMDCs with MHC-II expression in green. (**c**) Light microscopy shows individual unstimulated chBMDCs that were transferred to a Petri dish after gentle resuspension in DPBS$^{-/-}$. (**d**) Light microscopy shows lipopolysaccharide (LPS)-stimulated chBMDCs.

costimulatory molecules. Compared to MHC-II^{low} cells, MHC-II^{high} cells expressed higher levels of costimulatory receptors CD40 and CD80, CD1.1, and MRC1L-B (**Figures 2b**,**c** and **Supplementary Table S1**). Conversely, MHC-II^{low} cells expressed higher levels of CD11b/c, CSF1R, c-kit, and β2m. Taken together, MHC-II^{low} and MHC-II^{high} chBMDC subsets both showed a phenotype of antigen-presenting cells, but differentially expressed many myeloid markers.

Next, the expression patterns of CSF1R, MRC1L-B, and CD80 by MHC-II^{low} and MHC-II^{high} chBMDC subsets were evaluated by immunofluorescent confocal microscopy. The MHC-II^{low} subset expressed higher levels of CSF1R than the MHC-II^{high} subset (**Figure 3a**), in accordance with the flow cytometry data (**Figure 2c**). Therefore, CSF1R could be used as an additional marker to discriminate between chBMDC subsets. MHC-II^{low} CSF1R^{high} cells were found to be large and round and to have few protrusions, indicative of a macrophage-like morphology. Similar cells were observed by light microscopy (**Figure 1a**, stars). In contrast, MHC-II^{high} CSF1R^{low} cells showed irregular shapes with many protrusions, indicative of a DC-like morphology, and resemble the veiled cells that were observed by light microscopy (**Figure 1a**). MRC1L-B and CD80 expression levels were found to be highest on MHC-II^{high} chBMDCs (**Figures 3b**,**c**), in accordance with the flow cytometry data (**Figure 2c**). Nevertheless, MRC1L-B and CD80 did not colocalize with MHC-II. MRC1L-B and CD80 were mainly found in intracellular compartments, whereas MHC-II was found more on the

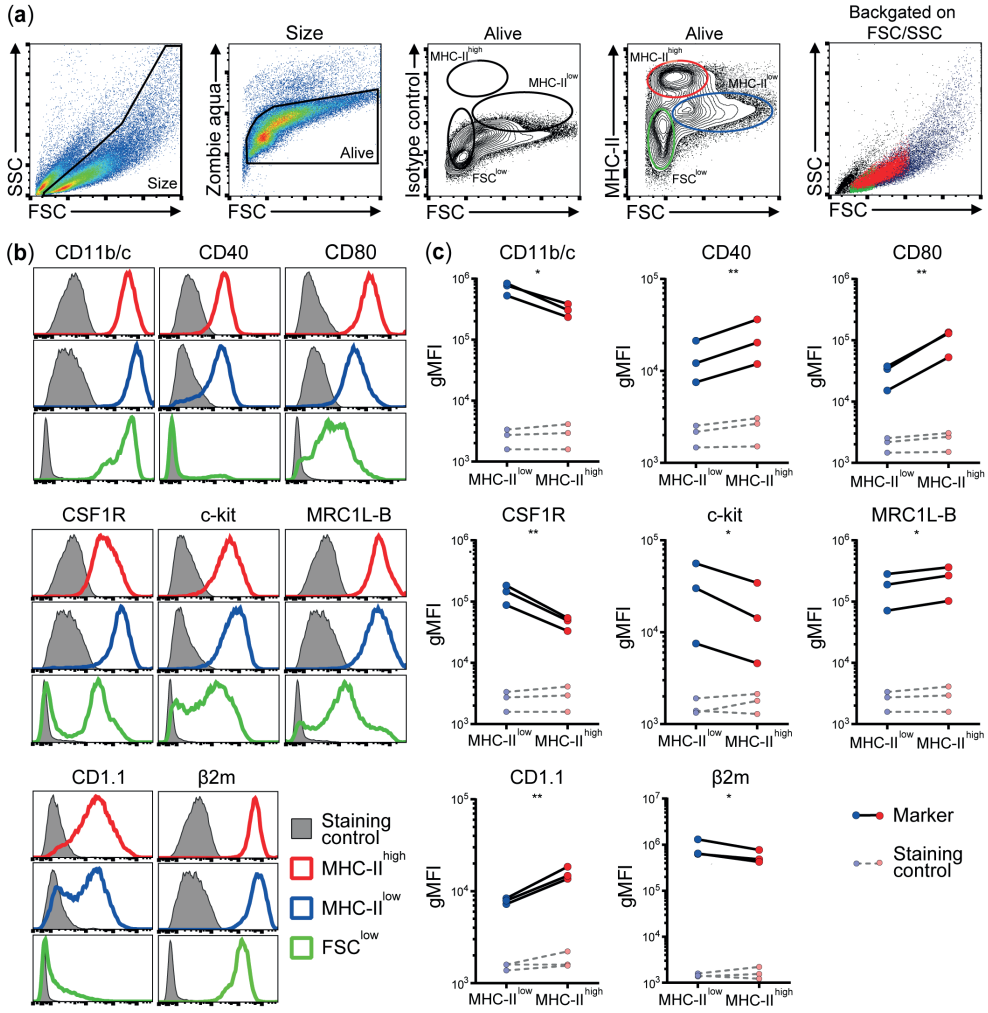

Figure 2. Phenotypic analysis of chicken bone marrow-derived dendritic cells (chBMDCs) shows the presence of different subpopulations. (**a**) The cells were gated, respectively, for their scatter profile [forward scatter (FSC)/side scatter (SSC)], viability (Zombie Aqua), and expression of major histocompatibility complex class II (MHC-II), as shown in the first, second, and fourth panels. The third panel shows the signal after staining the cells with an isotype control antibody that corresponds to the antibody against MHC-II. Three subpopulations were selected based on FSC and MHC-II expression: FSC^{low} $MHC\text{-}II^{-/low}$ (green), FSC^{int} $MHC\text{-}II^{high}$ (red), and FSC^{high} $MHC\text{-}II^{low}$ (blue). The last panel shows the scatter profiles of the different subsets in matched colors, which were overlaid on the first panel. (**b**) Expression levels of a set of phenotypic markers are shown by representative histograms for the three subpopulations in corresponding colors. Each marker addressed was stained with a combination of either unconjugated primary antibody and allophycocyanin (APC)-conjugated secondary antibody or biotinylated primary antibody and APC-conjugated streptavidin. Filled gray histograms represent cells that have been stained with APC-conjugated streptavidin or secondary antibody to show the background fluorescence. (**c**) For the $MHC\text{-}II^{low}$ and $MHC\text{-}II^{high}$ subpopulations, the expression levels of all phenotypic markers are expressed as the geometric mean fluorescent intensity (gMFI) for three independent replicates. The gray dashed lines represent corresponding controls that have

been stained with APC-conjugated streptavidin or secondary antibody alone. Statistically significant differences between chBMDC subsets are shown/indicated by *p < 0.05 and **p < 0.01.

cellular surface of MHC-II^{high}-expressing chBMDCs. A stronger colocalization was observed between MRC1L-B and CD80 (**Figure 3d**).

MHC-II^{low} chBMDCs have a higher capacity to phagocytose fluorescent beads compared to MHC-II^{high} cells

To assess whether the phenotypic distinction between MHC-II^{low} and MHC-II^{high} chBMDCs was functionally relevant, the subsets were assessed for their ability to phagocytose chicken IgY-coated fluorescent latex beads. First, the uptake of IgY-coated crimson fluorescent beads by chBMDCs was confirmed by showing that the beads localize beneath the surface of the plasma membrane, which was visualized using WGA (**Figure 4a** and **Supplementary Video S1**). chBMDCs were stained for MHC-II to identify the MHC-II^{high} subpopulation. Both MHC-II^{low} and MHC-II^{high} chBMDCs were found to take up beads as determined by confocal immunofluorescent microscopy. Next, the bead content of the chBMDC subsets was quantified by flow cytometry (**Figure 4b**). On average, MHC-II^{low} cells (0.54 beads per cell) contained 2.4 times more beads than MHC-II^{high} cells (0.23 beads per cell), which shows that the MHC-II^{low} and MHC-II^{high} chBMDC subsets differ in opsonophagocytic capacity (**Figure 4c**). A major part of IgY beads actually bound to the cells instead of being taken up, as shown in a separate experiment performed at 4°C (**Supplementary Figure S3**). However, when bound IgY beads from the experiment performed at 4°C were subtracted from the experiment performed at 41°C, MHC-II^{low} cells were still found to take up 2.7 times more beads than MHC-II^{high} cells. The FSC^{low} chBMDC subset showed little uptake of beads (0.048 beads per cell) (**Figure 4b**).

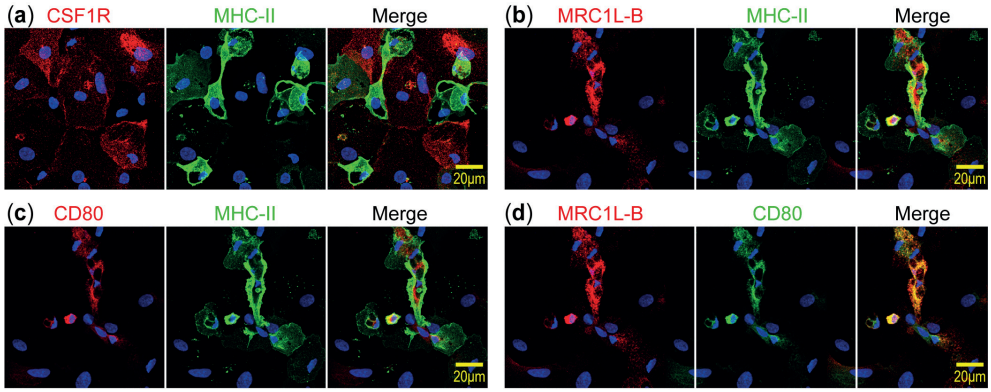

Figure 3. Mannose receptor C-type 1-like B (MRC1L-B) and CD80 are expressed by irregularly shaped MHC-II^{high} chicken bone marrow-derived dendritic cells (chBMDCs), whereas colony-stimulating factor 1 receptor (CSF1R) is expressed by large, round major histocompatibility complex class II (MHC-II)^{low} chBMDCs. (**a**) chBMDCs were stained for expression of CSF1R (red) and MHC-II (green). 4′,6-Diamidino-2-phenylindole (DAPI) (blue) was used as a nuclear staining. A merged image shows potential colocalization between CSF1R and MHC-II. Similar images show the colocalization between MRC1L-B (red) and MHC-II (green) (**b**), between CD80 (red) and MHC-II (green) (**c**), and between MRC1L-B (red) and CD80 (green) (**d**).

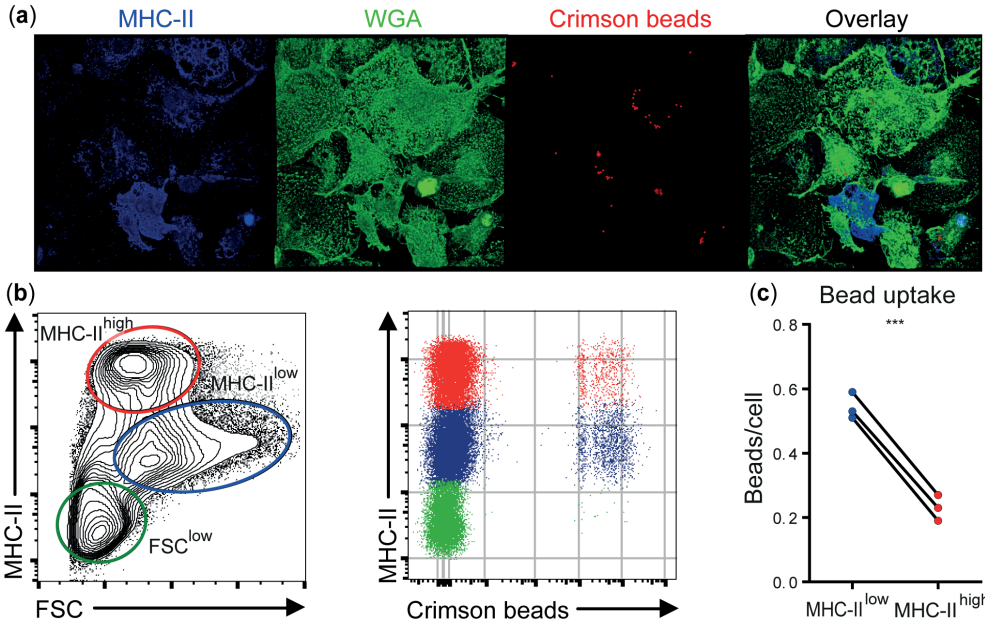

Figure 4. Major histocompatibility complex class II (MHC-II)^{low} chicken bone marrow-derived dendritic cells (chBMDCs) phagocytose IgY-coated beads more efficiently than MHC-II^{high} chBMDCs. (**a**) chBMDCs were stained with wheat germ agglutinin (WGA) to visualize the plasma membrane, shown in green, and MHC-II to identify MHC-II^{high} cells, shown in blue, by confocal microscopy. Fluorescent crimson beads are shown in red. A 3D image was constructed from 56 z-stacks over a total distance of 9.4 µm. Here, a top view on the 3D image is shown, whereas all dimensions are visible in **Supplementary Video S1**. (**b**) Next, the bead content was quantified by flow cytometry for the previously identified chBMDC subpopulations. (**c**) The average number of beads per cell was calculated for MHC-II^{low} and MHC-II^{high} subpopulations by dividing the mean fluorescent intensity (MFI) of the fluorescent beads by the MFI of cells that contained one bead (visible in B as the first positive line of cells). Each line represents an independent replicate (n = 3). A statistically significant difference between chBMDC subsets is shown/indicated by ***$p < 0.001$.

Addition of recombinant IL-4 to chBMDC cultures leads to a smaller proportion of MHC-II^{high} cells

The effect of recombinant IL-4 on the generation of chBMDC subsets was investigated, since this cytokine has been used to generate chBMDCs by others [14]. To confirm that recombinant IL-4, produced in COS-7 cells, was biologically active, its ability to induce PBMC proliferation was demonstrated (**Supplementary Figure S4**). Next, IL-4 was given to chBMDCs alone or in combination with GM-CSF. IL-4 alone led to few MHC-II-expressing clusters of chBMDCs (**Supplementary Figure S2**). IL-4 in combination with GM-CSF led to many MHC-II-expressing clusters, similar to GM-CSF alone. Next, the proportion of MHC-II^{low} and MHC-II^{high} cells was quantified by flow cytometry. The addition of IL-4 to the standard chBMDC culture with GM-CSF was found to increase the proportion of the MHC-II^{low} from 32.6 to 49.7% at the highest administered dose (1/25 dilution) (**Figure 5a**). The increase in the proportion of MHC-II^{low} cells occurred largely at the expense of the MHC-II^{high} subset, which changed proportionally from 35.0 to 23.0% at the highest administered dose of IL-4 (**Figure 5b**). The proportion of FSC^{low} cells remained fairly stable (*data not shown*).

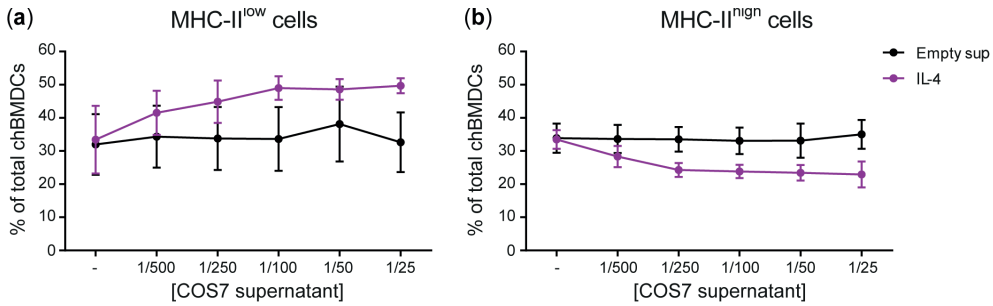

Figure 5. Addition of interleukin-4 (IL-4) to the culture medium during chicken bone marrow-derived dendritic cell (chBMDC) culture reduces the proportion of major histocompatibility complex class II (MHC-II)^{high} cells. The effect of IL-4 on MHC-II^{low} (**a**) and MHC-II^{high} chBMDC (**b**) subsets was assessed by flow cytometry. Supernatant from a COS-7 cell culture transfected with recombinant IL-4 (purple) was used as a source of IL-4, and supernatant from COS-7 cells transfected with an empty vector was used as a control (black). The graphs show the percentages of both chBMDC subsets within total chBMDCs after culturing the cells in the presence of titrated concentrations of IL-4 or empty supernatant control. Both panels show the mean of four independent replicates; the error bars of all panels show the SEM.

LPS stimulation affects the difference in phenotype between MHC-II^{low} and MHC-II^{high} chBMDCs

To determine the effect of LPS, commonly used to induce BMDC maturation, the cells cultured for 7 days were stimulated with 100 ng/ml of LPS for 24 h. MHC-II^{low} and MHC-II^{high} chBMDC subsets were still detected (**Figure 6a**). Both MHC-II^{low} and MHC-II^{high} chBMDCs upregulated CD40, CD1.1, and β2m expression, whereas the subsets downregulated c-kit and MRC1L-B expression (**Figures 6b**,**c**). CD11b/c, CSF1R, and c-kit expression on MHC-II^{low} cells decreased to levels similar to those on the MHC-II^{high} subset, while CD80 expression on MHC-II^{high} cells decreased to a level similar to that on the MHC-II^{low} subset. These expression patterns suggest that the phenotypes of the MHC-II^{low} and MHC-II^{high} subsets partially converged. Convergence in expression levels was also observed for CD40 and β2m, but not for MRC1L-B and CD1.1.

chBMDC subsets differ in maturation status rather than cell type

MHC-II^{low} and MHC-II^{high} chBMDC subsets were sorted by FACS to determine the differential expression of macrophage- and DC-related genes by RT-qPCR, since well-characterized monoclonal antibodies for these cell surface markers in chickens are scarce. Since MHC-II and CSF1R showed good discrimination between the subsets in confocal microscopy (**Figure 3**), these markers were used to separate the subsets by FACS (**Figure 7a**). In addition, chBMDCs were stained for CD80, which was found to be more highly expressed by the MHC-II^{high} CSF1R^{low} subset than the MHC-II^{low} CSF1R^{high} subset (**Figure 7a**), in accordance with previous results (**Figure 2c**). Both subsets were sorted to above 90% purity as determined by flow cytometric reanalysis after each sort (**Figure 7b**). Sorted cells were analyzed by fluorescent microscopy to confirm surface expression patterns of MHC-II, CSF1R, and CD80 (**Figure 7c**). In accordance with the flow cytometry data, MHC-II and CD80 were expressed by the MHC-II^{high} CSF1R^{low} sorted subset, but not by the MHC-II^{high} CSF1R^{low} subset. In contrast, CSF1R was shown to be present on both sorted

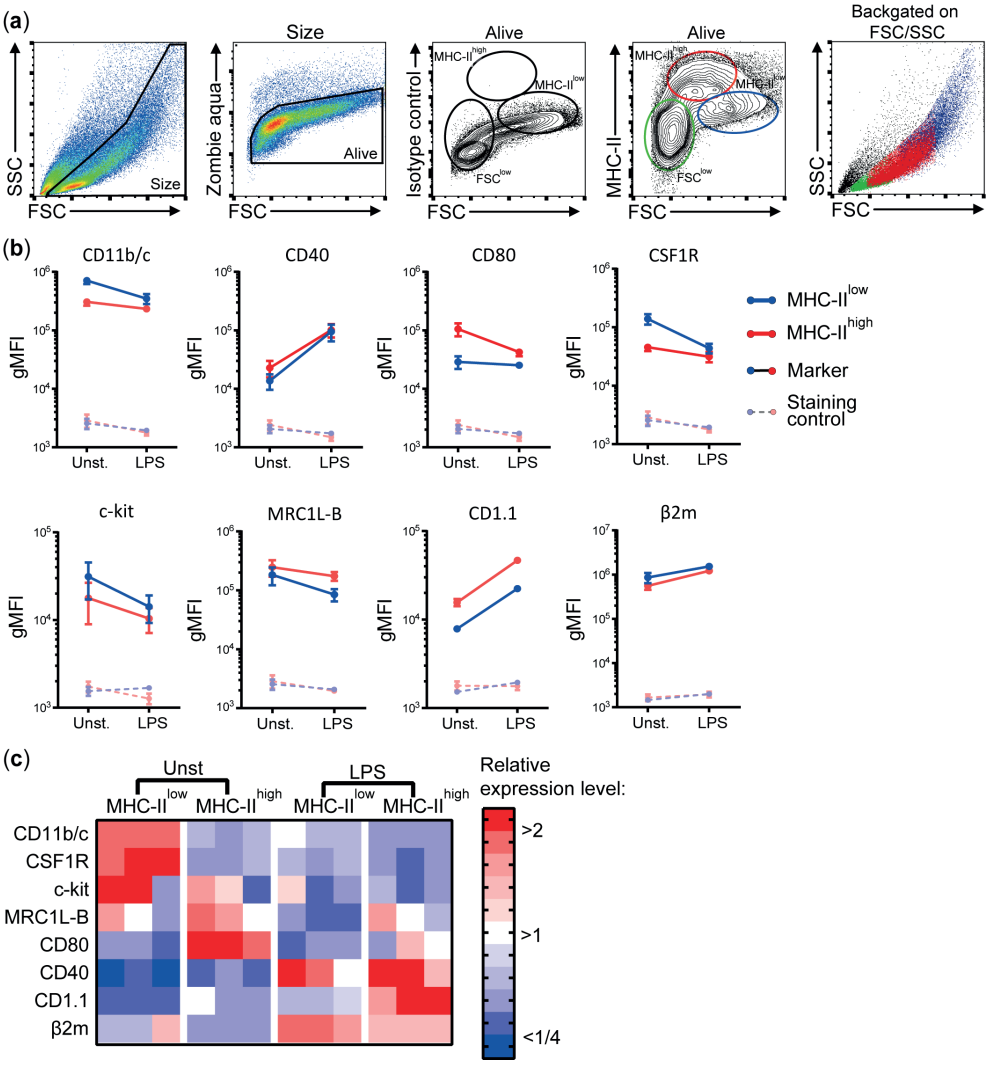

Figure 6. The phenotypic distinction between major histocompatibility complex class II (MHC-II)^{low} and MHC-II^{high} chicken bone marrow-derived dendritic cells (chBMDCs) is not retained after 24 h of lipopolysaccharide (LPS) stimulation. (**a**) LPS-stimulated chBMDCs were gated by a similar strategy as **Figure 2A**. (**b**) The change in myeloid marker expression upon LPS stimulation is shown for both MHC-II^{low} and MHC-II^{high} chBMDC subsets. The error bars show the SEM of three independent replicates. (**c**) The expression of phenotypic markers is shown in a heat-map for unstimulated and LPS-stimulated MHC-II^{low} and MHC-II^{high} chBMDCs. To obtain the relative expression level for each marker, the gMFI of each sample was normalized to the average gMFI of all samples.

subsets. Next, RNA was isolated from the sorted subsets, and RT-qPCR was performed to study the gene expression patterns of macrophage- and DC-enriched genes (**Figure 7d** and **Supplementary Table S2**). Tyrosine-protein kinase Mer (MERTK), toll-like receptor 4 (TLR4), TLR4 coreceptor CD14, and inducible nitric oxide synthase (iNOS) were used as macrophage-enriched genes, whereas zinc finger and BTB domain-containing protein 46 (ZBTB46), C-type lectins DEC205 and DC-SIGN, chemokine receptors C-C chemokine

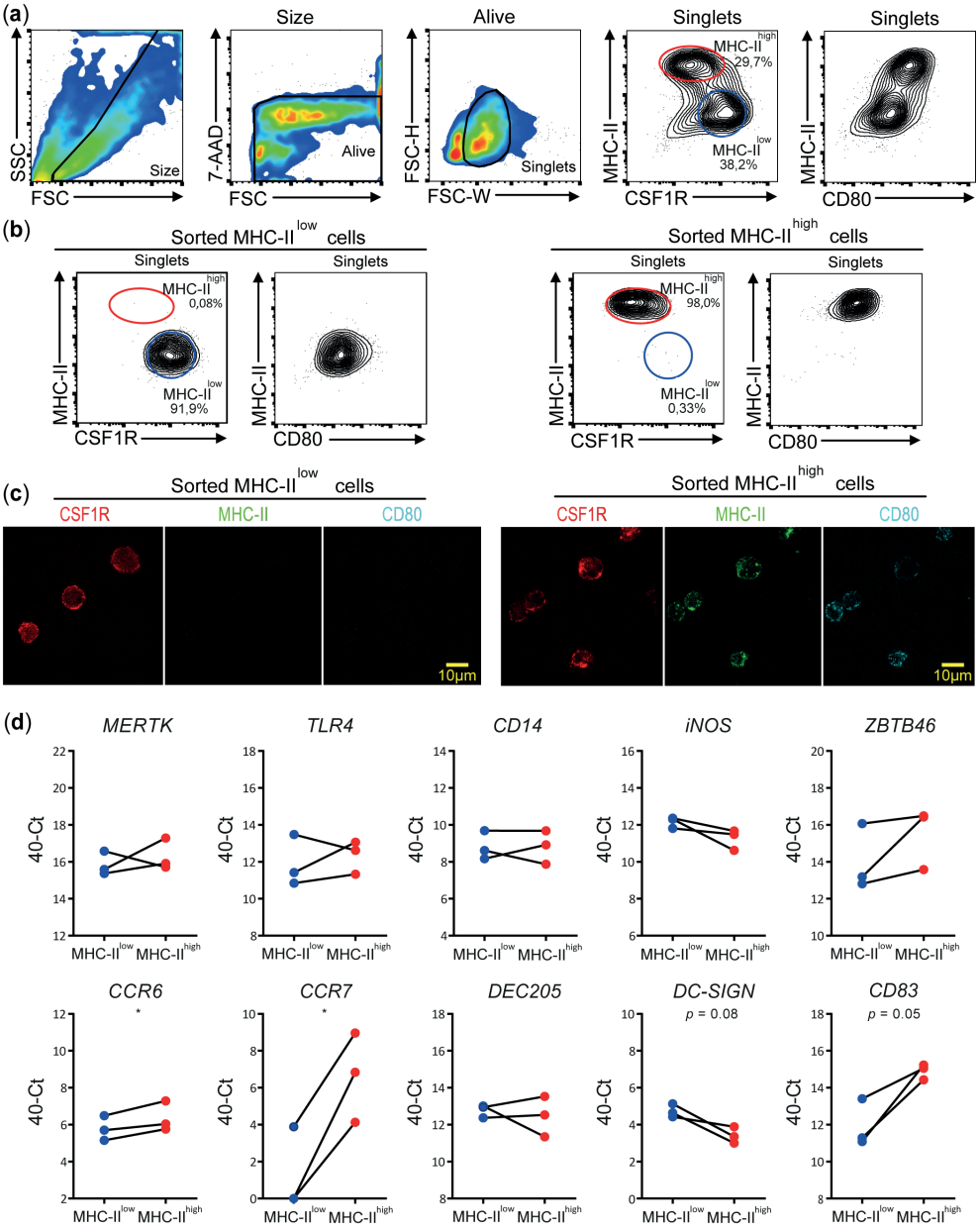

Figure 7. Quantitative real-time PCR (RT-qPCR) on sorted major histocompatibility complex class II (MHC-II)^{low} and MHC-II^{high} chicken bone marrow-derived dendritic cell (chBMDC) subpopulations shows clear differential expression of DC maturation markers, but not of other DC- or macrophage-specific phenotypic markers. (**a**) Pre-sort evaluation of chBMDCs shows the subpopulations that differ in their expression of MHC-II, CSF1R, and CD80. The subpopulations were sorted after gating for scatter profile [forward scatter (FSC)/side scatter (SSC)], viability [7-aminoactinomycin D (7-AAD)], single cells (FSC-W/FSC-H), and expression of MHC-II and colony-stimulating factor 1 receptor (CSF1R). (**b**) Post-sort analysis of sorted MHC-II^{low} and MHC-II^{high} chBMDC subpopulations shows that the sort led to populations of high purity. (**c**) Fluorescent microscopy shows CSF1R, MHC-II, and

CD80 expressions for sorted MHC-II^{low} and MHC-II^{high} chBMDCs. Overlays and individual channels are shown with CSF1R in red, MHC-II in green, and CD80 in cyan. (**d**) RT-qPCR was performed on sorted MHC-II^{low} and MHC-II^{high} chBMDCs. The results are expressed at the 40-Ct value with Ct being the number of cycles needed to reach a signal above the threshold. The results were normalized for RNA content by the average expression of housekeeping genes 28S and GAPDH. Each line represents an independent replicate (n = 3). Statistically significant differences between chBMDC subsets are shown/indicated by *p < 0.05. Non-significant *p*-values below 0.10 are given by the actual values to indicate trends close to significance.

receptor type 6 (CCR6) and 7 (CCR7), and costimulatory receptor CD83 were used as DC-enriched genes. Both subsets equally expressed MERTK (d = 0.46; p = 0.60), TLR4 (d = 0.42; p = 0.62), CD14 (d = 0; p > 0.99), and DEC205 (d = 0.31; p = 0.70). MHC-II^{low} cells showed slightly higher expressions of iNOS (d = 0.90; p = 0.16) and DC-SIGN (d = 1.32; p = 0.08), whereas MHC-II^{high} cells showed slightly higher expressions of ZBTB46 (d = 1.48; p = 0.24) and CCR6 (d = 0.58; p = 0.05). More strikingly, MHC-II^{high} cells showed much higher expressions of CCR7 (d = 5.35; p = 0.02) and CD83 (d = 2.98; p = 0.05). Thus, the differences between MHC-II^{low} cells and MHC-II^{high} cells were mainly found for DC-enriched genes, especially for CCR7 and CD83, which have been used by others before as maturation markers of chBMDCs [14, 30]. In contrast, differences in gene expression were hardly found for macrophage-enriched genes. Combined with the flow cytometric data (**Figure 2c**) that showed higher expressions of MHC-II, CD40, and CD80 by MHC-II^{high} chBMDCs compared to the MHC-II^{low} chBMDCs, the subsets seem to be DCs at different maturation states rather than different cell types.

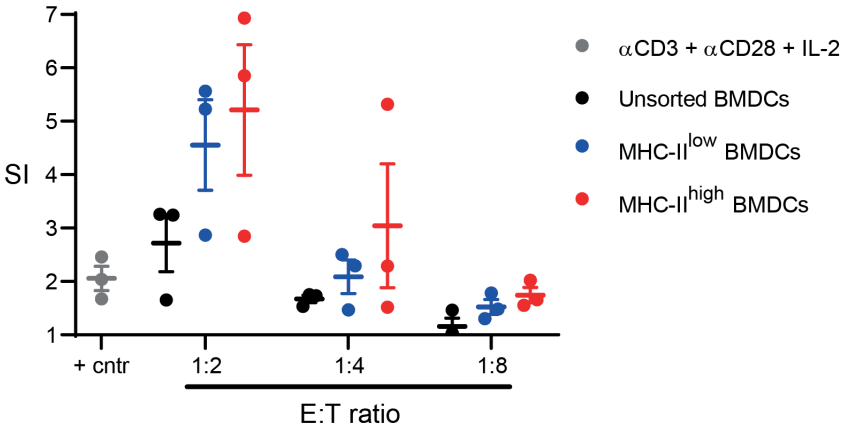

Figure 8. Sorted major histocompatibility complex class II (MHC-II)^{low} and MHC-II^{high} chBMDC subsets induce peripheral blood mononuclear cell (PBMC) proliferation in a mixed lymphocyte reaction. The MHC-II^{low} and MHC-II^{high} chicken bone marrow-derived dendritic cell (chBMDC) subsets were sorted using the gating strategy shown in **Figure 7a**. Unsorted and sorted chBMDC effector cells were cocultured with PBMCs from laying hens (n = 3) at different E:T ratios. In addition, PBMCs were stimulated with a combination of anti-CD3, anti-CD28, and IL-2 as a positive control. After 3 days of culture, proliferation was measured by ^{3}H-thymidine incorporation over a period of 18 h and expressed as a stimulation index (SI), which shows the ratio between stimulated and unstimulated PBMCs. All cultures were performed as three replicates.

To determine whether chBMDCs were able to induce T-cell proliferation, allogeneic mixed lymphocyte reactions were performed with sorted chBMDC subsets. Both MHC-II^{low} (SI = 4.6 at 1:2 E:T ratio) and MHC-II^{high} (SI = 5.2 at 1:2 E:T ratio) chBMDC subsets induced proliferation of PBMCs and did so more effectively than unsorted chBMDCs (SI = 2.7) or PBMCs stimulated with a combination of anti-CD3, anti-CD28, and IL-2 (SI = 2.0) (**Figure 8**).

MHC-II^{high} chBMDCs become MHC-II^{low} during prolonged incubation after sorting

MHC-II^{low} and MHC-II^{high} chBMDCs were found to differ in the expression of DC maturation markers, at both the protein and gene expression levels, but were similar in their ability to induce PBMCs to proliferate. To gain additional proof that MHC-II^{low} and MHC-II^{high} chBMDCs represent DCs at different maturation states rather than different cell types, sorted chBMDCs were reseeded for another 1 or 3 days of cell culture and stained again for MHC-II, CSF1R, and CD80. The MHC-II^{low} subset showed only minor changes in the expression of abovementioned markers (**Figures 9a**,**b**). In contrast, the MHC-II^{high} subset showed higher CSF1R, lower MHC-II, and lower CD80 expression and consequently became phenotypically more similar to the MHC-II^{low} subset. Therefore, both chBMDC subsets seem to consist of a DC-like cell type, but these appear to be different states of maturation with MHC-II^{high} chBMDCs being at a more mature but reversible state. In addition, the mRNA expression levels of CCR7 and CD83 were determined and found to be decreased for both subsets after prolonged cell culture (**Figure 9c**).

Discussion

The present study aimed to characterize GM-CSF-induced chBMDC cultures and address their heterogeneity. Despite the widespread use of *in vitro* grown DCs, there is still an ongoing debate about the representativeness of *in vitro* grown DCs for their *in vivo* counterparts [20–22, 24, 25, 31]. In research so far, discussion focused mainly on BMDCs from mice and less on those of farm and companion animals. Early studies already described the presence of macrophage and granulocyte "contaminants" in the murine BMDC culture [5, 32]. Moreover, murine [20, 33] and ovine [12] BMDC cultures, as well as bovine monocyte-derived DC cultures [34], have been shown to include CD11b^{low} MHC-II^{high} cells with a DC-like phenotype and CD11b^{high} MHC-II^{low} cells with a macrophage-like phenotype. The first study by Wu et al. describing the chBMDC culture already recognized its heterogeneity [14]. However, this study excluded adherent and relatively small cells from analysis, which may, respectively, represent the MHC-II^{low} chBMDCs and FSC^{low} undifferentiated monocytes of the current study. In our opinion, these neglected cells should be characterized to interpret responses of the chBMDC culture correctly when analyzed in bulk. This has been illustrated by studies using LPS-stimulated murine BMDCs, in which individual cell subsets [20] or individual cells [35] were shown to respond very differently in their maturation, cytokine expression profile, and capacity to induce T-cell proliferation. Moreover, virus infection studies with chBMDCs, including avian influenza virus [16, 26] and infectious bursal disease virus [36] were analyzed in bulk, while the different chBMDC subsets might vary in their susceptibility for viruses and influence the outcome of these studies.

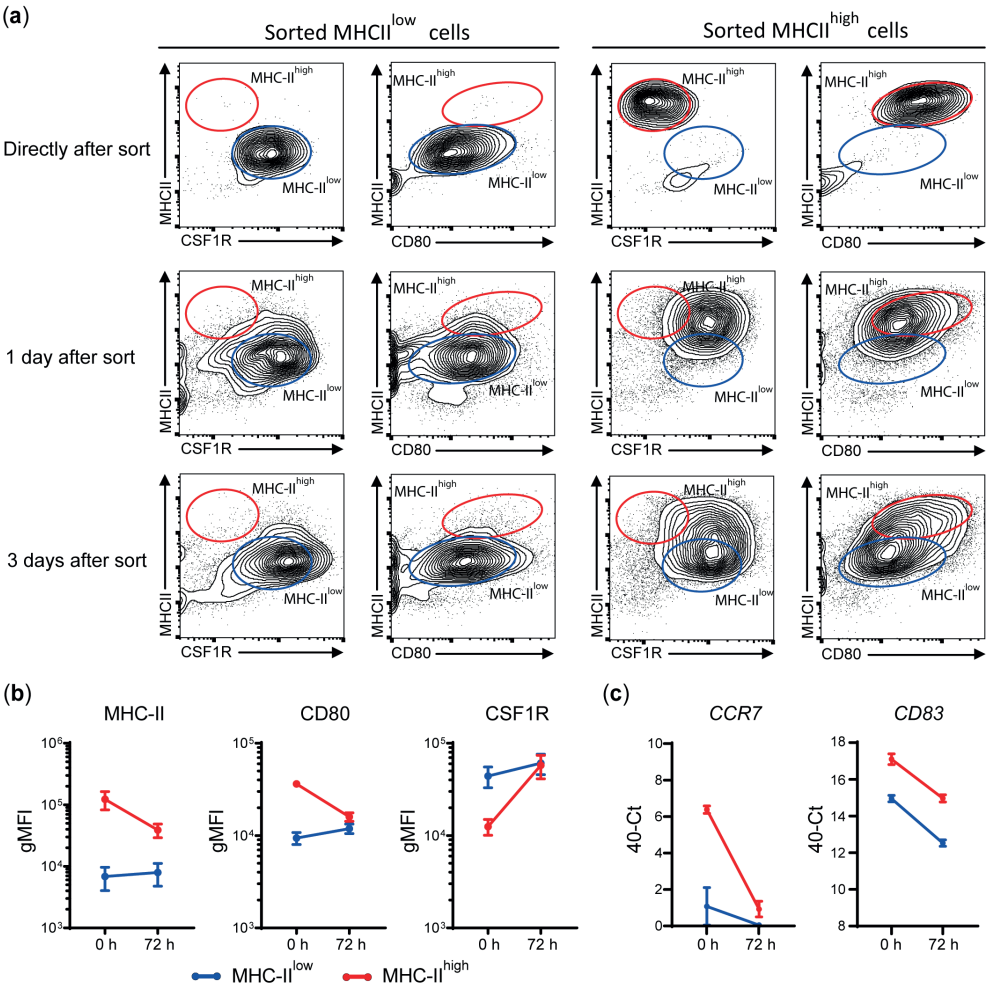

Figure 9. Sorted major histocompatibility complex class II (MHC-II)^{low} chicken bone marrow-derived dendritic cells (chBMDCs) maintain their phenotype, while sorted MHC-II^{high} chBMDCs become MHC-II^{low}. (**a**) The MHC-II^{low} and MHC-II^{high} chBMDC subsets were sorted using the gating strategy shown in **Figure 7A**. The cells were either reanalyzed directly after sorting or seeded for another 1 or 3 days of prolonged cell culture in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). After these periods, the cells were stained again using the same protocol that was used before the sort. The contour plots that are shown are representative for three independent technical replicates. (**b**) Surface expression levels of MHC-II, CD80, and colony-stimulating factor 1 receptor (CSF1R) are shown for MHC-II^{low} and MHC-II^{high} chBMDCs directly after sorting (0 h) and after the 3 days of prolonged cell culture (72 h) (n = 3). (C) Similarly, messenger RNA (mRNA) expression levels of CD83 and CCR7 are shown for both subsets at 0 and 72 h (one experiment, performed in triplicate).

In the present study, the chBMDC culture was found to contain MHC-II^{low} and MHC-II^{high} subsets, similar to BMDC and monocyte-derived DC cultures in other species [12, 20, 33, 34]. Expression of myeloid markers, morphology, and phagocytosis capacity differed between MHC-II^{low} and MHC-II^{high} subsets. Compared to the MHC-II^{high} subset, the MHC-II^{low} subset showed higher expression of CSF1R, contained larger and more granular cells based on flow cytometry scatter profile and light microscopy, and showed a higher capacity to phagocytose IgY-opsonized beads. These results suggested that the MHC-II^{low} chBMDC subset consists of macrophage-like cells, in agreement with studies in murine [20, 33] and ovine [12] BMDCs, as well as bovine monocyte-derived DCs [34]. In these studies, macrophage-like MHC-II^{low} cells showed high expression of CD11b, which contributed to the distinction between the MHC-II^{low} and MHC-II^{high} subsets of these BMDC cultures. Unfortunately, this was not possible for the chicken CD11b/c antigen, since the corresponding antibody has not yet been confirmed to recognize either CD11b or CD11c [26]. Compared to the MHC-II^{low} chBMDC subset, the MHC-II^{high} subset showed higher expression of costimulatory molecules CD40 and CD80, in agreement with the DC-like phenotype shown for MHC-II^{high} cells in murine BMDC [20, 33] and bovine monocyte-derived DC cultures [34]. In addition, the MHC-II^{high} chBMDC subset showed a relatively high expression of the non-classical MHC molecule CD1.1, which is also indicative of a DC-like phenotype [37]. Our phenotypical findings of the chBMDC subsets suggested that MHC-II^{low} and MHC-II^{high} subsets, respectively, represent macrophage- and DC-like cells, in agreement with studies that used murine, ovine, and bovine *in vitro* DC cultures [12, 20, 33, 34]. However, there is some discrepancy in the literature about murine BMDCs, since its MHC-II^{low} and MHC-II^{high} subsets have also been suggested to, respectively, represent an immature and mature phenotype of the same cell type [3, 38].

To further explore this alternative hypothesis, we investigated whether the chBMDC subsets were different cell types or DCs at different maturation states; MHC-II^{low} and MHC-II^{high} subsets were sorted to perform RT-qPCR for macrophage- and DC-specific markers. Both subsets showed similar expression of the macrophage-specific markers MERTK, TLR4, and CD14, which is in contrast to studies of murine BMDCs [20, 33, 39]. Moreover, no difference in expression of the DC-specific marker DEC205 was observed, while ZBTB46 and CCR6 were only moderately more highly expressed by the MHC-II^{high} subset. The most striking differences between the chBMDC subsets were higher CD83 and CCR7 expressions and lower DC-SIGN expression for the MHC-II^{high} subset. Increased CD83 and CCR7 expression and decreased DC-SIGN expression have been reported as maturation signatures of human and murine monocyte-derived DCs [20, 40, 41], suggesting that the MHC-II^{high} chBMDC subset represents a mature DC phenotype. Moreover, it has been shown that CCR7 expression is upregulated by chBMDCs shortly after LPS stimulation [36]. Taken together, these results suggest that, rather than being different cell types, MHC-II^{low} and MHC-II^{high} chBMDC subsets are DCs at different maturation states with MHC-II^{high} chBMDC being more mature. Nonetheless, MHC-II^{low} and MHC-II^{high} chBMDCs induced similar levels of PBMC proliferation in an allogeneic mixed lymphocyte reaction. Therefore, we hypothesize that of MHC-II^{high} chBMDCs are in a semi-mature state, which is described in literature as the state at which DCs express high levels of MHC-II and costimulatory molecules but do not produce elevated levels of pro-inflammatory cytokines or optimally stimulate T-cell proliferation [42, 43]. Reseeding sorted chBMDCs showed that the immature phenotype of the MHC-II^{low} subset remained stable over time, whereas

the semi-mature MHC-II^{high} subset decreased MHC-II and CD80 expressions and increased CSF1R expression, indicating the plasticity and reversibility of this semi-mature phenotype. The chBMDC subsets differed in phagocytosis capacity, with the MHC-II^{low} subset being more efficient in bead uptake. This finding can also be explained by a different maturation status of the subsets, since mature DCs generally have a lower phagocytotic capacity [44, 45]. Others observed that LPS-induced maturation diminished the phagocytosis capacity of chBMDCs [14], which was not the case for the semi-mature MHC-II^{high} chBMDC subset of the present study.

The semi-mature phenotype of the MHC-II^{high} chBMDCs must have been induced by the culture conditions that were used, since the cells were not intentionally stimulated. Since it is common practice to include IL-4 cytokine in BMDC differentiation protocols of different species, including chBMDCs [10–12, 14, 46, 47], the effect of IL-4 on the development of chBMDC subsets was investigated. When IL-4 alone was added to the culture, we observed that few chBMDC aggregates appeared, in agreement with previous studies [17]. Surprisingly, addition of IL-4 led to a lower proportion of MHC-II^{high} cells, inhibiting chBMDC maturation, though the number of observed chBMDC aggregates remained unaffected. To the best of our knowledge, there are no earlier reports that show an inhibitory effect of IL-4 on maturation of the chBMDC culture in terms of MHC-II expression. Previous studies have only observed the occurrence of chBMDC aggregates when investigating the effects of IL-4 [14, 17]. Studies with murine and rat BMDCs have reported that IL-4 supplementation leads to proportionally larger MHC-II^{high} subsets [20, 47], in contrast to our findings for the chBMDC culture. Another study has shown that IL-4 has no effect on ovine BMDC yield or phenotype [12]. Therefore, the effect of IL-4 on BMDC cultures seems to differ between species. Another parameter affecting BMDCs was the source of the serum used in the culture. FBS was found to lead to a large MHC-II^{high} CD80^{+} chBMDC population (**Supplementary Figure S5**), whereas chicken serum led to the immature MHC-II^{low} and semi-mature MHC-II^{high} chBMDCs of the present study.

BMDCs are often stimulated by LPS to induce maturation. In the chBMDC culture, LPS stimulation led to a striking increase in CD40 and CD1.1 expression by both chBMDC subsets, whereas expression of maturation marker CD80 by the MHC-II^{high} subset was unexpectedly decreased. Of note, LPS stimulation led to smaller differences between the subsets in their expression of MHC-II, CD80, and CSF1R. Overall, MHC-II^{low} and MHC-II^{high} chBMDCs responded similarly to the LPS stimulus, which favored the hypothesis that the subsets reflected one cell type at different states. In contrast, murine MHC-II^{low} and MHC-II^{high} BMDC subsets were shown to maintain differential gene expression profiles after LPS stimulation, which provided additional proof that the murine subsets were truly different cell types [20].

In conclusion, this study describes the heterogeneity of the GM-CSF-differentiated chBMDC culture, which comprised MHC-II^{low} and MHC-II^{high} subsets that both possess features of antigen-presenting cells. These populations were found to differ in phenotype, morphology, and their phagocytosis capacity, whereas their ability to induce PBMC proliferation was similar. Based on higher expressions of maturation markers MHC-II, CD40, CD80, CD83, and CCR7 by MHC-II^{high} chBMDCs compared to MHC-II^{low} chBMDCs, the MHC-II^{low} and MHC-II^{high} subsets were found to, respectively, represent immature and semi-

mature chBMDCs. The semi-mature phenotype of the MHC-II^{high} subset was found to be reversible, since reseeding and prolonged culture of these cells led to a transition toward the immature phenotype of the MHC-II^{low} cells. Taken together, these results yield a thorough characterization of the chBMDC culture and explain its heterogeneity by the simultaneous presence of immature and mature subsets. Our findings are instrumental for the interpretation of experiments that use this culture in future research.

Acknowledgments

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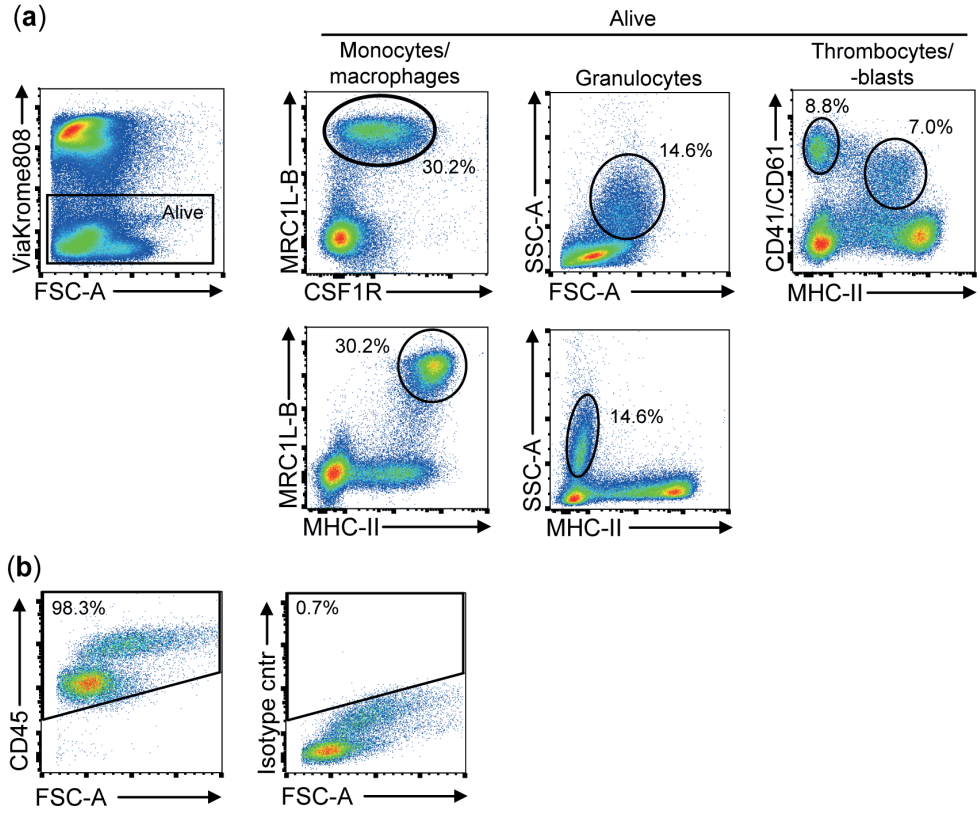
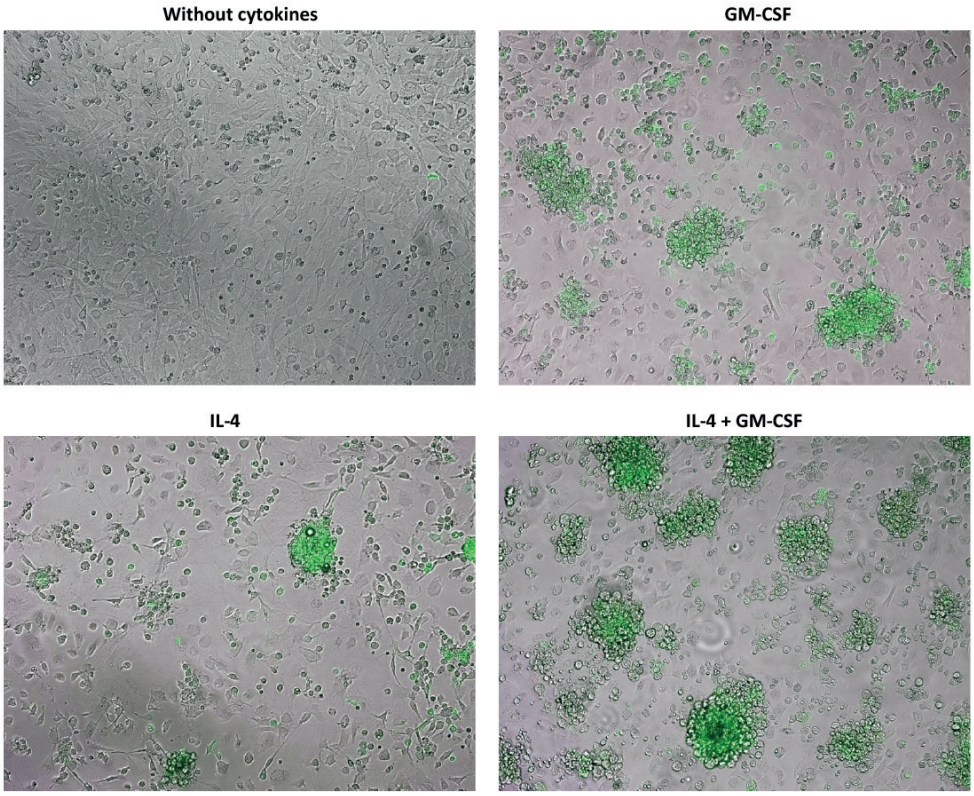
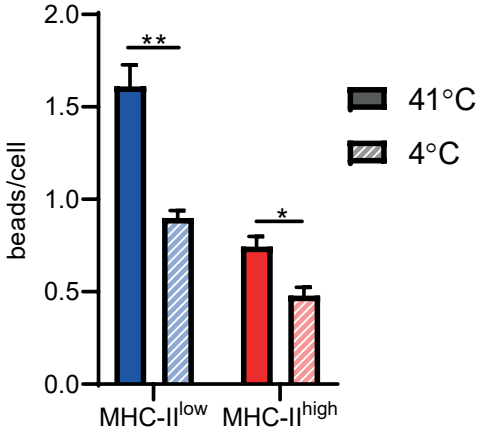
Supplementary Materials



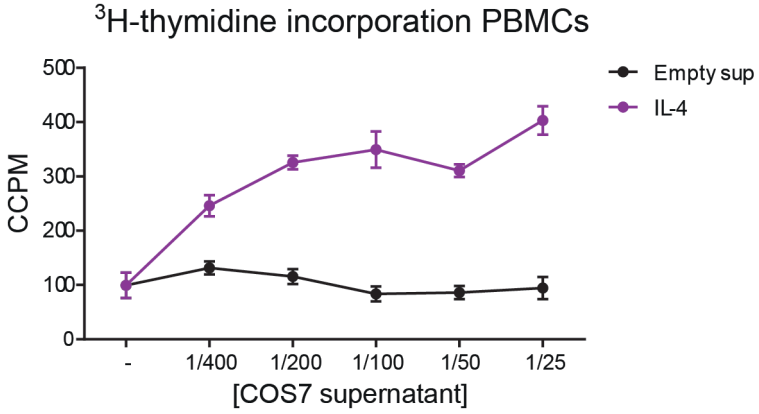
Supplementary Figure S1. Characterization of cells in an embryonic bone marrow isolate. (**a**) Embryonic bone marrow was characterized by flowcytometry to determine the presence of different subsets of myeloid cells. Cells from the monocyte/macrophage lineage were defined as MRC1L-B^{+} CSF1R^{+} MHC-II^{+}, granulocytes as SSC^{high}, thrombocytes as CD41/CD61^{+} MHC-II-, and thromboblasts as CD41/CD61^{+} MHC-II^{+}. (**b**) After chBMDCs were cultured for 7 days in the presence of GM-CSF the proportion of hematopoietic cells was determined by CD45 staining.



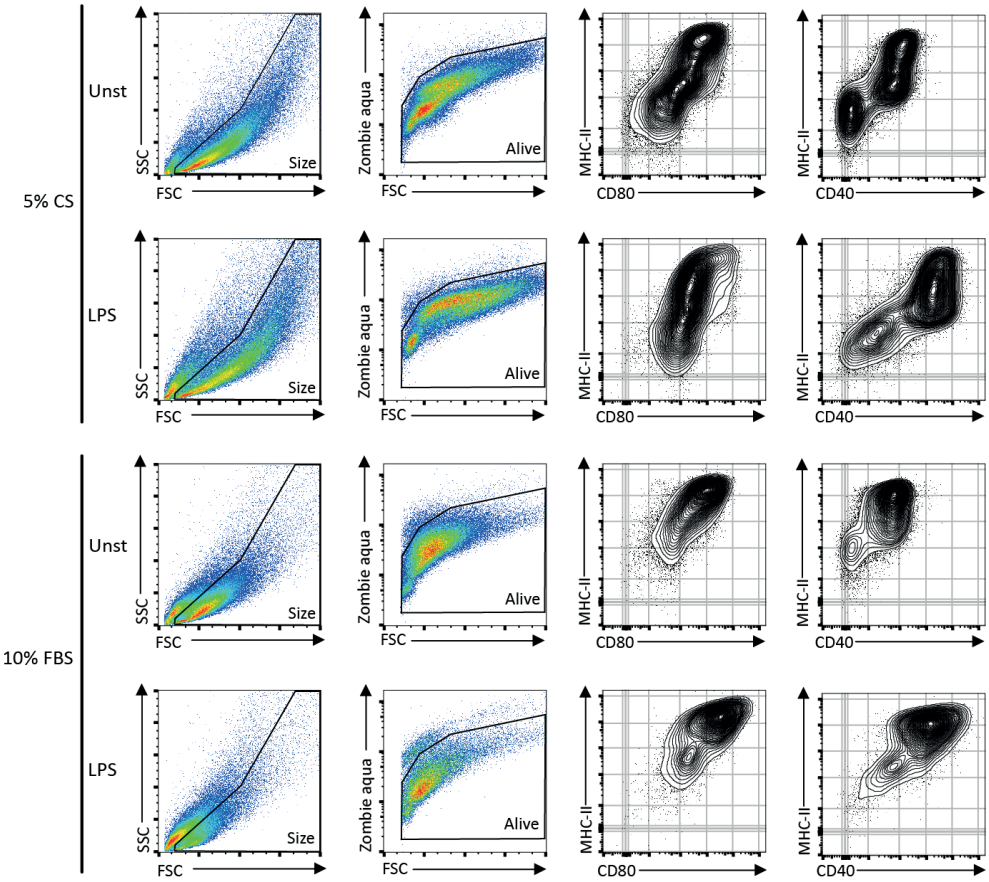
Supplementary Figure S2. The effects of GM-CSF and IL-4 on the morphology and MHC-II expression of the chBMDC culture. The chBMDC culture was performed in the absence of cytokines and in the presence of GM-CSF alone, IL-4 alone, or the combination of GM-CSF and IL-4. COS-7 cell culture supernatant containing the cytokines was added at 2 µl/ml for GM-CSF and at 5 µl/ml for IL-4. The chBMDC culture conditions were one or both cytokines were absent received an equivalent volume of supernatant from a COS-7 cell culture transfected with an empty pCI-neo vector. At the end of the culture (day 8), an AlexaFluor488-conjugated mouse anti-chicken MHC-II monoclonal antibody was added to the cultures at 2 µg/ml and incubated for 30 min at 4°C. Next, the cells were washed twice with DPBS with calcium and magnesium and analyzed using an EVOS FL microscope.



Supplementary Figure S3. Increased phagocytosis of IgY-opsonized beads by chBMDCs upon incubation at 41°C compared to 4°C. IgY-opsonized beads were added to chBMDCs, followed by an incubation period of 4 h at either 41 °C or 4 °C. Next, bead content of MHC-II^{low} and MHC-II^{high} chBMDC subsets was quantified by flow cytometry. The error bars show the SEM of an experiment performed in triplo. Unpaired t-tests were performed to test for statistically significant differences between the experiments performed at 41 °C or 4 °C for both chBMDC subsets. A statistically significant difference between the experiments is shown by * for $p < 0.05$ and ** for $p < 0.01$.



Supplementary Figure S4. IL-4 induced PBMC proliferation. PBMC proliferation was assessed by ^{3}H-thymidine incorporation over a period of 18 h after 4 days of stimulation with COS-7 supernatant containing recombinant chicken IL-4 (purple) or empty COS-7 supernatant as a control (black) (n=4).



Supplementary Figure S5. The phenotypical outcome of chBMDCs is affected by serum supplements in the cell culture medium. 10% FBS supplemented cell culture medium led to a more homogeneous chBMDC population with higher expression of MHC-II and CD80 compared to chBMDCs grown in cell culture medium supplemented with 5% chicken serum. The FSC^{low} and MHC-II^{low} subsets from the chBMDC culture were almost absent in the adapted protocol that includes FBS supplementation. Moreover, LPS stimulation of chBMDCs in the presence of 10% FBS led to an increase in both CD40 and CD80 expression, whereas only CD40 expression was found to be increased after stimulation of chBMDCs that were cultured according to the standard protocol that includes 5% chicken serum.

Supplementary Table S1. The quantification of flow cytometry data. The average geometric mean fluorescent intensity (gMFI) of three independent replicates is shown for each marker for the MHC-II^{low} and MHC-II^{high} chBMDC subsets. In addition, the ratio between the gMFIs of both subsets are shown for each marker.

Marker	gMFI values per subset		Ratio MHCII^{low}/MHCII^{high}
	MHCII^{low}	MHC^{high}	
CD11b/c	$7.1x10^5$	$3.1x10^5$	2.31
CD40	$1.4x10^4$	$2.3x10^4$	0.60
CD80	$2.9x10^4$	$1.1x10^5$	0.27
CSF1R	$1.4x10^5$	$4.5x10^4$	3.05
c-Kit	$3.1x10^4$	$1.8x10^4$	1.76
MRC1L-B	$1.8x10^5$	$2.4x10^5$	0.74
CD1.1	$7.8x10^3$	$1.6x10^4$	0.50
β2m	$8.6x10^5$	$5.6x10^5$	1.55

Supplementary Table S2. The quantification of RT-qPCR data. The average 40-Ct-value of three independent replicates is shown for each marker for the MHC-II^{low} and MHC-II^{high} chBMDC subsets. In addition, the difference between the gMFIs of both subsets are shown for each marker.

Marker	40-Ct values per subset		Δ MHCII^{low} - MHCII^{high}
	MHCII^{low}	MHC^{high}	
MerTK	15.85	16.30	-0.46
CD14	8.82	8.82	0.00
TLR4	11.92	12.34	-0.42
iNOS	12.16	11.26	0.90
Zbtb46	14.02	15.50	-1.48
CCR6	5.78	6.36	-0.58
DEC205	12.78	12.47	0.31
DC-SIGN	4.73	3.41	1.32
CCR7	1.30	6.64	-5.35
CD83	11.93	14.90	-2.98

Supplementary Video S1. This shows a 3D constructed model created by confocal microscopy from chBMDCs that phagocytosed fluorescent beads (in red) for 4 h. The cells from the chBMDC culture were stained for MHC-II (in blue) and the plasma membrane by using WGA (in green). This movie is available online at https://doi.org/10.3389/fimmu.2020.00141.

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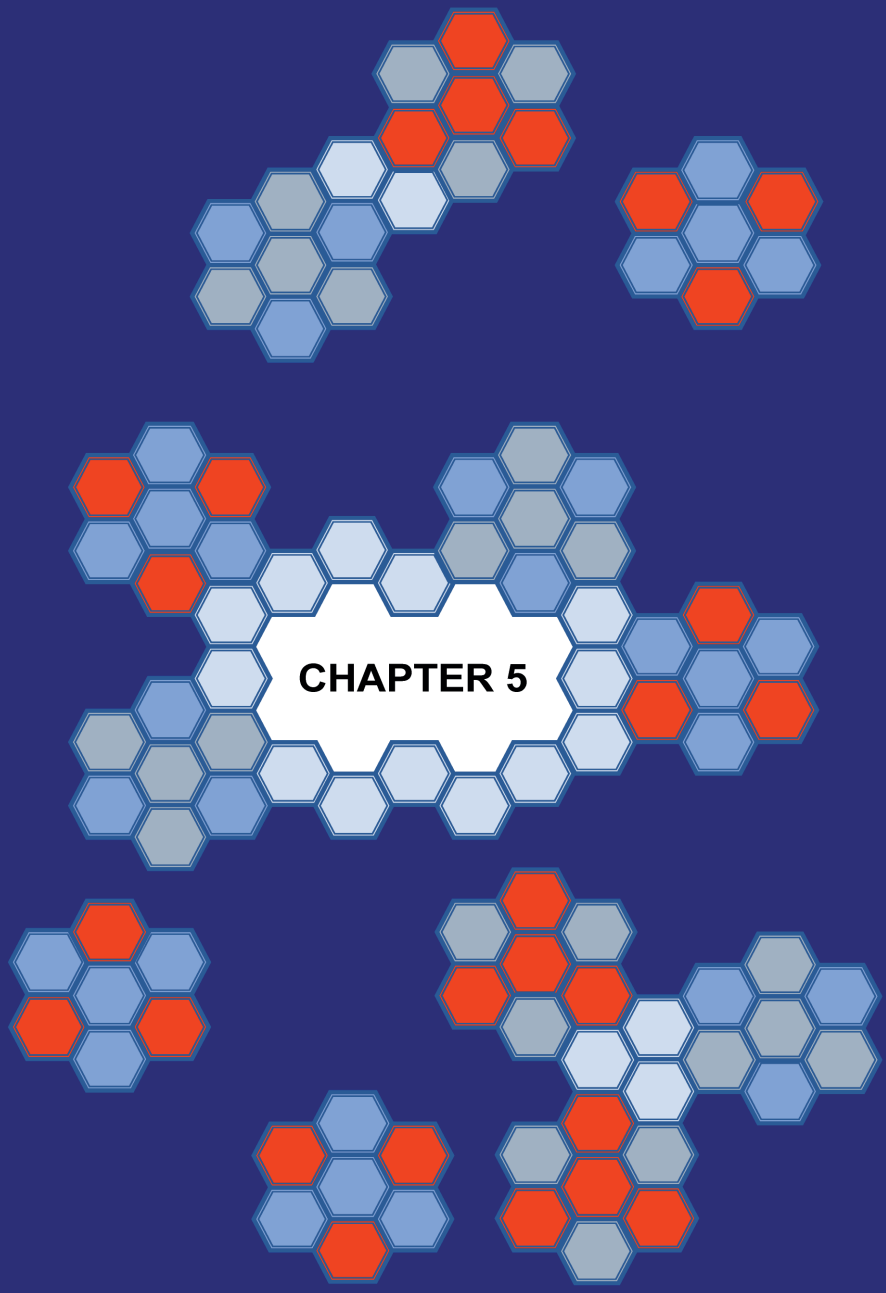
CHAPTER 5

Proteomic analysis of chicken bone marrow-derived dendritic cells in response to an inactivated IBV+NDV poultry vaccine

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Abstract

Inactivated poultry vaccines are subject to routine potency testing for batch release, requiring large numbers of animals. The replacement of *in vivo* tests for cell-based alternatives can be facilitated by the identification of biomarkers for vaccine-induced immune responses. In this study, chicken bone marrow-derived dendritic cells were stimulated with an inactivated vaccine for infectious bronchitis virus and Newcastle disease virus, as well as inactivated infectious bronchitis virus only, and lipopolysaccharides as controls. Next, the cells were lysed and subjected to proteomic analysis. Stimulation with the vaccine resulted in 66 differentially expressed proteins associated with mRNA translation, immune responses, lipid metabolism and the proteasome. For the eight most significantly upregulated proteins, mRNA expression levels were assessed. Markers that showed increased expression at both mRNA and protein levels included PLIN2 and PSMB1. Stimulation with infectious bronchitis virus only resulted in 25 differentially expressed proteins, which were mostly proteins containing Src homology 2 domains. Stimulation with lipopolysaccharides resulted in 118 differentially expressed proteins associated with dendritic cell maturation and antimicrobial activity. This study provides leads to a better understanding of the activation of dendritic cells by an inactivated poultry vaccine, and identified PLIN2 and PSMB1 as potential biomarkers for cell-based potency testing.

Introduction

The safety and potency of poultry vaccines has traditionally been assessed through *in vivo* vaccination and challenge tests [1]. However, there is a global intent to replace *in vivo* vaccine tests for *in vitro* alternatives [2]. This has already led to the development of an enzyme-linked immunosorbent assay (ELISA) as an *in vitro* antigen quantification method to assess inactivated vaccines for Newcastle disease virus for potency [3]. However, other inactivated poultry vaccines still require *in vivo* tests to prove their potency and are thus in need for *in vitro* alternatives. The development of *in vitro* tests for inactivated poultry vaccines can be facilitated by the identification of biomarkers that may be inherent to the vaccine, demonstrating a consistent product profile of different vaccine batches [4–6], or be part of the vaccine-induced immune response that correlates with protection [7–10]. In particular, dendritic cells are ideal study targets because of their central role in the initiation of immune responsiveness [11]. One approach to discover biomarkers of immune responsiveness to inactivated poultry vaccines is the investigation of *in vitro* vaccine-stimulated immune cells by proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Both infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) cause respiratory tract infections that can disseminate to other tissues, leading to reduced egg production and mortality in chickens [12,13]. In this study, primary chicken bone marrow-derived dendritic cells (chBMDCs), which have been characterized in previous studies [14,15], were stimulated with a commercially available inactivated poultry vaccine against IBV and NDV in a mineral oil adjuvant in a water-in-oil formulation, and analyzed by LC-MS/MS to evaluate changes in the proteome In addition, chBDMCs were stimulated with inactivated IBV antigens only to discriminate between the effects of a single inactivated antigen and a vaccine. Stimulation with *E. coli* lipopolysaccharide (LPS) was included in this study as a strong stimulator of innate immune cells to validate the methodology used. Previous studies have shown that LPS stimulates maturation of chicken DCs, including increased surface expression of MHC class II molecules and costimulatory molecules [14–19], decreased endocytosis and phagocytosis [14,15], increased expression of proinflammatory cytokines and cytokines that promote the differentiation of naïve helper T cells [14, 17,19], and an increased capacity to stimulate naïve T cells [14,15,18]. The primary aim of this study was to search for biomarkers of vaccine-stimulated dendritic cells to use as targets for *in vitro* cell-based quality assessment of an inactivated IBV + NDV poultry vaccine. Secondly, this study may generate new hypotheses about the mechanisms by which an inactivated viral poultry vaccine activates chicken DCs, as well as new insights in the cellular processes involved in chBMDCs maturation after stimulation with LPS.

Materials and Methods

Generation and stimulation of chBMDCs

Bone marrow was isolated from eighteen-day-old embryonated NOVOgen Brown eggs (Verbeek Broederij, Zeewolde, the Netherlands) as described previously [15]. Experiments with embryonated chicken eggs are not considered animal experiments according to European legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). Briefly, the bone marrow cells were obtained from the tibiae and femurs

of 200 chicken embryos, which were pooled and resuspended with 2.5-5 x 10^7 cells/ml in Roswell Park Memorial Institute (RPMI)-1640 cell culture medium supplemented with GlutaMAX™-I, phenol red, HEPES (RPMI-1640; Gibco™, Life Technologies Limited, Paisley, UK), 50% chicken serum (Gibco™) and 10% dimethyl sulfoxide (Honeywell, Bucharest, Romania). The cells were viably frozen with 2.5–5 x 10^7 cells per cryotube and stored at -140 °C until further use.

Frozen bone marrow cells were thawed and cultured in Costar® 24-well plates (Corning®, Corning B.V. Life Sciences, Amsterdam, the Netherlands) in 1 ml/well RPMI-1640 cell culture medium supplemented with 5% chicken serum and 50 U/ml of penicillin–streptomycin (all from Gibco™) in the presence of 2 µl/ml recombinant GM-CSF at 41 °C, 5% CO_2 [15]. Recombinant GM-CSF was produced using COS-7 cells transfected with pCI-neo (Promega Corporation, Madison, WI, USA) expressing the relevant cytokine gene, a kind gift from P. Kaiser and L. Rothwell (Roslin Institute, Edinburgh, UK). GM-CSF was used at the concentration (2 µl/ml) that, after titration, resulted in the highest percentage of MHC-II$^+$ CD40$^+$ CD80$^+$ cells, as determined using flow cytometry analysis [15]. In the morning of day 3, culture medium with non-adherent cells was removed, and 1 ml of fresh RPMI-1640 medium with GM-CSF was added. Approximately 32 hours later (±2 hours), on day 4, an additional 1 ml RPMI-1640 medium with GM-CSF was added to the cultures. At day 7, chBMDCs were stimulated with 30 µl/ml IBV + NDV vaccine, 10 µl/ml IBV antigens (both were kind gifts from Boehringer Ingelheim, Ingelheim am Rhein, Germany), or 100 ng/ml of LPS from *E. coli* O127:B8 (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h or left untreated. To create sample volumes with sufficient protein or RNA content, six wells of a 24-well plate were used for each condition. After 24 h, the cell culture supernatant with floating cells was first collected. Subsequently, the cultures were washed with Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS; Lonza, Basel, Switzerland) and the non-adherent cells were collected. The remaining adherent cells were incubated in PBS supplemented with 5 mM UltraPure EDTA (Invitrogen™, Life Technologies Europe BV, Bleiswijk, the Netherlands) for 10 min at room temperature and harvested. All cell-containing fluids per culture condition (cell culture medium, PBS and PBS 5 mM EDTA) obtained during the harvest procedure were pooled for subsequent experiments. The samples were spun at 400 x *g* for 5 min and the cells were resuspended in 100 mM phosphate buffer (Sigma-Aldrich) and stored at -80 °C for subsequent LC-MS/MS analysis or lysed in RLT buffer (Qiagen GmbH, Hilden, Deutschland) and stored at -20°C for RNA isolation. The preparation of chBMDC samples, for both the LC-MS/MS and gene expression analysis, was performed three times independently.

Protein isolation, digestion and labelling

Frozen samples were thawed, and the cells were lysed in 100 mM phosphate buffer with 0.1% (w/v) RapiGest SF Surfactant (Waters Corporation, Milford, MA, USA) by vortexing and incubation at 80 °C for 30 min. Next, the protein content was determined by the Pierce BCA assay (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The proteins were digested with 0.05 mg/ml Lys-C (Roche, Merck, Kenilworth, NJ, USA) at an enzyme:substrate ratio of 1:400 for 4 h at 37 °C followed by digestion with 0.2 mg/ml trypsin (Promega) overnight at 37 °C at an enzyme:substrate ratio of 1:100. Next, aliquots from the digests were labelled with native formaldehyde (CH_2O with M = 30.03 g/mol, hereinafter referred to as "light") (Sigma-Aldrich) together with sodium

cyanoborohydride ($NaCNBH_3$) (Sigma-Aldrich), both at final concentrations of 30 mM, and incubation for 2 h at 37 °C. In addition, an internal standard was created by pooling digest aliquots of all stimulated samples (LPS, IBV antigen or IBV + NDV vaccine) and corresponding unstimulated samples, each of equal peptide mass as determined before digestion. This so-called "Common Reference" was labelled with deuterated formaldehyde (CD_2O with M = 32.04 g/mol, hereinafter referred to as "heavy") (Sigma-Aldrich), together with $NaCNBH_3$, both at final concentrations of 30 mM, and incubation for 2 h at 37 °C.

LC-MS/MS analysis and data processing

Each "light"-labelled sample was mixed with an aliquot of the "heavy"-labelled Common Reference in a 1:1 ratio (based on the mass of the peptides). The resulting mixtures were desalted on a GX 271 Automated Solid Phase Extraction system (Gilson International B.V., The Hague, The Netherlands). Briefly, each mixture was loaded onto a 1 ml capacity C18 cartridge (Waters) and the absorbed peptides were washed with 600 µl of water containing 0.1 vol% of formic acid and subsequently eluted with 600 µl of 60 vol% of acetonitrile in water, also containing 0.1 vol% of formic acid. Each eluate was dried by centrifugation under reduced pressure (Eppendorf Vacuum Concentrator Plus, Eppendorf, Germany) and reconstituted in 100 µl of water containing 5 vol% of dimethyl sulfoxide and 0.1 vol% of formic acid for nanoscale LC MS/MS analysis.

The nanoscale LC-MS system comprised an Agilent 1290 Infinity UPLC system (Agilent, Waldbronn, Germany) coupled to a Tribrid Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific). The system was setup essentially as described [20] containing an inhouse-made trapping column (20 mm length x 100 µm I.D., Reprosil-Pur® C18 AQ 5 µm particle size, 120 Å pore size, Dr. Maisch, Ammerbuch, Germany) and an analytical column (32.4 cm length x 50 µm I.D., Reprosil-Pur® C18-AQ 3 µm particle size, 120 Å pore size, dr. Maisch GmbH) made in-house at Intravacc, the Netherlands. Solvent A was 0.1 vol% of formic acid in water and solvent B was 0.1 vol% of formic acid in acetonitrile. The peptides were trapped on the trapping column for 10 min at a flow rate of 5 µl/min of 100% solvent A. The subsequent separation of the peptides was performed at a flow rate of 125 nl/min in a 95-min non-linear gradient (8% solvent B to 28% solvent B in 0.25%/min, followed by an increase to 43% solvent B in 1.5%/min and a step to 85% solvent B hold for 5 min). The column effluent was electrosprayed into the MS with an inhouse-made gold/carbon-coated fused silica spray tip (360 µm O.D. x 25 µm I.D., manually tapered to a 3.5-µm tip I.D.), butt-connected to the analytical column. Full scan mass spectra were acquired with an Orbitrap readout at a 120,000 FWHM resolution with an m/z scan range of 300-1,500 Da, a maximum ion injection time of 50 ms and the Automatic Gain Control (AGC) set to 200,000. Internal mass calibration with fluoranthene was enabled. CID fragmentation scans with an Ion Trap readout were acquired for z = 1 and z = 2-5 positively charged ions with intensity thresholds of $1x10^6$ or 5,000 counts, respectively. Dynamic exclusion was set to 2 for a 45-sec time window. Default instrument settings were used for MS/MS acquisition.

Acquired data were processed with PEAKS® X (Bioinformatics Solutions Inc., Waterloo, Canada) against the *Gallus gallus* domesticus database (Taxonomy ID=9031, www.uniprot.org) containing 34,930 entries for protein identification. Enzyme specificity was set to Trypsin (semispecific) with a maximum of 3 miscleavages. Mass error tolerances for parent ions and fragment ions were set to 20 ppm and 0.6 Da, respectively. Fixed modifications

were carbamidomethylating on Cys (+57.0215 Da) and dimethylation on Lys (+28.0313 Da and +32.0564 Da for Light and Heavy, respectively). The variable modifications were deamidation on Asn and Gln (+0.9840 Da) and oxidation on Met (+15.9949 Da). Peak area intensities of the precursor ions of the identified peptides were used for relative protein quantification.

Analysis of relative protein abundance

Data processing and statistical analysis was done using R statistical software, version 3.6.0. The relative quantification of the samples was based on the comparison of all samples against the Common References (**Supplementary Table S1**) acting as an internal standard between samples, in accordance with a previous publication [21]. The resulting sample / Common Reference ratios were $\log_2$-transformed. Next, the values were normalized for variations between measurements by performing a median correction, in which all relative protein expression values were divided by median protein expression value of each run (**Supplementary Table S2**), in accordance with previous publications [22,23]. Proteins were included as quantitative differentially expressed proteins (DEPs) if they were detected in at least two out of three replicates for both stimulated and unstimulated samples, showed an average upregulation or downregulation of at least 1.5-fold upon stimulation, and change was statistically significant ($p < 0.05$) according to a Student's T-test. Moreover, *de novo*-expressed proteins (*i.e.* uniquely expressed proteins detected in all samples of the stimulated group and in no samples of the unstimulated group, or the reverse situation) were also included as qualitative DEPs in the results. Although statistics could not be performed for these *de novo*-expressed proteins with expression levels were below the detection limit in one of the treatment groups, the qualitative differences were still considered to be of practical significance. Finally, some proteins appeared more than once in the results, while showing the same expression values. These redundant appearances were removed resulting in a list of unique proteins.

Volcano plots were made using Perseus software [24] to visualize the selection of DEPs. The selected DEPs were further analysed for protein-protein association networks using the STRING database (www.string-db.org) [25]. The database was searched for physical or functional protein-protein interactions with a minimum required interaction score of 0.700 (high confidence) among the DEPs. The resulting STRING network clusters were functionally classified using annotations with the lowest Benjamini-Hochberg false-discovery rates from integrated Reactome (www.reactome.org), KEGG (www.kegg.jp), GO (www.ebi.ac.uk/QuickGo/), PFAM (www.pfam.xfam.org) and SMART (www.smart.embl-heidelberg.de) databases. Additionally, the STRING network was exported as tabular text output, and combined with protein expression data in Microsoft Excel. The resulting files were loaded in Cytoscape [26] version 3.7.2 for visualization.

The subcellular localization of DEPs was determined based on GO terms for cellular component using QuickGO (https://www.ebi.ac.uk/QuickGO/). The search was limited to a selected number of gene ontology terms for cellular components listed in **Supplementary Table S3**. The UniProtKB database was used as a reference for chicken proteins by choosing taxon identifier 9031 (*Gallus gallus*). The DEPs of stimulated chBMDCs were tested for enrichment for cellular components as compared to the reference using a one-tailed binominal test. Next, the Benjamini-Hochberg method was used to control false discovery

rates (FDR) as a result of multiple comparisons [27]. An FDR < 0.05 was considered significant.

To create KEGG pathway maps, the KEGG Mapper tool (http://www.genome.jp/kegg/mapper.html) [28] was used to modify the maps gga03050 (Proteasome – *Gallus gallus* [chicken]), gga01100 (Metabolic pathways – *Gallus gallus* [chicken]) and gga04142 (Lysosome – *Gallus gallus* [chicken]). Permission was granted by Kanehisa Laboratories to publish the KEGG pathway map images both in print and digital under the CC BY 4.0 open access license.

Gene expression analysis

RNA isolation was performed with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, including a DNase treatment step using the RNase-Free DNase Set (Qiagen). Next, cDNA was prepared using the reverse transcriptase from the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) according to the manufacturer's instructions. RT-qPCRs were performed for several genes of the most significantly upregulated DEPs with primers listed in **Table 1** and SYBR Green Master Mix (both from Life Technologies). RT-qPCRs were performed with a CFX Connect and analyzed with the CFX Maestro software (both from Bio-Rad). All RT-qPCRs were evaluated for proper amplification efficiency (90–110%) using serial dilutions of reference cDNA obtained from the chicken macrophage-like cell line HD11 after 3 h stimulation with LPS. RT-qPCRs were performed in triplicate for every sample. Relative gene expression levels after stimulation of chBMDCs were normalized using the housekeeping gene GAPDH as the reference gene expressed as $\log_2$-transformed fold changes compared to unstimulated chBMDCs. Genes were considered significantly differentially expressed if at least two out of three stimulated replicates were upregulated or downregulated compared to unstimulated controls, the change was at least 1.5-fold after stimulation, and the difference was statistically significant ($p < 0.05$) according to a Student's T-test.

Results

Differentially expressed proteins identified in chBMDCs stimulated with IBV + NDV vaccine did not show overlap with differentially expressed proteins after stimulation with IBV antigen only

Quantitative proteome analysis was performed for chBMDCs stimulated with a IBV + NDV vaccine, IBV antigen or LPS to identify the respective potential biomarkers. Initially, LC-MS/MS led to the identification of 2,828 proteins (**Supplementary Table S1**), some of which were identified multiple times, because several UniProtKB accession codes were present for these individual protein sequences, and these were manually removed. The resulting list contained 154 quantitative DEPs, which were found to be differentially expressed with statistical significance (≥ 1.5-fold with $p \leq 0.05$) after stimulation with IBV + NDV vaccine, IBV antigen or LPS (**Supplementary Tables S2** and **S3**). In addition, 45 proteins were differentially expressed at a qualitative level, *i.e.* these proteins were detected only in either unstimulated or any of the stimulated cultures of chBMDCs (**Supplementary Tables S2 and S3**). We found 33 upregulated and 34 downregulated DEPs after stimulation with the IBV + NDV vaccine (**Figure 1a**). Less DEPs, 14 upregulated and 11 downregulated proteins,

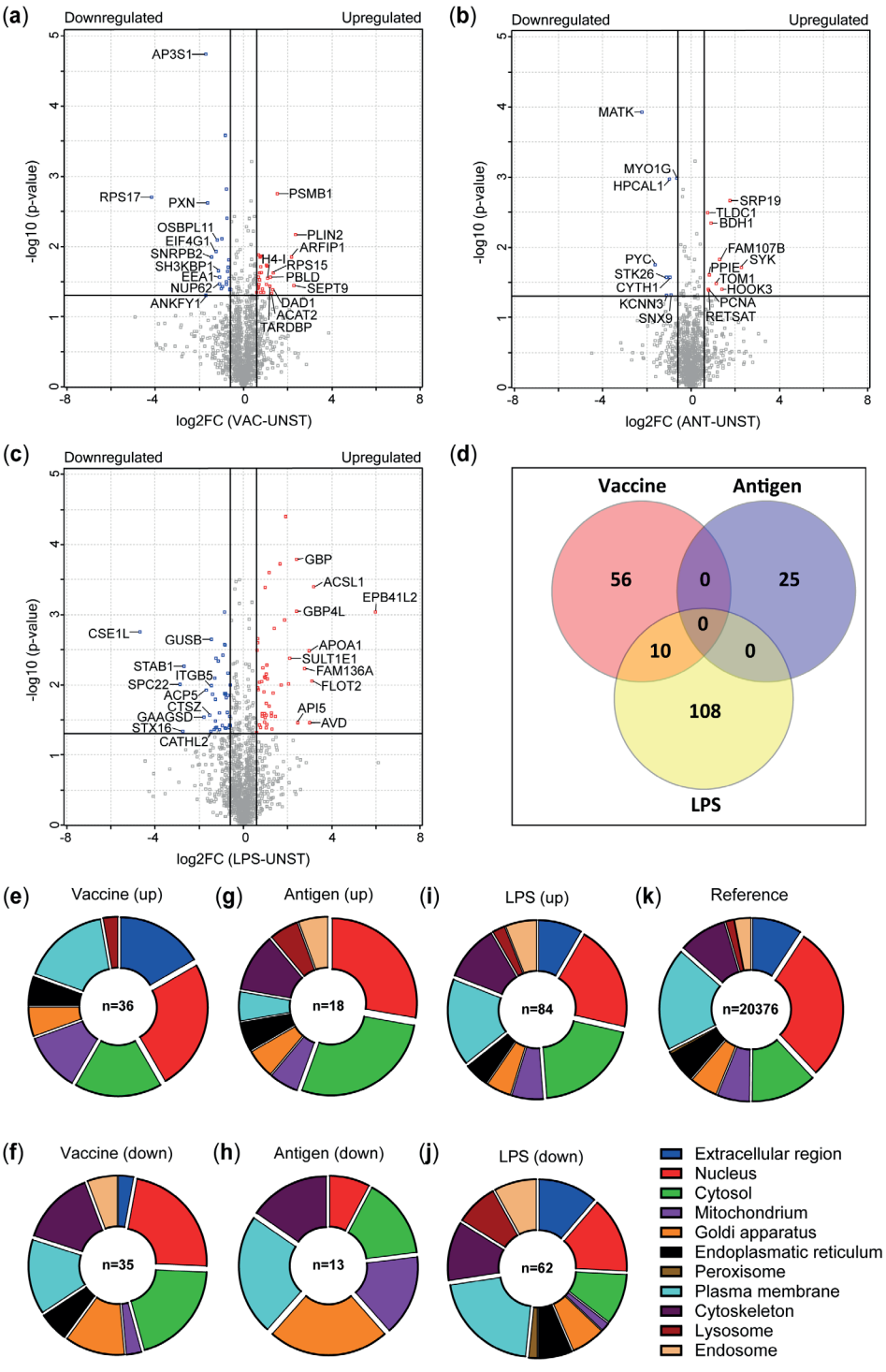
(a)
Downregulated
Upregulated
AP3S1
RPS17
PXN
PSMB1
OSBPL11
EIF4G1
SNRPB2
SH3KBP1
EEA1
NUP62
ANKFY1
PLIN2
ARFIP1
H4-I
RPS15
PBLD
SEPT9
DAD1
ACAT2
TARDBP
-log10 (p-value)
log2FC (VAC-UNST)
(b)
Downregulated
Upregulated
MATK
MYO1G
HPCAL1
SRP19
TLDC1
BDH1
FAM107B
SYK
PPIE
TOM1
HOOK3
PYC
STK26
CYTH1
KCNN3
SNX9
PCNA
RETSAT
-log10 (p-value)
log2FC (ANT-UNST)
(c)
Downregulated
Upregulated
GBP
ACSL1
EPB41L2
GBP4L
CSE1L
GUSB
APOA1
STAB1
SULT1E1
ITGB5
FAM136A
SPC22
FLOT2
ACP5
CTSZ
API5
GAAGSD
AVD
STX16
CATHL2
-log10 (p-value)
log2FC (LPS-UNST)
(d)
Vaccine
Antigen
56
0
25
0
10
0
108
LPS
(e)
Vaccine (up)
n=36
(g)
Antigen (up)
n=18
(i)
LPS (up)
n=84
(k)
Reference
n=20376
(f)
Vaccine (down)
n=35
(h)
Antigen (down)
n=13
(j)
LPS (down)
n=62
Extracellular region
Nucleus
Cytosol
Mitochondrium
Goldi apparatus
Endoplasmatic reticulum
Peroxisome
Plasma membrane
Cytoskeleton
Lysosome
Endosome

Figure 1. Differentially expressed proteins identified by LC-MS/MS after stimulation of chBMDCs. Volcano plots show differentially expressed proteins of chBMDCs after stimulation with LPS (**a**), an *IBV + NDV* vaccine (**b**) and IBV antigens only (**c**). Proteins that are ≥ 1.5-fold upregulated or downregulated and significantly different from the unstimulated controls are shown respectively in red and blue. The protein names are shown for up to 10 of the most upregulated and downregulated proteins for each condition. (**d**) A total of 199 proteins were found to be differentially expressed after the different stimulations. The overlap in differentially expressed proteins between stimulation conditions is given by a Venn diagram, which shows an overlap of 10 proteins between LPS- and vaccine-stimulated chBMDCs. The subcellular localization of differentially expressed proteins after stimulation with LPS (**e** and **f**), *IBV + NDV* vaccine (**g** and **h**) and IBV antigen only (**i** and **j**) was determined using gene ontology terms for cellular compartments (GOCC). A distinction was made between upregulated (**e**, **g** and **i**) and downregulated (**f**, **h** and **j**) differentially expressed proteins. The GOCC terms of all proteins from the UniProtKB reference database are shown as well (**k**). Not all proteins could be annotated with gene ontology terms for cellular compartments and some proteins were annotated with multiple entries.

were found after stimulation with IBV antigen (**Figure 1b**). Stimulation with LPS induced a total of 66 upregulated and 52 downregulated DEPs (**Figure 1c**). The Venn diagram (**Figure 1d**) shows overlaps of DEPs induced by the different stimuli. The overlap between DEPs from IBV + NDV vaccine- and LPS-stimulated chBMDCs comprised 10 proteins of which five showed a change into the same direction for both stimuli (**Supplementary Table S4**). No DEPs were shared between IBV + NDV vaccine and IBV antigen, or LPS and IBV antigen.

The DEPs after stimulation with IBV + NDV vaccine (**Figures 1e** and **f**), IBV antigen (**Figures 1g** and **h**) or LPS (**Figures 1i** and **j**) were annotated for subcellular localization using gene ontology terms for cellular compartments. The DEPs were distributed throughout all major cellular compartments. Overall, most DEPs were associated with the nucleus, plasma membrane and the cytosol, which is similar to the overall distribution of annotated proteins in the UniProtKB reference database (**Figure 1k**). Compared to the reference, upregulated DEPs of vaccine-stimulated chBMDCs were relatively often associated with the extracellular region (FDR = 0.054) (**Figure 1e**), whereas downregulated DEPs were more associated with the cytosol (FDR = 0.059) (**Figure 1f**). Stimulation with IBV antigen resulted in a significant enrichment of upregulated DEPs associated with the cytosol (FDR = 0.046) and downregulated DEPs associated with the Golgi apparatus (FDR < 0.050) (**Figures 1g** and **h**). LPS stimulation of chBMDCs resulted in a significantly large proportion of upregulated DEPs associated with the cytosol (FDR < 0.001) and downregulated DEPs associated with the plasma membrane (FDR = 0.043), lysosome (FDR = 0.003) and endosome (FDR = 0.037) (**Figures 1i** and **j**).

Differentially expressed proteins of vaccine-stimulated chBMDCs cluster in functional networks related to mRNA translation, immune responses, metabolism and the proteasome

As a next step, an enrichment analysis of protein-protein interaction networks was performed using STRING. A STRING network was created using the DEPs found for the different stimuli. From the 199 DEPs that were included in the analysis we found 91 DEPs that formed 136 high confidence physical or functional protein-protein interactions (**Figures 2**, **5** and **6**). These DEPs formed 16 clusters comprising 2 to 14 proteins (**Supplementary Table S5**).

Table 1. Primer sequences for RT-qPCR to assess gene expression of selected upregulated DEPs.

Gene	Reference	Type	Sequences (5'-3')
PBLD	ENSGALT00000006268.6	Forward	CGGTTTGTCTGCTCGAAAATGAC
		Reverse	AACCATCGGAGTCCAAAGCG
ACAT2	ENSGALT00000019060.5	Forward	CCGGATCGTATCTTGGGCTCA
		Reverse	AGTCCAGCCGGCTTTCTCAATA
RPS15	ENSGALT00000070660.2	Forward	CCAAGATGGCAGAAGTGGAGCA
		Reverse	AGGACATATCGAGCAGCTGGT
DAD1	ENSGALT00000048785.2	Forward	AGCTTCATCCTCGGCGTTTGT
		Reverse	CCCTCTCTGGTGAAATGCCTTGG
PSMB1	ENSGALT00000018217.5	Forward	GCCATTTTGCCGTGAGACAGC
		Reverse	ACACAGTCCCGCCGTTGAAG
ARFIP1	ENSGALT00000065974.3	Forward	AGGTTCCTCAGCATCACCCA
		Reverse	ACCGGTCCACTCTTCTGCTG
SEPT9	ENSGALT00000047767.2	Forward	GAAGGTCCAGAGGCCGTTCC
		Reverse	GCATCGATCCCCACGTAGCC
PLIN2	ENSGALT00000083439.2	Forward	CCGCTCAACTGGCTGGTTCC
		Reverse	GGGCCATTTGTGCGTGAAAAGT
SRP19	ENSGALT00000000294.6	Forward	GGTAGGCCTGGTCGTTAGCA
		Reverse	AAATCTCTCCTTGTCGGCCGG
HOOK3	ENSGALT00000024828.6	Forward	ACAACCATGAGATTCTAGGGCAAC
		Reverse	GCATTCTTCCAAGCTCCGCA
SYK	ENSGALT00000032807.5	Forward	CCCTCTGGCAGTTAGTTGAGCA
		Reverse	TCACTTTCTGAGCCATGCCGT
GBP4L	ENSGALT00000069561.2	Forward	AGCGGCCCATCAGTGAGGAT
		Reverse	GAGCCGCGTCCCATTGTAGT
APOA1	ENSGALT00000011524.6	Forward	CTGTGCTCTTCCTGACGGG
		Reverse	GAGCCATGTCCTCACGCA
ACSL1	ENSGALT00000017294.4	Forward	AGCCCAGGGAGAGTACATAGCA
		Reverse	AGGCCTGCAAGCTCTCTCCA
EPB41L2	XM_025148931.1	Forward	CATCGGGCCGCTAAGAGGTT
		Reverse	TGTCGTGTCTGTGCTTGCGT
GAPDH	ENSGALT00000023323.6	Forward	GTGGTGCTAAGCGTGTTATC
		Reverse	GCATGGACAGTGGTCATAAG

The DEPs of IBV + NDV vaccine-stimulated chBMDCs were found overrepresented in clusters **a** (8/12), **c** (7/14), **d** (4/8) and **h** (3/6) (**Figure 2**). For cluster **a**, the top ranking functional enrichments were "mRNA translation" (Reactome: GGA-72766, FDR = $1.6x10^{-12}$; GO: 0006412, FDR = $2.3x10^{-4}$) representing all proteins except NME2, an enzyme required for synthesis of nucleoside triphosphates, and ETF1, which plays a role in the termination of translation. Cluster **a** is linked to cluster **b** through COPS4, a subunit of the COP9 signalosome complex. After stimulation with IBV + NDV vaccine, 3/7 proteins of cluster **b** were identified as DEPs. The highest ranking functional enrichments for cluster **b** were "DNA repair" (Reactome: GGA-73894, FDR = $1.3x10^{-8}$), "transcription-coupled nucleotide excision repair [TC-NER]" (Reactome: GGA-6781827, FDR = $7.9x10^{-7}$) and mRNA splicing (Reactome: GGA-72163, FDR = $6.5x10^{-6}$), which together comprised all DEPs of this cluster. Cluster **c** was most functionally enriched for "immune system" (Reactome: GGA-168256; FDR = $4.5x10^{-8}$), followed by the more specific description "innate immune system" (Reactome: GGA-168249; FDR = $2.7x10^{-7}$). Except for PXN, which is involved in focal adhesion, all DEPs found in cluster **c** after IBV + NDV vaccine stimulation were described to be involved in immune responses. Within cluster **d**, two upregulated DEPs (OXCT1 and ACAT2) are part of lipid metabolism and another two upregulated DEPs (SUCLG2 and IDH3G) are part of the citrate cycle (**Figure 3**). Cluster **f** was functionally enriched for "proteasome" (KEGG: gga03050; FDR = $6.4x10^{-12}$) and comprised subunits of this protein complex that has a critical role in cellular proteolysis, including in the processing of antigens for presentation on the cell surface to the adaptive immune system. Proteins of cluster **f**

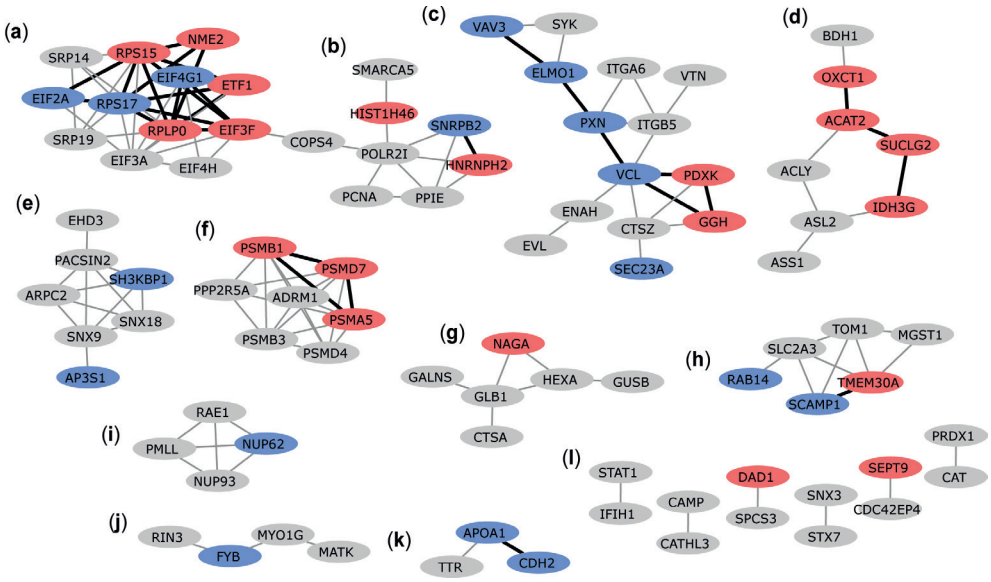

Figure 2. Cytoscape STRING network of differentially expressed proteins shows clusters of differentially expressed proteins after *IBV + NDV* vaccine stimulation. Upregulated differentially expressed proteins are shown in red and downregulated differentially expressed proteins are shown in blue. Proteins in grey were not significantly differentially expressed after *IBV + NDV* vaccine stimulation. Networks are only shown for proteins with a minimum required interaction score of 0.700 (high confidence). STRING interactions between proteins that are both differentially expressed by vaccine exposure are shown in black, otherwise in grey. The letters indicate different clusters with cluster **l** representing six clusters comprising two proteins each.

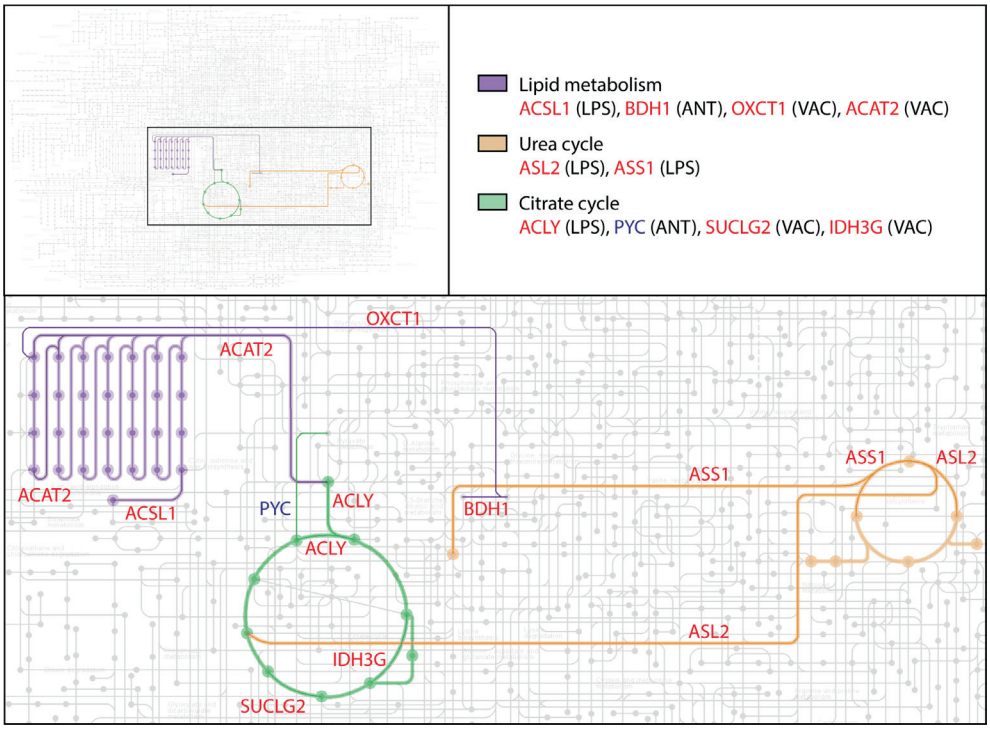

Figure 3. Interconnected metabolic pathways of chBMDCs change upon stimulation. The KEGG metabolic pathways map (gga01100) was modified to show differentially expressed proteins after stimulation with LPS, IBV antigen or *IBV + NDV* vaccine. Downregulated proteins are shown in blue and upregulated proteins are shown in red. The differentially expressed proteins could be categorized in members of lipid metabolism (purple), the urea cycle (orange) or citrate cycle (green).

were mapped to KEGG proteasome pathway (**Figure 4**), which demonstrated that two upregulated DEPs of chBMDCs stimulated with IBV + NDV vaccine, PSMA5 (α5) and PSMB1 (β6), are part of the proteasomal 20S core particle. The third DEP, PSMD7 (Rpn8), is part of the Rpn8-Rpn11 heterodimer with a function in removal of polyubiquitin tags before protein degradation in the proteasome [29]. A proteasomal subunit in close proximity to the Rpn8-Rpn11 heterodimer is PSMD13 (Rpn9), which showed a trend towards upregulation (fold change = 4.2; p = 0.07). Finally, the polyubiquitin-capturing protein PSMD4 (Rpn10) also showed a trend towards downregulation (fold change = 1.6; p = 0.07).

Besides the proteins of cluster **c**, more proteins that were differentially expressed by chBMDCs after stimulation with IBV + NDV vaccine were found to be part of the Reactome "immune system" pathways (GGA-168256). This includes the proteasomal proteins of cluster **f**, but also BLMH, FYB, RAB14, TCEB1, SCAMP1 and MIF. Although the "immune system" annotation was not assigned to IFITM3, this protein has a known role in antiviral immune responses [30].

The small number of differentially expressed proteins of antigen-stimulated chBMDCs showed limited clustering

A limited number of DEPs was found for IBV antigen-stimulated chBMDCs. The only cluster with a majority of DEPs after stimulation with IBV antigen was cluster **j** (**Figure 5**). This cluster was annotated for Src homology 2 (SMART: SM00252; FDR = $1.5x10^{-3}$) and 3 (SMART: SM00326; FDR = $3.2x10^{-3}$) domains. The DEPs RIN3, MATK and SYK possess a Src homology 2 domain. Apart from this, IBV antigen seemed to have little impact on functional pathways of chBMDCs.

Differentially expressed proteins of LPS-stimulated chBMDCs cluster in functional networks related to immune responses, metabolism, endocytosis, the proteasome and lysosomal enzymes

The DEPs of LPS-stimulated chBMDCs were found overrepresented in clusters **c** (8/14), **d** (4/8), **e** (4/7), **f** (4/7), **g** (6/6), **h** (3/6) and **i** (3/4) (**Figure 6**). Similar to stimulation with IBV + NDV vaccine, cluster **c** contained many DEPs after stimulation with LPS. Within this cluster, two DEPs (ELMO1 and PDXK) were shared between IBV + NDV vaccine- and LPS-stimulated chBMDCs. Furthermore, "regulation of actin cytoskeleton" (KEGG: gga04810; FDR = $3.3x10^{-6}$) and "focal adhesion" (KEGG: gga04510; FDR = $3.3x10^{-6}$) were found as important functional enrichments. All proteins of cluster d were part of "metabolic pathways" (KEGG: gga01100; FDR = $4.6x10^{-7}$), more specifically they were part of the citrate cycle, urea cycle or fatty acid metabolism (**Figure 3**). Cluster **e** was functionally enriched for proteins containing "Src homology 3 domains" (PFAM: PF14604; FDR = $2.2x10^{-6}$), "clathrin-mediated endocytosis" (Reactome: GGA-8856828; FDR = $1.6x10^{-6}$) and "membrane trafficking"

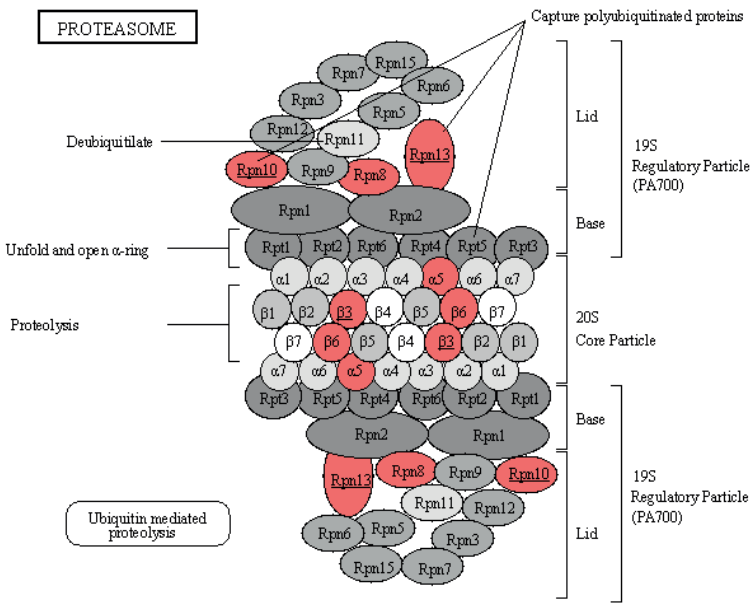

Figure 4. Differential expression of several proteasomal subunits in chBMDCs upon stimulation with LPS or *IBV + NDV* vaccine. The KEGG proteasome map (gga03050) shows upregulated proteins in red and others in grayscale. Underlined subunits PSMB3 (β3), PSMD4 (Rpn10) and ADRM1 (Rpn13) were upregulated after LPS stimulation, whereas the non-underlined subunits PSMA5 (α5), PSMB1 (β6) and PSMD7 (Rpn8) were upregulated after stimulation with *IBV + NDV* vaccine.

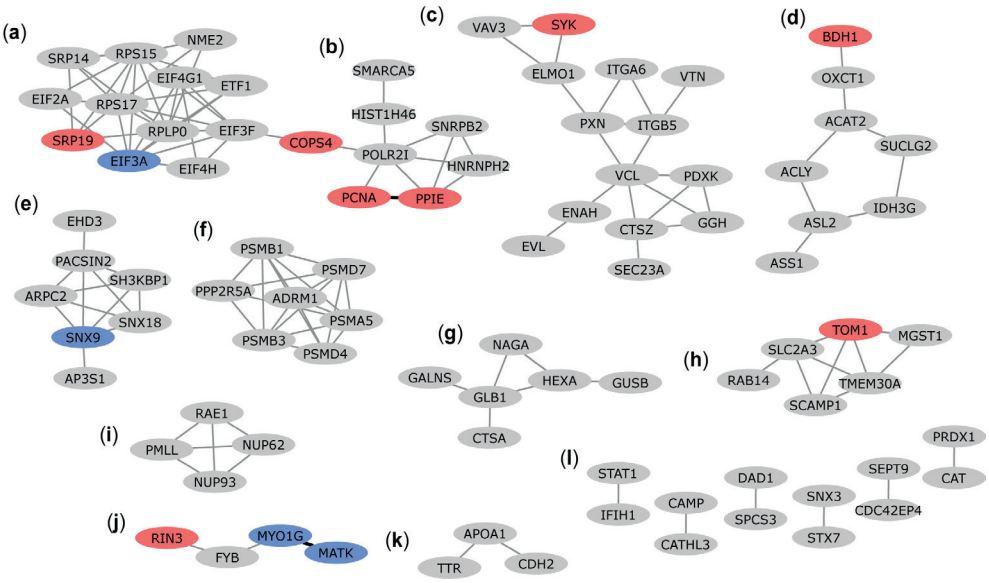

Figure 5. Cytoscape STRING network of differentially expressed proteins shows clusters of differentially expressed proteins after IBV antigen stimulation. Upregulated differentially expressed proteins are shown in red and downregulated differentially expressed proteins are shown in blue. Proteins in grey were not significantly differentially expressed after IBV antigen stimulation. Networks are only shown for proteins with a minimum required interaction score of 0.700 (high confidence). STRING interactions between proteins that are both differentially expressed by antigen exposure are shown in black, otherwise in grey. The letters indicate different clusters with cluster **l** representing six clusters comprising two proteins each.

(Reactome: GGA-199991; FDR = 4.6×10^{-6}). Similar to stimulation with IBV + NDV vaccine, cluster **f** contained DEPs after stimulation with LPS, including the non-ATPase regulatory proteasomal subunits PSMD4 (Rpn10) and ADRM1 (Rpn13) with a function in capturing polyubiquitinated proteins for proteolysis (**Figure 4**). A third proteasomal subunit, PSMC3 (Rpt5), with a function in the capture of polyubiquitinated proteins showed a trend of being upregulated after LPS stimulation (fold change = 1.3; p = 0.053). The peptide transporter TAP2, which is responsible for the translocation of proteasome-processed peptides into the endoplasmatic reticulum for loading on MHC molecules, was found upregulated after LPS stimulation (**Supplementary Table S2**). Cluster **g** comprised lysosomal enzymes (KEGG: gga04142; FDR = 8.4×10^{-13}) and was completely downregulated after LPS stimulation. In addition to the proteins of cluster **g**, other lysosomal enzymes were downregulated upon LPS stimulation (**Supplementary Figure S1**), including GAAGSD, DNaseII, ACP5, and SMPD1. Furthermore, the minor lysosomal membrane protein NPC was downregulated. Cluster **h** was functionally enriched for "organic substance transport" (GO: 0071702; FDR = 1.7×10^{-5}) and "neutrophil degranulation" (Reactome: GGA-6798695; FDR = 4.8×10^{-5}). Cluster **i** comprised four proteins that were found to be related to small ubiquitin-related modifier (SUMO) E3 ligases (Reactome: GGA-3108232; FDR = 1.1×10^{-8}).

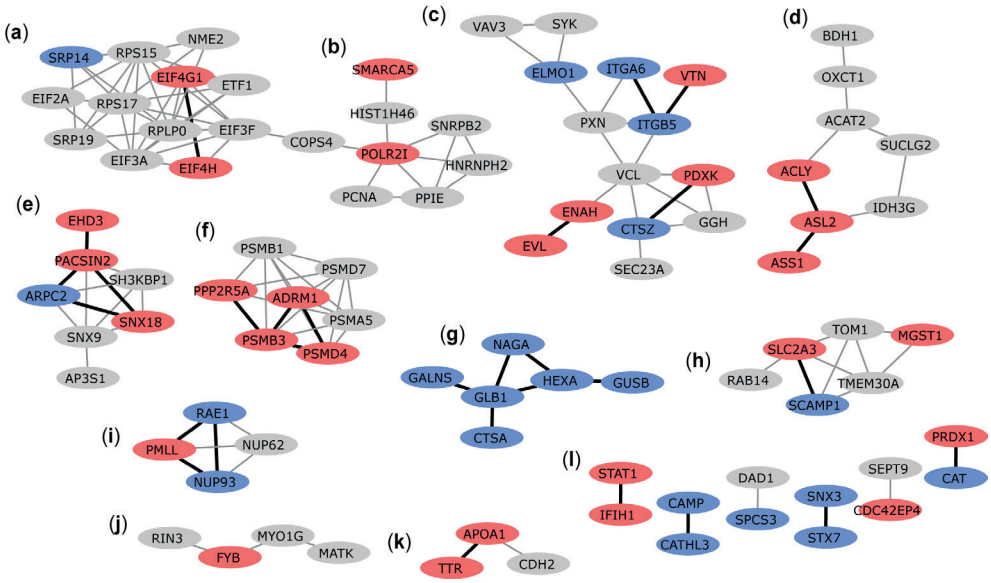

Figure 6. Cytoscape STRING network of differentially expressed proteins shows clusters of differentially expressed proteins after LPS stimulation. Upregulated differentially expressed proteins are shown in red and downregulated differentially expressed proteins are shown in blue. Proteins in grey were not significantly differentially expressed after LPS stimulation. Networks are only shown for proteins with a minimum required interaction score of 0.700 (high confidence). STRING interactions between proteins that are both differentially expressed by LPS exposure are shown in black, otherwise in grey. The letters indicate different clusters with cluster **l** representing six clusters comprising two proteins each.

Gene expression analysis shows partial overlap with mass spectrometry data, resulting in candidate vaccine-associated biomarkers PSMB1 and PLIN2

Finally, we set out to investigate whether any of the DEPs could also be detected as differentially expressed genes (DEGs) by RT-qPCR, a method that could directly be used to evaluate biomarker expression in chickens immune cells. Similar to the proteomics data, chBMDCs were stimulated for 24 h with IBV + NDV vaccine, IBV antigen or LPS. The eight most significantly upregulated DEPs of IBV + NDV vaccine-stimulated chBMDCs were selected to evaluate gene expression levels (**Figure 7a**). Moreover, three DEPs were selected for IBV antigen-stimulated chBMDCs (**Figure 7b**) and four DEPs for LPS-stimulated chBMDCs (**Figure 7c**). Analysis of gene expression showed that the three out of eight tested vaccine-related DEPs were also found upregulated on mRNA, including PSMB1 ($p = 0.042$) and PLIN2 ($p = 0.002$), which were significantly upregulated, (**Figure 7d**) and SEPT9, which tended to be upregulated ($p = 0.055$). None of the selected DEPs of antigen-stimulated chBMDCs were differentially expressed on mRNA level (**Figure 7e**). Two out of four DEPs of LPS-stimulated chBMDCs, GBP4L and ACSL1, were found as upregulated DEGs (**Figure 7f**). Analyzing mRNA expression levels of all DEP after 8 h rather than 24 h stimulation showed an upregulation of PLIN2 (1.96-fold) of vaccine-stimulated chBMDCs, an upregulation of GBP4L (8.5-fold) and ACSL1 (9.8-fold) of LPS-stimulated chBMDCs, and a downregulation of EPB41L2 (3.0-fold) of LPS-stimulated chBMDCs, while other genes were unaffected (*data not shown*).

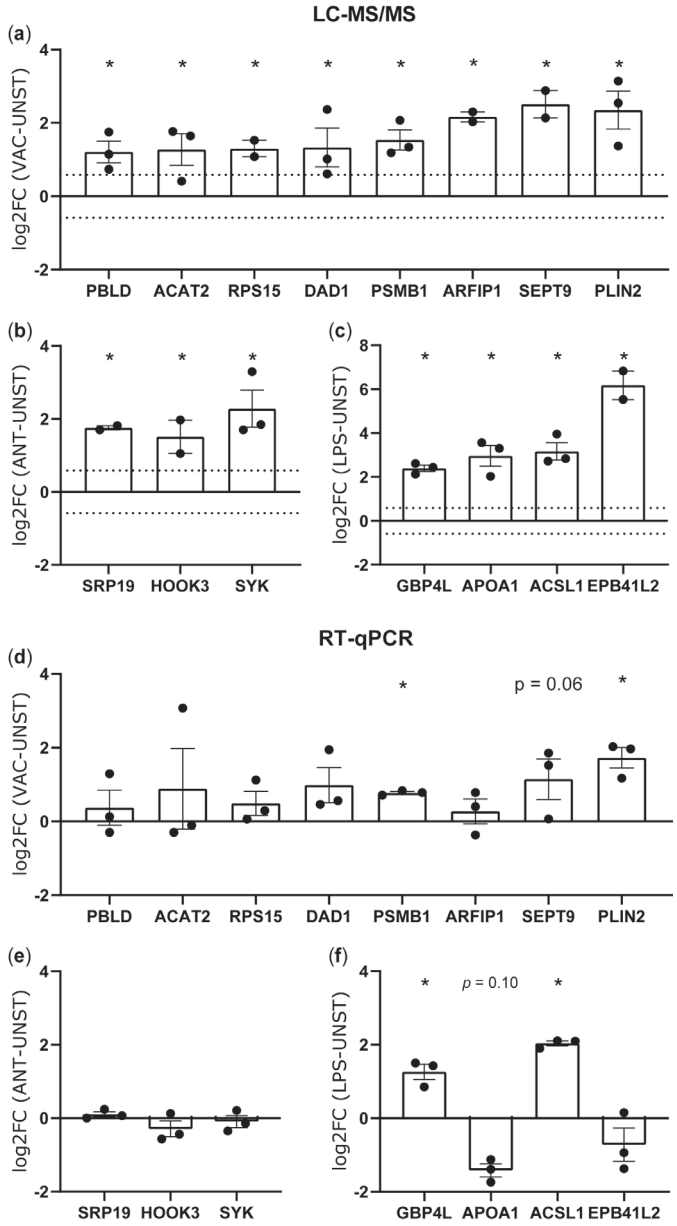

Figure 7. Comparison of protein and gene expression levels of differentially expressed proteins found to be most upregulated in mass spectrometry. Differentially expressed proteins of (**a**) *IBV + NDV* vaccine-, (**b**) IBV antigen- (**c**) and LPS-stimulated chBMDCs were selected for gene expression analysis using qPCR (resp. **d-f**). The expression of proteins and genes was analyzed for three independent replicates. The error bars show the SEM, and the dotted lines represent the cutoff-values of 1.5-fold change that was used to select differentially expressed proteins in mass spectrometry. Statistically significant differences between stimulated and unstimulated chBMDCs are indicated by * for $p < 0.05$.

Discussion

To our best knowledge, this study was the first attempt to discover biomarkers for inactivated poultry vaccines by investigating vaccine-induced immune responses with proteomics. To do so, we stimulated chBMDCs with an inactivated IBV + NDV poultry vaccine containing a mineral oil adjuvant or inactivated IBV antigen only and we analyzed the cell lysates by LC-MS/MS, which resulted in a list of DEPs that were also evaluated for expression on mRNA level using RT-qPCR. Furthermore, chBMDCs were stimulated with *E. coli* LPS as a strong stimulator of innate immune cells, as demonstrated in previous studies [14–19,31,32], and was used to validate our methodology and to generate new insights in cellular processes involved in LPS driven maturation of chBMDCs.

Mass spectrometry analysis led to the identification of 118 DEPs in chBMDCs after stimulation with LPS, 66 DEPs after stimulation with IBV + NDV vaccine and 25 DEPs after stimulation with IBV antigen. For each stimulus, 20-25% of the DEPs were found to be uniquely expressed in either the unstimulated group or any of the stimulated groups, whereas the protein expression levels were absent or below the detection limit of the mass spectrometer in the other groups. Although statistics could not be performed for these proteins, the qualitative differences were still considered to be of practical significance. In the STRING analysis, DEPs based on qualitative differences made up 23% and were therefore not over- or underrepresented compared to DEPS based on quantitative differences. Moreover, DEPs based on qualitative differences were randomly distributed among the clusters of the STRING network (**Supplementary Table S5**). Therefore, inclusion of DEPs based on qualitative differences strengthened our STRING enrichment analysis without significantly changing the clusters observed.

Stimulation of chBMDCs with *E. coli* LPS resulted in most DEPs, which were associated with the (innate) immune system, regulation of the cytoskeleton, metabolic pathways, endocytosis, the lysosome, the proteasome, and SUMO E3 ligases. A previous study reported *in vivo* effects of LPS at the transcriptomic level [33], showing increased transcription of genes associated with inflammatory responses, regulation of the cytoskeleton and cell migration in the bursa of Fabricius, a lymphoid organ only found in birds. This corresponds with the DEPs of the current study, annotated for functions in the innate immune system and regulation of the cytoskeleton. Moreover, many DEPs of LPS-stimulated chBMDCs, including proteins with functions in the innate immune system, have previously been described to be significantly increased at the gene expression level in the chicken caecum and spleen upon bacterial infection with *S. enteritidis* [34,35]. Finally, the SUMO E3 ligase PMLL was upregulated by LPS stimulation, which is in agreement with a study about the mammalian homologue PML that showed its importance for immune cell activation by LPS [36].

The results of this study shed light on mechanistic aspects of LPS stimulation that result in chBMDC maturation [14–19,31,32]. First of all, increased expression of STAT1 was observed, in agreement with its requirement for DC maturation [37]. Secondly, we observed a strong downregulation of lysosomal hydrolases after LPS stimulation, in line with previous reports showing a decreased uptake of antigens by endocytosis or phagocytosis after LPS stimulation [14]. This effect is in accordance with studies in human and murine BMDCs showing reduced recruitment of lysosomal hydrolases to phagosomes, reduced phagosomal acidification and

reduced phagolysosomal activity that together preserve exogenous antigens for (cross-) presentation by mature DCs [38–41]. Thirdly, we have observed an upregulation of the non-ATPase regulatory subunits PSMD4 and ADRM1 of the proteasome. The increased expression of regulatory proteasomal subunits after LPS stimulation has been reported to facilitate more efficient antigen processing in murine BMDCs [41]. The downregulation of lysosomal activity, the upregulation of proteasomal activity and the upregulation of TAP2, which is required for the transport of proteasome-processed peptides to the endoplasmic reticulum for loading on MHC molecules, suggest that antigen-presentation by chBMDCs increased as a consequence of DC maturation after LPS stimulation, similar to murine BMDCs [39,41].

Several DEPs of LPS-stimulated chBMDCs are involved in antimicrobial activity, even though these proteins did not cluster into one STRING network. Four of these proteins were upregulated metabolic enzymes involved in the production of inflammatory mediators, namely ASL2, ASS1, IRG1 and ACSL1. The DEPs ASL2 and ASS1 are involved in nitric oxide production [42], ACSL1, has a role in the synthesis of the inflammatory mediator prostaglandin E2 [43], and IRG1 produces itaconate [44], all of which are inflammatory mediators with antimicrobial effects. IRG1 [41] and ACSL [45] were previously shown to be upregulated in murine BMDCs stimulated with LPS or bacteria. Furthermore, the upregulated DEPs AVD and GBP4L have direct antimicrobial effects [44]. GBP4L was also found as DEG by RT-qPCR and is involved in the activation of innate immune cells by LPS and bacteria [46]. In contrast, the antimicrobial cathelicidins CATHL2 and CATHL3, known to neutralize LPS [47,48], were found downregulated in the chBMDCs. Taken together, our proteomics data showed that LPS stimulation induced changes associated with cellular processes including DC maturation and antimicrobial activity that have not been described before at the protein level for chicken immune cells. In addition to its function as a strong immunostimulatory control, thereby verifying our methodology, LPS stimulation led to increased insight in relevant changes in protein expression in chBMDCs.

For IBV antigen-stimulated chBMDCs, a limited number of DEPs was observed. Of these, SYK was the strongest upregulated DEP. SYK was previously shown to be upregulated in response to uric acid crystals in humans [49]. Uric acid crystals are also present in the IBV antigens, since these are dissolved in allantoic fluid from embryonated chicken eggs [50]. Previously, we have shown that allantoic fluid has immunostimulatory properties, potentially through the presence of uric acid crystals, leading to increased Fc receptor-mediated phagocytosis in macrophages [51], in which SYK is likely to be involved [52].

The STRING analysis for vaccine-stimulated chBMDCs showed that the DEPs identified were largely related to immune response, the proteasome, metabolism, and mRNA translation. Since there was no overlap between chBMDCs stimulated with the inactivated IBV antigen and those that were stimulated with vaccine, the effects of the vaccine are likely to be induced by either the mineral oil adjuvant or inactivated NDV antigen as the other components of the bivalent vaccine. Due to its slow metabolization, mineral oil acts as a depot that releases antigen over a long period of time, during which strong humoral immune responses can develop [53–57]. Some upregulated DEPs of vaccine-stimulated chBMDCs involved in the storage (PLIN2 and TMEM23A) and metabolism (LIPA, OCXT1 and ACAT1) of lipids, or part of the citric acid cycle (SUCLG2 and IDH3G), may together contribute to

the metabolism of internalized mineral oil adjuvant and hence to the release of antigen in course of time. Two more upregulated proteins involved in lipid storage, SYAP1 and RAB18, were very close to significance ($p < 0.06$). In contrast, OSBP11L and APOA1 involved in lipid transport were found as downregulated DEPs. The storage of lipids in IBV + NDV-stimulated chBMDCs is in agreement with microscopic observations (**Supplementary Figure S2d**). Lipid droplets have been described as important for the induction of cross-presentation by saponin-based adjuvants [58]. PLIN2 was also found as a DEG and might therefore be particularly useful as a biomarker of the mineral oil-adjuvanted vaccine since its expression can be evaluated with RT-qPCR in addition to mass spectrometry.

Studies in mice suggest that water-in-oil-adjuvanted vaccines induce the release of damage-associated molecular patterns (DAMPs) by stressed or damaged cells, which promotes the differentiation of follicular helper T cells [59] and the induction of humoral adaptive immune responses [60]. In our present study, we found significantly DEPs in chBMDCs stimulated with the IBV + NDV vaccine involved in the innate immune response. The DEPs VAV3 and ELMO1, both involved in the formation of phagocytic cups, were downregulated, which fits to the functional change of DCs, from uptake of antigens to presentation of antigens, during DC maturation. Moreover, the proteasomal subunits PSMA5, PSMB1 and PSMD7 play a role in antigen processing and were found significantly upregulated. Of these, PSMB1 was also found as an upregulated DEG. In addition, the upregulated DEP BLMH has been implicated in antigen-processing for presentation on MHC-I molecules, downstream of the proteasome [61]. Finally, the pro-inflammatory cytokine MIF, which acts as a DAMP [62], was found to be significantly upregulated in response to the vaccine.

The aim of this study was to identify protein targets to be used for *in vitro* quality assessment of inactivated poultry vaccines and to generate new insights in aspects of the mechanisms-of-action by which these vaccines as well as LPS activate chicken DCs. Stimulation with LPS led to decrease expression of DEPs associated with phagolysosomal activity, thereby preserving exogenous antigens for (cross-)presentation, and to increased expression of DEPs associated with proteasomal antigen processing, which together suggests that antigen presentation increases during chBMDC maturation. Furthermore, LPS stimulation led to an increase in DEPs associated with antimicrobial activity. For IBV antigen- and IBV + NDV vaccine-stimulated chBMDCs, no overlap was observed between the DEPs, which suggests that the DEPs of IBV + NDV vaccine-stimulated chBMDCs were induced by the mineral oil adjuvant, NDV antigen or the combination of both. The DEPs of vaccine-stimulated chBMDCs were found largely associated with mRNA translation, immune responses, lipid metabolism and proteasomal degradation, which provides new insights about the effects that inactivated poultry vaccines have on chicken DCs. Furthermore, two significantly upregulated DEPs of vaccine-stimulated chBMDCs included PLIN2, involved in the storage of lipid droplets, and PSMB1, a proteasomal subunit, and were also found as upregulated DEGs using RT-qPCR. Future studies should include different conforming as well as non-conforming vaccine batches to assess the ability of the identified DEPs and DEGs to discriminate between batches of good and poor quality. The identification of these vaccine-associated biomarkers may facilitate the transition from *in vivo* to cell-based quality assessment of inactivated poultry vaccines.

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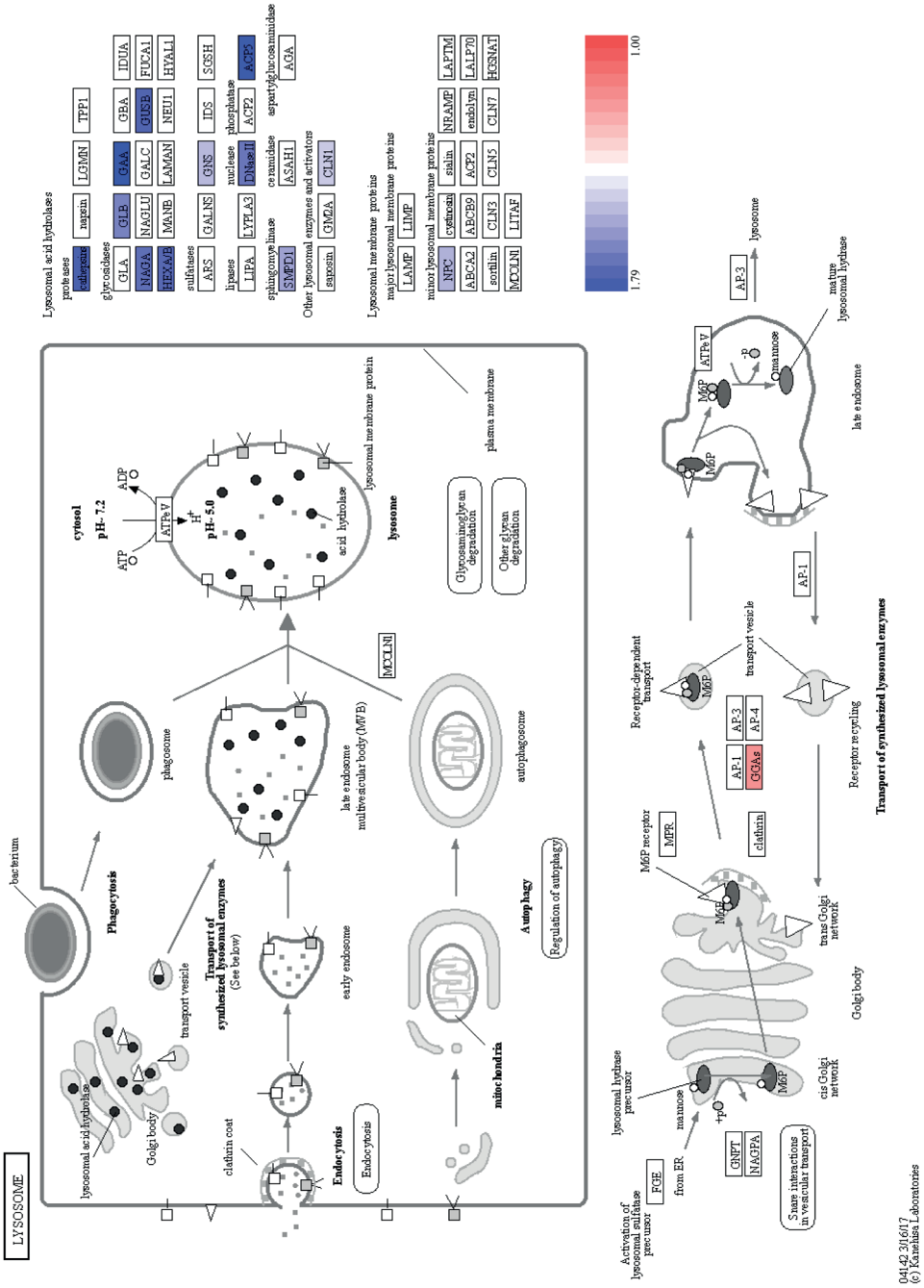
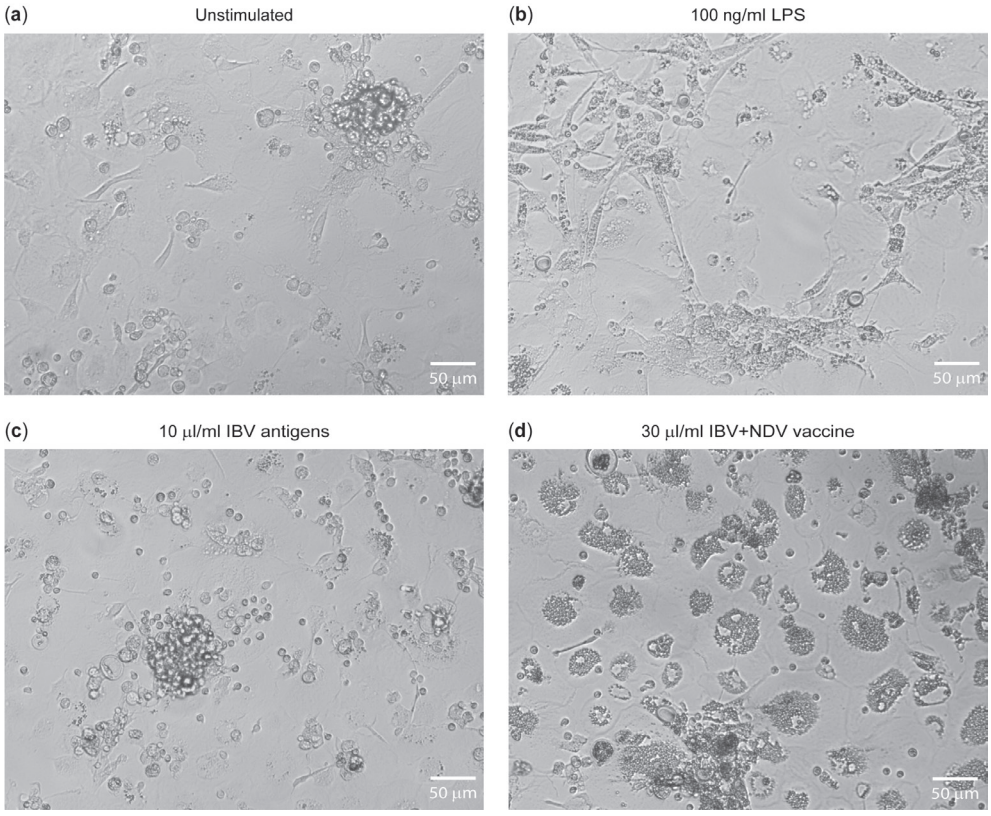
Supplementary Materials



Supplementary Figure S1. Stimulation of chBMDCs with LPS led to the significant downregulation of lysosomal enzymes. The KEGG lysosome map (gga04142) shows differentially expressed proteins of LPS-stimulated chBMDCs in the lysosome. Downregulated proteins are shown in blue and upregulated proteins are shown in red.



Supplementary Figure S2. The morphology of chBMDCs changes upon stimulation. The chBMDC cultures were left unstimulated (**a**) or were stimulated with 100 ng/ml LPS (**b**), 10 µl/ml IBV antigen (**c**) or 30 µl/ml *IBV + NDV* vaccine (**d**). Subsequently, the cell morphology of the cultures was captured with brightfield light microscopy at 100x magnification.

Supplementary Tables S1-S5. These datafiles can be provided by the authors on request.

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CHAPTER 6

Assessment of antigen-specific T cell responses in chickens after vaccination with an inactivated vaccine for infectious bronchitis virus and Newcastle disease virus

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Abstract

Early in life, chickens are vaccinated for infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) using live attenuated vaccines. Before the laying period, layers as well as broiler breeders receive additional inactivated booster vaccines for IBV and NDV to enhance protective immunity against these diseases during the laying period. Batch quality control of inactivated poultry vaccines is often assessed in serological assays. The role of cell-mediated immunity (CMI) in the protection induced by these vaccines is less well understood. In this study, we investigated the suitability of a T cell proliferation assay in routine vaccine quality control of inactivated IBV/NDV vaccines. Blood was collected from laying hens before and three weeks after vaccination with an inactivated IBV/NDV vaccine. Peripheral blood mononuclear cells were isolated and stimulated *ex vivo* with inactivated IBV and NDV antigens, and a bivalent IBV and NDV vaccine, after which T cell proliferation was assessed. Proliferation of T cells was readily observed after polyclonal stimulation. Furthermore, proliferation of T cells was observed upon stimulation with inactivated IBV antigens before but not after vaccination. Thus, although T cell proliferation was readily observed upon polyclonal stimulation, IBV-specific T cell proliferative responses were limited and not affected by the additional booster vaccination. Finally, proliferation was not observed upon stimulation with inactivated NDV antigens or the bivalent vaccine, neither before nor after vaccination. Based on the present findings, we have concluded that the T cell proliferation assay was not sensitive enough for routine quality control of the inactivated IBV/NDV vaccine. Whether the absence of T cell proliferation in response to IBV or NDV after additional vaccination with an inactivated vaccine has a technical or biological explanation needs to be further elucidated by future studies.

Introduction

Vaccines are important to maintain poultry health and welfare, and to prevent economic losses in the poultry industry due to infectious diseases. Chickens are vaccinated against a plethora of infectious diseases including infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) before or soon after hatch using live attenuated vaccines, or alternatives like recombinant viral vector-based vaccines or DNA vaccines [1,2]. Later in life, laying hens and broiler breeders may receive additional inactivated booster vaccines for IBV and NDV to enhance protective immunity against these viruses during the laying period.

Quality of batches of inactivated poultry vaccines is evaluated routinely by assessing their performance in *in vivo* vaccination-challenge studies or in serological tests that evaluate the induction of antigen-specific antibodies, which is an important aspect of vaccine-mediated protection against IBV and NDV. Antibodies also protect the offspring of broiler breeders from infectious diseases during early life [3]. Whole inactivated vaccines against IBV and NDV are adjuvanted with mineral oil formulated as water-in-oil emulsions to further enhance the induction of protective antibodies [4,5]. The induction of IBV- [6] and NDV-specific T cells [7] has also been demonstrated to be important for protective immunity in infection studies.

Evaluation of antigen-specific T cell proliferation *ex vivo* could potentially be used to demonstrate CMI in routine vaccine quality control and complement serological assays. However, whether inactivated poultry vaccines have the capacity to stimulate antigen-specific T cells remains unclear. Some studies were able to demonstrate the induction of CMI using T cell proliferation assays in chickens that received live attenuated vaccines against NDV [8,9] or a combination of live attenuated and inactivated vaccines against NDV [8,10], whereas another study could not [11]. With regard to IBV vaccines, one study demonstrated CMI in chickens that received a single dose of inactivated vaccine by a small proliferative response of T cells upon stimulation with IBV *ex vivo* [12], but the results have not been repeated since. As a consequence, knowledge about the extent to which inactivated poultry vaccines induce CMI is still limited.

In a previous study, a T cell proliferation assay was used to demonstrate CMI upon infection with *Salmonella enteritidis* in broiler chickens (Meijerink, *submitted*). Using the same methods, we investigated whether this T cell proliferation assay is suitable in routine vaccine quality control of inactivated vaccines against IBV and NDV.

Materials and Methods

Animals and experimental design

Ten Lohmann Brown Classic laying hens of 33 weeks old were housed at the animal facilities of the department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University (Dutch Central Authority for Scientific Procedures on Animals registration number AVD108002016642-1). The chickens were daily monitored by animal caretakers for signs of diseases, which were absent. All chickens in this study had been subjected to the standard vaccination regimen for laying hens before the onset of this study. In addition, the chickens received the inactivated booster vaccine Nobilis IB Multi (strains M41 and D274)

+ ND by intramuscular injection (*musculus pectoralis superficialis*). The complete vaccination scheme is given in **Table 1**.

Peripheral blood mononuclear cell isolation

For nine out of ten chickens, 3 ml heparinized blood was taken from the wing (*vena ulnaris*) and collected in 50 ml Falcon tubes containing 200 µl heparin (5000 IE/ml; LEO Pharma A/S, Copenhagen, Denmark) both before and 21 days after vaccination, and kept at room temperature. For one chicken, only approximately 1 ml blood was obtained. The heparinized blood, was diluted 1:1 in PBS (Lonza, Basel, Switzerland) and the peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA) according to standard procedures.

Labeling of PBMCs with CellTrace Violet proliferation dye

To measure proliferation by flow cytometry, PBMCs were labelled with CellTrace Violet (CTV; Invitrogen™, Life Technologies Europe BV, Bleiswijk, the Netherlands) by resuspending $5x10^6$ cells/ml in PBS with 5 µM CTV and incubation for 20 min at RT, while the

Table 1. Vaccination history of the laying hens. HVT = herpesvirus of turkeys; NDV = Newcastle disease virus; F = fusion protein; IBDV = infectious bursal disease virus; VP2 = minor capsid protein VP2; MDV = Marek's disease virus; IBV = infectious bronchitis virus; *S.* = *Salmonella*; TRT = turkey rhinotracheitis; AEV = avian encephalomyelitis virus; EDSV = egg-drop syndrome virus; *E.* = *Escherichia*; *P.* = *Pasteurella*; ILTV = infectious laryngotracheitis virus.

Age	Vaccine	Method
Day 1	Live recombinant HVT + NDV F and IBDV VP2 (HVP360)	Injection
	MDV (Rispens)	Injection
	Live IBV (stain 4-91)	Spray course
	Live IBV (stain Ma5)	Spray course
	Eimeria spores	Spray course
Day 7	Live *S. enteritidis* and *S. typhimurium*	Drinking water
Day 37	Live NDV	Spray
Day 48	Live IBV (stains H120 and D274)	Spray
Day 50	Live *S. enteritidis* and *S. typhimurium*	Drinking water
Day 56	Live TRT	Spray
Day 69	Live IBV (stain QX)	Spray
Day 70	Live AEV	Drinking water
Day 74	Inactivated TRT, IBV (strains M41, 249g), EDSV and NDV	Injection
	Inactivated *E. coli*	Injection
	Live ILTV	Eye drop
	Inactivated P. multicoca	Injection
Day 105	Live IBV (stain 4-91)	Spray course
	Live IBV (stain Ma5)	Spray course
Day 115	Live *S. enteritidis* and *S. typhimurium*	Drinking water
Day 232	Inactivated IBV (strain M41, D274) and NDV	Injection

cell suspension was mixed by gentle shaking every 5 min. Next, unbound dye was quenched by addition of 5 ml RPMI-1640 cell culture medium supplemented with GlutaMAX™-I, phenol red, HEPES, 10% fetal bovine serum and 50 U/ml of penicillin – streptomycin (all from Gibco™, Life Technologies Limited, Paisley, UK) for every ml of CTV staining solution and incubated for 5 min at room temperature. Cells were centrifuged for 5 min at 400x *g* and resuspended at $2.5x10^6$ cells/ml in X-VIVO 15 cell culture medium (Lonza) with 50 U/ml penicillin and streptomycin, and 50 µM 2-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA). The PBMCs ($5x10^5$) were seeded in 200 µl/well in 96 wells round-bottom cell culture plates (Costar™, Corning B.V. Life Sciences, Amsterdam, the Netherlands).

Ex vivo stimulation

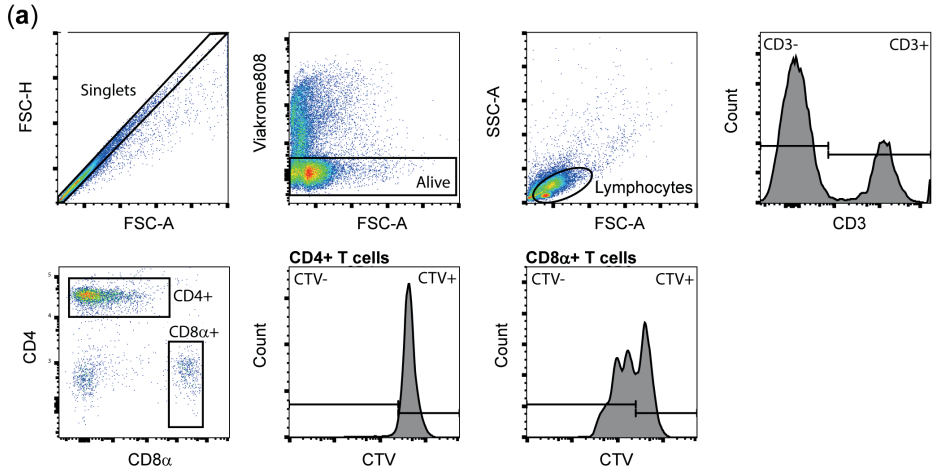
PBMC were stimulated *ex vivo* with 2 µl/ml formaldehyde-inactivated IBV and NDV dissolved in allantoic fluid (MSD Animal Health, Boxmeer, the Netherlands), collected from infected embryonated chicken eggs. In addition, cells were stimulated with 2 µl/ml bivalent vaccine (MSD Animal Health), containing inactivated IBV M41 and NDV clone 30 antigens, and emulsified (water-in-oil) mineral oil as adjuvant. As a negative control, 2 µl/ml allantoic fluid without inactivated viruses (MSD Animal Health) was added to the PBMCs. As a positive control, PBMCs were stimulated with 1 µg/ml mouse anti-chicken CD3, 1 µg/ml, mouse anti-chicken CD28 (both from SouthernBiotech, Birmingham, AL, USA) and 1:50 conditioned medium from the supernatant of a COS-7 cell culture transfected with a pcDNA1 vector (Invitrogen™) encoding for recombinant chicken IL-2 (kind gift from prof. Pete Kaiser and dr. Lisa Rothwell) [13]. The plates containing the cells were wrapped in aluminum foil and incubated for 4 days at 41°C and 5% CO_2.

Flow cytometric assessment of proliferation

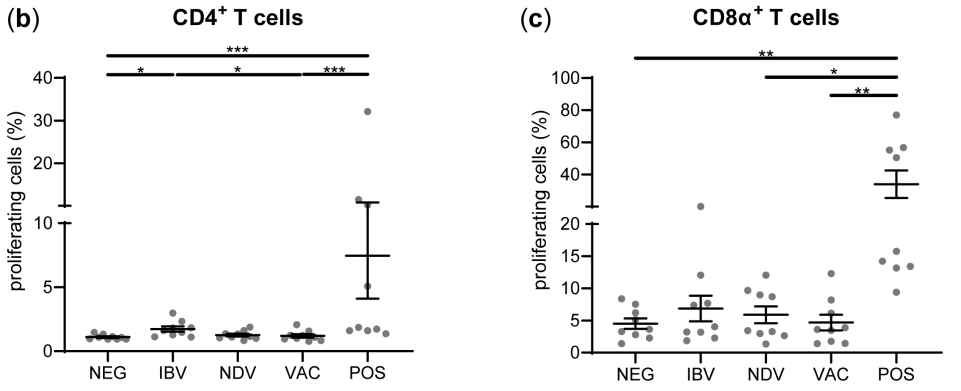
After incubation, cells were resuspended and transferred to a 96 wells V-bottom plate, centrifuged for 3 min at 400x *g*, and stained in 30 µl PBS with ViaKrome 808 viability dye (Beckman Coulter Inc., Brea, CA, USA) for 20 min at 4°C in the dark. The cells were centrifuged for 3 min at 400x *g* and washed once in FACS buffer (PBS containing 0.5% BSA and 0.005% NaN3, both from Sigma-Aldrich). The cells were stained in FACS buffer containing the monoclonal antibodies mouse anti-chicken CD3-PE (clone CT-3, 2 µg/ml), mouse anti-chicken CD4-FITC (clone CT-4, 1.25 µg/ml) and mouse anti-chicken CD8α-APC (clone CT-8, 0.25 µg/ml) (all from SouthernBiotech) for 20 min at 4°C in the dark. Finally, the cells were washed twice in FACS buffer, centrifuged for 3 min at 400x *g* in between, and resuspended in 100 µl FACS buffer. From each sample, 80 µl (containing at least $1.2x10^5$ and an average of $5.1x10^5$ cells) was analyzed using a CytoFLEX LX flow cytometer using 375-, 488-, 561-, 638-, and 808-nm lasers (Beckman Coulter Inc., Brea, CA, USA) and FlowJo Software v. 10.5 (FlowJo LCC, Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). The data were tested for the assumptions of normally distributed data using D'Agostino & Pearson test, which demonstrated that the results of several groups contained skewed data. Next, the results were tested for significant differences between groups using the Friedman test for nonparametric and matched data and Dunn's multiple comparisons test. Differences with *p*-values of < 0.05 were considered significant.



Before vaccination



After vaccination

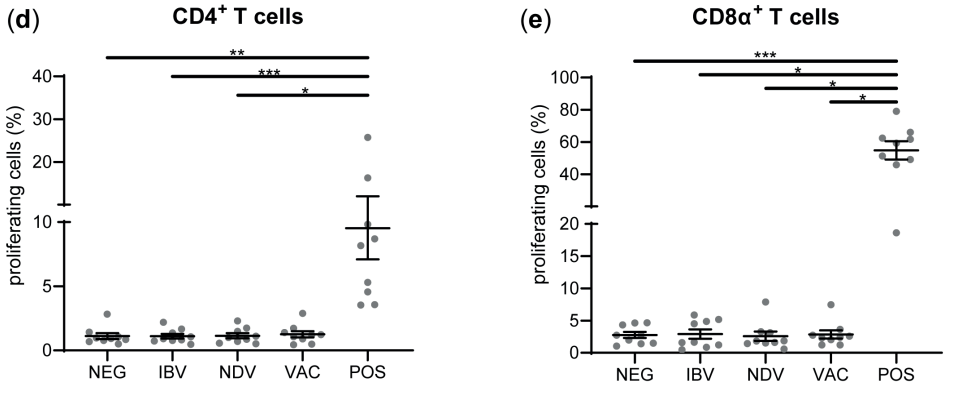


Figure 1. T cell proliferation in response to inactivated IBV and NDV, before and after vaccination. PBMCs were isolated from blood of chickens, labelled and analyzed by flow cytometry after 4 days of stimulation. The gating strategy that was used includes gating for single cells (FSC-A/FSC-H), live cells (ViaKrome 808-), lymphocytes (FSC-A/SSC-A), T cells ($CD3^+$), and $CD4^+$ T cells or $CD8\alpha^+$ T cells (**a**). Finally, the percentages of $CD4^+$ (**b** and **d**) or $CD8\alpha^+$ T cells (**c** and **e**) that proliferated at least once were determined by gating the cells that showed reduced fluorescence of CTV. Proliferation was assessed just before (**b** and **c**) and three weeks after booster vaccination (**d** and **e**). Stimulation was performed with allantoic fluid as a negative control (NEG), inactivated IBV antigens, inactivated NDV antigens, an inactivated bivalent *IBV + NDV* vaccine (VAC), and a combination of mouse anti-chicken CD3, mouse anti-chicken CD28 and recombinant chicken IL-2 as a positive control (POS). Every scatter plot shows the results of nine chickens. The error bars show the standard error of the mean. Statistical significance is shown by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Results

T cell proliferation after *ex vivo* stimulation was analyzed for 9 out of 10 chickens, since the amount of blood from 1 chicken was not sufficient to isolate the number of cells required to perform the assay. Proliferation of $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry. The gating strategy used to determine the percentages of proliferating $CD4^+$ T cells and $CD8\alpha^+$ T cells is shown in **Figure 1a**.

Before vaccination, *ex vivo* stimulation with allantoic fluid as negative control resulted in 1.1% (standard error of the mean: ±0.1%) proliferating $CD4^+$ T cells (**Figure 1b**) and 4.5% (±0.1%) proliferating $CD8\alpha^+$ T cells (**Figure 1c**). T cell proliferation significantly increased after stimulation with the positive control, both for $CD4^+$ T cells (7.5% ±3.3%) and $CD8\alpha^+$ T cells (34% ±8.6%). The percentage of proliferating $CD4^+$ T cells increased significantly to 1.7% (±0.2%) after stimulation with IBV antigens, compared to the negative control. Proliferation of $CD8\alpha^+$ T cells only tended to increase upon stimulation with IBV antigens, which resulted in 6.9% (±2.0%) proliferating cells. Upon stimulation with inactivated NDV antigen or the inactivated IBV + NDV vaccine, no significant changes in proliferation were observed.

Three weeks after the booster vaccination with the inactivated vaccine, stimulation with allantoic fluid as negative control resulted in 1.1% (±0.2%) proliferating $CD4^+$ T cells (**Figure 1d**) and 2.8% (±0.5%) proliferating $CD8^+$ T cells (**Figure 1e**). Again, stimulation with the positive control resulted in a significant increase in the proliferation of $CD4^+$ T cells (9.5% ±2.4%) and $CD8\alpha^+$ T cells (55% ±5.6%). In contrast to before vaccination, no significant changes were observed in the proliferation of $CD4^+$ T cells and $CD8\alpha^+$ T cells upon stimulation with inactivated IBV antigens. Moreover, no significant changes were observed in the proliferation of T cells upon stimulation with inactivated NDV antigens or the inactivated IBV + NDV vaccine.

Taken together, although T cell proliferation was readily observed upon polyclonal stimulation and, though to a lower extent, after IBV-specific stimulation before vaccination, neither IBV-specific nor NDV-specific proliferation was increased 3 weeks by the booster vaccination.

Discussion

The aim of the present study was to evaluate *ex vivo* proliferation of T cells isolated from the blood of laying hens vaccinated against IBV and NDV as an indication for the suitability of T cell proliferation assays for routine vaccine quality control. Since the laying hens of the current study were previously subjected to a standard vaccination regimen, the conditions were similar to the field situation in which application of inactivated (booster) vaccines is preceded by that of live attenuated vaccines to prime the immune response early in life. The set-up of the proliferation assay used in this study was previously optimized and used to demonstrate the induction of *Salmonella enteritidis*-specific T cells after infection (Meijerink et al., *in preparation*).

We observed T cell proliferation upon *ex vivo* stimulation with inactivated IBV antigen in PBMCs from 33-weeks-old layers exposed to a standard vaccination regimen. Unexpectedly, this response was no longer observed three weeks after an additional booster vaccination with an inactivated IBV/NDV vaccine. A previous study using 3-week-old specific-pathogen-free (SPF) laying hens showed that vaccination with a single inactivated IBV vaccine resulted in a small increase in T cell proliferation up to 33 days [12]. Similar results were obtained after vaccination chickens with a live attenuated vaccine against IBV followed by an inactivated vaccine after three moths [12]. Thus, vaccination with an inactivated vaccine against IBV results in a limited induction of CMI. In contrast, infection with virulent IBV leads to a strong induction of CMI between 2 and 6 weeks after infection, as demonstrated by T cell proliferation upon stimulation with inactivated IBV *ex vivo* [14]. Eighteen weeks before the chickens entered the current study they were vaccinated with a live attenuated vaccine for IBV. Likely, the IBV-specific T cell response that we observed *ex vivo* before vaccination at 33 weeks resulted from previous vaccination with live attenuated vaccines. Three weeks later, at 36 weeks, the frequency of IBV-specific T cells might have declined to undetectable levels. This rapid decline in IBV-specific T cells after vaccination or infection has been observed by others before [12].

Ex vivo stimulation of T cells with NDV or a bivalent vaccine against IBV and NDV did not result in increased T cell proliferation. Previous studies that evaluated CMI in chickens vaccinated with NDV showed different outcomes. Some indicated CMI in chickens that received live attenuated vaccines [8,9] or in chickens that were vaccinated with a combination of live attenuated and inactivated vaccines [8,10], however, without specifically looking at the effect of the inactivated vaccine. The induction of T cell proliferation was shown to be highly dependent on MHC haplotype as well as highly variable even within haplotypes [10]. Another study evaluated the effect of vaccination of naive chickens with an inactivated vaccine against NDV between 1 and 4 weeks after vaccination, but observed only limited interferon-γ production after 4 weeks and no T cell proliferation *ex vivo* [11]. Although the authors suggested that more than a single vaccination might be required to detect CMI, we were not able to demonstrate CMI in chickens that were vaccinated multiple times against NDV.

This study shows that although T cell proliferation was readily observed upon polyclonal stimulation, it remains difficult to detect proliferation of low frequencies of IBV- or NDV-specific (memory) T cells upon *ex vivo* stimulation. Further optimization of the assay

methods may be needed to enable measurement of low frequencies of antigen-specific T cells by proliferation. During optimization of the T cell proliferation assay, we did already assess whether antigen-presenting cells were present in the culture, which was confirmed by the presence of cells expressing major histocompatibility complex class II at both the start and the end of the culture (*not shown*). Secondly, like in a previous study we showed that X-VIVO 15 culture medium, the medium that was used in this study, is suitable to perform chicken T cell proliferation assays [8]. Thirdly, the 4-day stimulation period that was used in the T cell proliferation assay of the current study was relatively short, though in line with a previous study showing CMI after vaccination against NDV [10]. Since a previous study has demonstrated that the proliferative capacity of T cells declines with ageing [12], the age of the chickens used in our study might explain our difficulty to detect CMI by T cell proliferation. Furthermore, doses of the antigens and the vaccine used for stimulation might have been insufficient to induce proliferation of antigen-specific T cells, but higher concentrations were found to be toxic for the cells in optimization experiments prior to this study. Purified or recombinant antigens could be used in future studies to reduce toxicity. Alternatively, an explanation can be that the inactivated poultry vaccine against IBV and NDV does not strongly induce CMI, but rather depends on the induction of antibody-mediated responses. This needs to be addressed by future studies.

Based on the results of this study, the presented T cell proliferation, assay, even though functioning properly after polyclonal stimulation, was not sensitive enough for routine quality control of inactivated poultry vaccines for IBV and NDV. Future studies with slightly younger chickens or antigens of higher dose are warranted to potentially optimize the assay.

Acknowledgements

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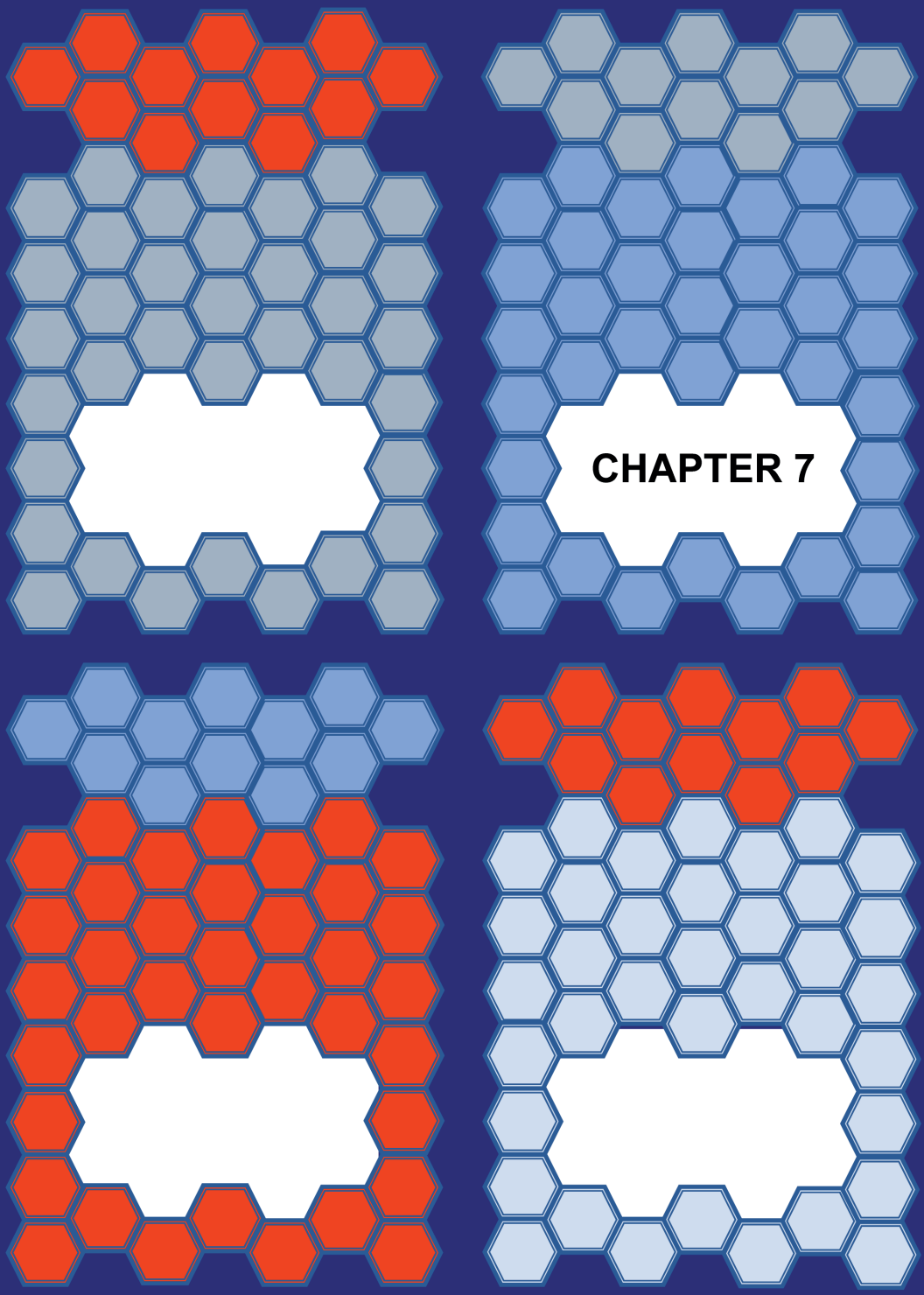
CHAPTER 7

Overcoming scientific barriers in the transition from *in vivo* to non-animal batch testing of human and veterinary vaccines

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(Submitted)

Abstract

Before their release on the market, vaccine batches are assessed for quality to evaluate whether they meet the product specifications. Batch tests of many human and veterinary vaccines, and in particular of inactivated and toxoid vaccines, still largely rely on *in vivo* methods. Improved vaccine production processes, ethical concerns, and suboptimal performance of some *in vivo* tests have led to a global intent to develop *in vitro* alternatives. Currently, some *in vivo* potency and safety tests have been replaced by *in vitro* assays. However, for many *in vivo* tests such alternatives are not yet available or poorly implemented. In this review, we focused on scientific constraints that need to be overcome for replacement of *in vivo* batch tests of vaccines, as well as potential solutions. Topics include the critical quality attributes of vaccines that require testing, the use of cell-based assays to mimic relevant aspects of *in vivo* vaccine-induced immune responses, how difficulties with testing adjuvanted vaccines *in vitro* can be overcome, the use of altered batches to validate new *in vitro* test methods, and how cooperation between different stakeholders is key to move the transition forward.

Background

Vaccination is a cost-effective strategy to prevent infectious diseases among humans and livestock. Vaccines are biologicals that are used to immunize large groups of healthy individuals and they may be subject to inherent batch-to-batch variation, which is why newly produced vaccine batches require quality assessment before being released to the market. Vaccines have traditionally been generated using trial and error approaches involving *in vivo* experiments [1]. Subsequently produced batches of such established vaccines are often still tested using *in vivo* methods to confirm that the quality of the new vaccine batch meets the specifications as defined in the marketing authorization. These specifications concern purity (i.e., freedom from extraneous matter), potency (i.e., the capacity of a vaccine batch to exert its effect), safety (i.e., relative freedom of harmful effects) and efficacy (i.e., effect of vaccination on the target species/population under ideal circumstances) of the vaccine [2]. Over one million animals were used for batch potency and safety testing of medicinal products in the EU in 2017, which corresponds to approximately 12% of total animal use for scientific purposes in the EU [3]. Nowadays, there are several reasons to move away from the use of these *in vivo* potency and safety tests to assess the quality of vaccine batches. Firstly, significant improvements in and standardization of the vaccine production process, adherence to good manufacturing practice (GMP) guidelines and in-process controls have resulted in less batch-to-batch variation and a lower risk of producing unsafe or ineffective products [4]. Secondly, the use of large numbers of animals in experiments that may inflict pain and distress is not in line with the ethics of contemporary research and the 3Rs principles of Replacement, Reduction and Refinement [5–7]. Thirdly, the relevance of some *in vivo* tests is disputed because the test results show high variability and poor reproducibility [7–12]. Fourthly, the use of animal models is expensive, time consuming and risky for personnel when models involve exposure to viable pathogenic organisms [7].

The consistency approach was proposed as a strategy to enable the transition of *in vivo* to *in vitro* batch testing of vaccines [13,14]. This approach is based on the principle that quality is the consequence of consistent production of subsequent batches monitored by a GMP quality system [15]. The evaluation of a number of pre-defined vaccine parameters using *in vitro* physicochemical, immunochemical and cell-based test methods should demonstrate that final batches are of consistent quality, making the use of *in vivo* tests unnecessary.

Major steps have been taken to promote the use of *in vitro* alternatives for vaccine batch testing, including the creation of a legal and logistic framework. For instance, Directive 2010/63/EU on the protection of animals used for scientific purposes, which includes regulatory testing of vaccines, states "The use of animals for scientific or educational purposes should only be considered where a non-animal alternative is unavailable" and thus promotes the use of novel *in vitro* test methods [16]. In addition, a general chapter on the "Substitution of *in vivo* methods by *in vitro* methods for the quality control of vaccines" has been incorporated in the European Pharmacopoeia to provide guidance on the implementation of the consistency approach in vaccine quality testing (5.2.14; [17]). Bodies like the Biological Standardisation Programme of the European Directorate for the Quality of Medicines & Healthcare (EDQM), which facilitates multi-center validation studies [18], and the European Centre for Validation of Alternative Methods (ECVAM), which acts as a reference laboratory of the European Union [16], provide the logistic framework needed to

validate novel *in vitro* methods. Meanwhile, some *in vivo* tests of vaccines have been replaced by *in vitro* alternatives, whereas others are no longer used as will be discussed further below.

In September 2015, industrial, regulatory and scientific experts gathered for a workshop in the Netherlands to identify drivers and barriers for the implementation of the consistency approach, of which the results were published [15]. Newer generation vaccines, including recombinant subunit vaccines (e.g. human papillomavirus and hepatitis B) or conjugate vaccines (e.g. *Haemophilus influenzae* type b, meningococcus and pneumococcus) are well defined and can be evaluated for quality using *in vitro* methods [17,19,20], which is why the workshop focused on established live attenuated, inactivated and toxoid vaccines that were in part still tested for quality using *in vivo* methods. Identification and development of *in vitro* methods that provide alternatives to currently used *in vivo* batch tests of vaccines is hampered by several scientific and other constraints including the following points [15]:

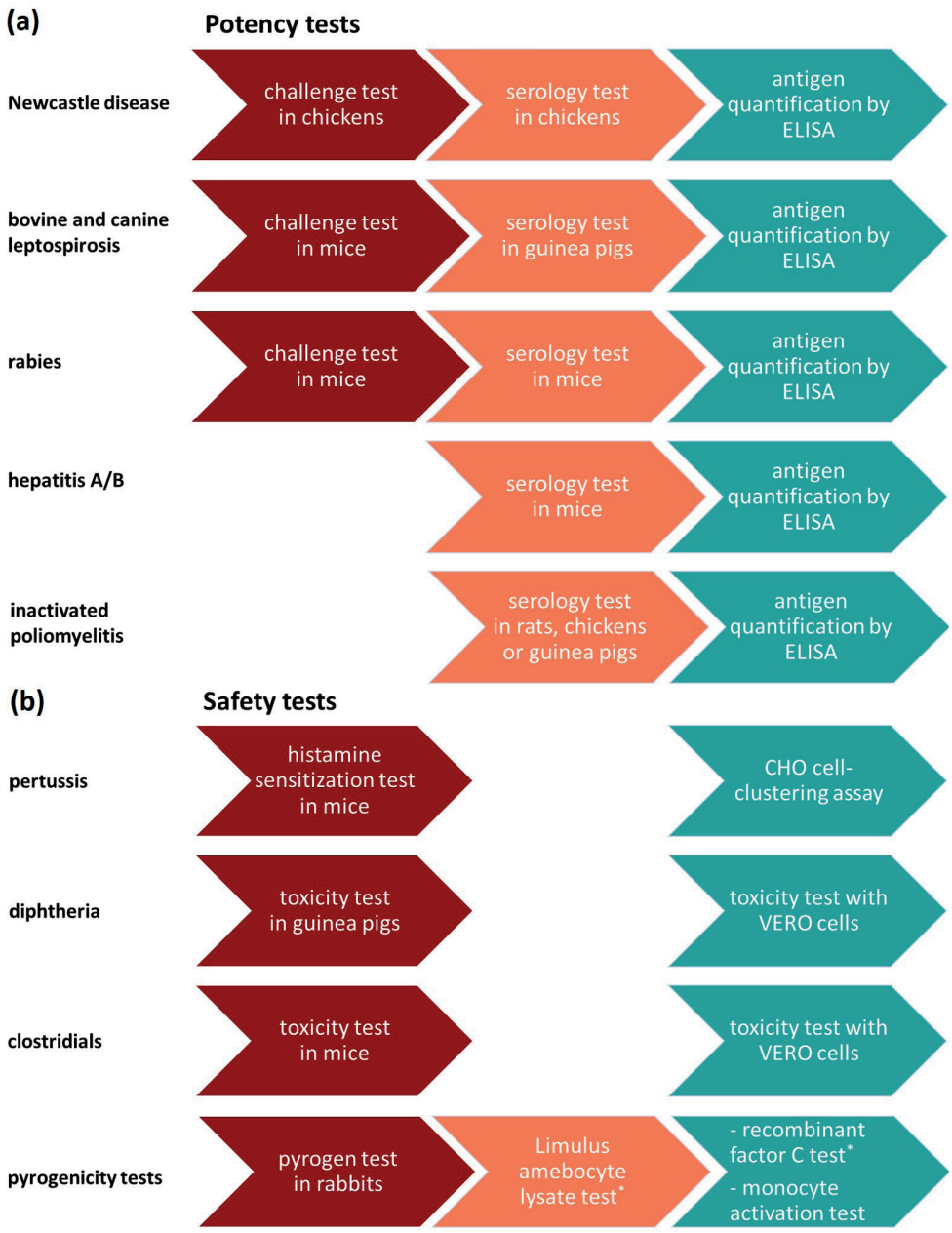
- It is difficult to mimic vaccine-induced immune responses using *in vitro* test methods.
- Measuring vaccine properties of adjuvanted vaccines is complex.
- There is a need to create subpotent formulations for method validation due to a lack of appropriate non-compliant batches to test (and validate) *in vitro* methods.
- Research into *in vitro* test methods needs to be prioritized and financed based on number of test animals, level of severity, and performance of the *in vivo* test.
- Knowledge about the critical quality attributes of vaccines and critical process parameters of vaccine production is often limited.

In the present review we will discuss the scientific barriers that have been overcome since the workshop or still need to be dealt with to enable a complete transition from *in vivo* to *in vitro* vaccine quality testing.

Current repertoire of *in vitro* vaccine quality tests

Despite the constraints described above [13], several human and veterinary vaccines already underwent the transition from *in vivo* to *in vitro* batch testing (**Figure 1**). These vaccines could provide a roadmap to guide the transition to *in vitro* for the vaccines that are currently still tested for potency and safety using *in vivo* methods.

For live attenuated vaccines, comprising bacteria or viruses, the use of *in vivo* batch tests is in general not demanded in the European Pharmacopoeia (**Supplementary Table 1**) [17]. Due to their replicative nature, live attenuated vaccines can be tested for potency by viral or bacterial titration. Some live attenuated viral vaccines (e.g. influenza virus and viral poultry vaccines) are propagated and titrated on embryonated chicken eggs when suitable cell lines are not available. These chicken embryos are not regarded as laboratory animals in view of the European act on the protection of animals used of scientific purposes (Directive 2010/63/EU) is not applicable to fetal stages of birds [16]. However, the embryos may experience pain [21] and cell-based alternatives to propagate and titrate these viruses are therefore desirable whenever available. Moreover, virus propagation through cell lines allows faster upscaling during epidemics and is better standardized than propagation through eggs [22]. Finally, the transportation of chicken eggs may be prohibited by governments during avian influenza outbreaks.



* These alternatives only test for endotoxin pyrogens.

Figure 1. For several established inactivated and toxoid vaccines for human and veterinary use there has already been a transition from *in vivo* to *in vitro* batch testing in Europe [5]. Advancements have been made for both potency (**a**) as well as safety tests (**b**). Conventional *in vivo* tests are shown in red. Alternative *in vivo* tests with reduced and refined use (i.e., less animals and less discomfort) of animals are shown in orange. Alternative *in vitro* tests are shown in green.

Unlike live attenuated vaccines, inactivated vaccines and toxoid vaccines mostly rely on vaccination-challenge or vaccination-serology potency tests, unless antigen quantification methods are available as *in vitro* alternatives (**Supplementary Table 1**). These antigen quantification methods may be physiochemical or immunochemical assays that are specific for one or more dominant antigens of the vaccines. In particular, the development of enzyme-linked immunosorbent assays (ELISAs) for antigen quantification has been successful for the replacement of *in vivo* potency tests of several vaccines in Europe, including those against Newcastle disease in poultry [23], foot-and-mouth disease in cattle [24], leptospirosis in cattle and dogs (monographs 0447 and 1939; [17]), rabies [25] in both animals and humans, and hepatitis A [26] and B [20] in humans (**Figure 1a**). Other ELISAs for antigen quantification have shown to be successful in determining the potency of some but not all inactivated vaccines and toxoid vaccines, but these are not yet approved by the regulatory authorities in Europe. These include tests for infectious bronchitis virus and infectious bursal disease virus vaccines for poultry [27], furunculosis vaccines for salmonids [28], tetanus vaccines for human [29] and veterinary [30] use, and diphtheria vaccines for human use [31,32]. In addition to the ELISA, another important antigen quantification method is the immunodiffusion test, which is used to determine the content of hemagglutinin antigens of inactivated or subunit influenza vaccines [33]. For the whole-cell and acellular pertussis vaccines, there have been efforts to develop *in vitro* ELISA [34], Luminex [35], cell-based [36,37] and proteomic assays [38] as alternatives.

With respect to safety, alternative *in vitro* methods have also become available in Europe (**Figure 1b**). Originally, vaccines were tested for pyrogenicity using the Rabbit Pyrogen Test (RPT), in which temperature changes in rabbits are evaluated after injection of a vaccine. To specifically quantify endotoxin pyrogens, comprising bacterial lipopolysaccharides (LPS), the Limulus Amebocyte Lysate (LAL) test is used. Although the LAL test is legally not regarded as an animal test, 100,000 horseshoe crabs die annually due to the bleeding [39], which impacts the survival of the species classified as threatened by the International Union for Conservation of Nature [40]. Nowadays, the blood clotting factor C (rFC) from the horseshoe crab is commercially available as a recombinant protein [39], but the rFC test has been implemented to a limited extent despite its proven efficacy [39] and its adoption in the European Pharmacopoeia as alternative reagent in endotoxin tests (general text 2.6.14, 2.6.32 and 5.1.10; [17]). The Monocyte Activation Test (MAT) was developed to detect both endotoxin and non-endotoxin pyrogens [41–43]. In this test human whole blood or (cryopreserved) PBMCs are stimulated with the vaccine of interest after which the production of pro-inflammatory cytokines (TNF, IL-1β and IL-6) is assessed in ELISAs. The European Pharmacopoeia states that whenever possible and after product-specific validation, the RPT test should be replaced by the MAT (general text 2.6.8; [17]). To justify the use of the LAL test or the rFC test as alternatives for the RPT test, a risk assessment using the MAT is recommended to rule out the presence of any non-endotoxin pyrogens in a vaccine (general text 5.1.10). These statements promote the use of the MAT as a safety test for pyrogenicity or bacterial endotoxins. The MAT test was recently optimized for pyrogenicity testing of the Encepur vaccine against tick-borne encephalitis [41].

To test for residual toxicity of toxoid vaccines *in vitro* alternatives have been developed including the Chinese hamster ovary (CHO) cell clustering assay as an alternative to the *in vivo* Histamine Sensitization Test (HIST) for acellular pertussis vaccines [44]. In addition,

VERO cells toxicity assays to use instead of mouse toxicity tests for veterinary *Clostridium septicum* vaccines are currently being validated [45]. Finally, the binding and cleavage (BINACLE) assay evaluates residual tetanus toxicity of human tetanus vaccines [46], as an alternative for the currently used test in guinea pigs, and is currently being validated as part of the European Biological Standardisation Programme.

In summary, although the quality of many inactivated vaccines and toxoid vaccines is still tested with *in vivo* potency tests, an increasing number of *in vitro* alternatives are being developed to achieve their replacement. These alternatives include immunochemical assays to quantify antigen, but also tests for other critical properties of vaccines, as will be discussed in the next section. For safety tests, many *in vitro* alternatives are already available in Europe and implementation of these methods has now become important to move the transition to *in vitro* safety testing forward.

Critical quality attributes of vaccines

Critical quality attributes of vaccines are physical, chemical or microbiological properties of vaccines that should be within certain limits to ensure vaccine quality [47]. The *in vitro* methods that are currently in place for potency testing of vaccine batches are mainly used to evaluate antigen identity, quantity and integrity (**Figure 1a**). However, antigen quantification methods often depend on the integrity of a single antigen [48], while at population level vaccination efficiency hardly ever depends on a single dominant epitope. Furthermore, the ability of immunochemical assays to measure the relevant epitopes of antigens inactivated with agents like formaldehyde and β-propiolactone should be validated. The inactivation may alter or hide specific epitopes of antigens and hence affect potency as measured *in vitro* [48–51], even when the potency as measured *in vivo* remains the same [48,50,51]. The use of a pool of monoclonal antibodies in ELISAs can result in more consistent and standardized quality testing of vaccines, without depending too much on single epitopes [48]. In addition to antigen identity, quantity and integrity characteristics, the potency of vaccines may depend on critical quality attributes like vaccine composition, susceptibility of the antigen to proteolytic degradation, the spatial organization (i.e., three-dimensional structure) of the antigen, or the presence of additional immunostimulatory molecules. A combination of assays addressing these different critical quality attributes may thus be required to sufficiently demonstrate batch-to-batch consistency for some vaccines.

The vaccine composition depends on production processes, and inconsistencies in these processes may affect the potency of the vaccine. For instance, for whole-cell pertussis vaccines it was shown that disturbances of the bacterial culture conditions may results in the downregulation of important virulence proteins [52]. Mass spectrometry-based proteome analysis was proposed as a method to evaluate the whole protein composition of vaccines to detect these disturbances [38]. Another applicability of mass spectrometry is the assessment of the stability of antigens and their susceptibility to enzymatic degradation as an important part of antigen processing by antigen-presenting cells [53]. Inactivation by formaldehyde [54] and heat exposure [55] were shown to affect the degradation kinetics of a model antigen and tetanus toxoid, respectively. The importance of the spatial organization of antigens has been demonstrated for influenza vaccines by showing that during a priming vaccination whole-inactivated influenza vaccines induced higher antibody titers, both in mice

[56,57] and humans [58], and superior T cell responses in humans [59] as compared to the less reactogenic split vaccines with spatially disrupted antigens [60]. The antigens of the split vaccine lack the proper spatial organization to efficiently induce antibody production [61]. Similar results have been found for detergent-treated inactivated vaccines against Newcastle disease virus for use in poultry [62]. Finally, the presence of immunostimulatory molecules, which may include exogenous adjuvants or endogenous pathogen-associated molecular patterns (PAMPs), is important for vaccine potency. The contribution of PAMPs to vaccine potency has been demonstrated for both viral (e.g. influenza [57]) and bacterial (e.g. pertussis [36,63]) vaccines. Similar to the antigens, PAMPs can be destroyed or become less accessible by inactivating agents like formaldehyde or β-propiolactone [49,64]. The presence of immunostimulatory molecules can be evaluated using cell-based assays, as discussed in more detail below. The described quality attributes may require additional testing when antigen identity, quantity and integrity characteristics are insufficient to predict the potency of a vaccine.

The previous section included *in vitro* alternatives to test vaccines for pyrogenicity and to test toxoid vaccines for residual toxicity. Furthermore, toxicity tests that were found to be unnecessary based on historical data have recently been deleted from the European Pharmacopoeia, including abnormal toxicity tests (to detect any unexpected hazards), some specific toxicity tests of human vaccines, and some residual toxicity tests of veterinary vaccines [65,66]. However, the BINACLE assay is not suitable for use with adjuvanted tetanus toxoids [46]. In contrast, the porcine actinobacillosis vaccine, porcine progressive atrophic rhinitis vaccine, and tetanus vaccine for veterinary use still require animal-based toxicity tests [17,65]. Moreover, safety tests of the Bacillus Calmette-Guérin vaccine, used to protect against tuberculosis, include the virulent mycobacteria test and the excessive dermal reactivity test, both in guinea pigs [17], to show absence of virulence and excessive reactogenicity, respectively. An alternative safety assay based on the proliferation of lymphocytes from sensitized guinea pigs has been proposed instead of the currently used excessive dermal reactivity test [67]. A vaccine for rabbit hemorrhagic disease still requires a residual live virus safety test in rabbits [17]. Some live attenuated viral vaccines (e.g. smallpox and poliomyelitis) require neurovirulence safety testing in monkeys or transgenic mice [17], although deep sequencing methods have been proposed as an alternative strategy to test these vaccines for genetic instability and to prevent the occurrence of neurovirulent viral mutants [68,69]. Recently, a model based on brain cells in a transwell system, named the BBB-Minibrain culture device, was developed as a next step in search for an alternative neurovirulence test [70]. Finally, batch release of pertussis vaccines still requires a test for residual dermonecrotic toxin in mice [17]. Recently, the use of liquid chromatography mass spectrometry to quantify dermonecrotic toxin has been proposed as an alternative *in vitro* method [71].

To summarize this section, antigen identity, quantity and integrity can be considered the most important critical quality attributes of inactivated and toxoid vaccines. However, additional quality attributes including vaccine composition, the spatial organization of the antigen, or the presence of additional immunostimulatory molecules may be critical for specific vaccines. Importantly, it is increasingly recognized that one-to-one replacement of an *in vivo* test for an *in vitro* test will be difficult and that a combination of assays to demonstrate batch-to-batch consistency may be needed for some vaccines [11,72].

Mimicking *in vivo* vaccine-induced immune responses using *in vitro* cell-based assays

The use of immunochemical and physicochemical methods for potency testing are based on evidence showing that consistency of batches, with regard to one or more aspects like antigen identity, antigen quantity, antigen integrity, antigen structure, vaccine composition, and quantity of immunostimulatory molecules will lead to consistent outcomes *in vivo*. For vaccines that are well defined (e.g. recombinant and subunit vaccines) the use of these methods may indeed be sufficient to guarantee the potency and safety of a vaccine. However, for less defined inactivated and perhaps even toxoid vaccines, additional information on the immunostimulatory capacity of vaccines may be needed to generate sufficient evidence about vaccine quality when *in vitro* methods are used. Cell-based assays can assess aspects of vaccine-induced immune reactivity and may identify critical quality attributes of vaccine.

An immune response is initiated by innate immune cells (**Figure 2**; step 1), among which professional antigen-presenting cells, including dendritic cells, macrophages and B cells process and present antigen, and in addition express co-stimulatory molecules and release cytokines to activate T cells (**Figure 2**; step 2). Several methods have been proposed to evaluate vaccine potency in cell-based assays with immune cells using either primary cells or immortalized cell lines [37,73–77]. Primary dendritic cell-based assays use monocyte-derived dendritic cells (moDCs) [37,75–77] or bone marrow-derived dendritic cells (BMDCs) [74]. Whereas primary cells more closely represent the physiological nature of immune cells, they are collected from animals or human donors, often have a limited lifespan, are available in limited numbers, and may show variable responses due to genetic diversity of the individual donors. Cell lines do not have these disadvantages and are thus being explored for use in a vaccine quality control setting (**Table 1**), even though they may be less representative for the *in vivo* situation than primary cells. Most cell-based potency assays measure vaccine-induced activation of dendritic cell-, monocyte- and macrophage-like cells by expression of co-stimulatory molecules (e.g. CD40, CD80, CD83, CD86) or pro-inflammatory cytokines. Furthermore, dendritic cells have been used in *in vitro* antigen degradation assays, in which the susceptibility of antigens for proteolytic degradation is used as a biomarker for immunogenicity [53]. Reporter cell lines like PRR-expressing human embryonic kidney 293 (HEK)-Blue cells have been used to evaluate the immunostimulatory properties of clinical isolates of *Bordetella pertussis* [78,79] and may also be useful to test vaccines for potency.

Assays with innate immune cells have also been used to evaluate safety aspects of vaccines. Vaccine pyrogenicity can be evaluated by the MAT using monocytic cell lines like the MM6 cell line [86]. Interestingly, the MM6 cell line has been explored for use in *in vitro* safety tests [81], as well as potency tests [37], both using secretion of the pleiotropic cytokines IL-1β and IL-6 as readouts. The use of IL-1β and IL-6 as readouts for potency is in line with the role of these cytokines in T cell activation and differentiation [87,88], whereas their use as readouts for safety is in line with their ability to induce fever and other potential side effects of vaccination [81]. Thus, potency and safety limits of pro-inflammatory cytokines need to be identified beforehand to discriminate between potent and potentially dangerous levels of vaccine-induced immune responses [81].

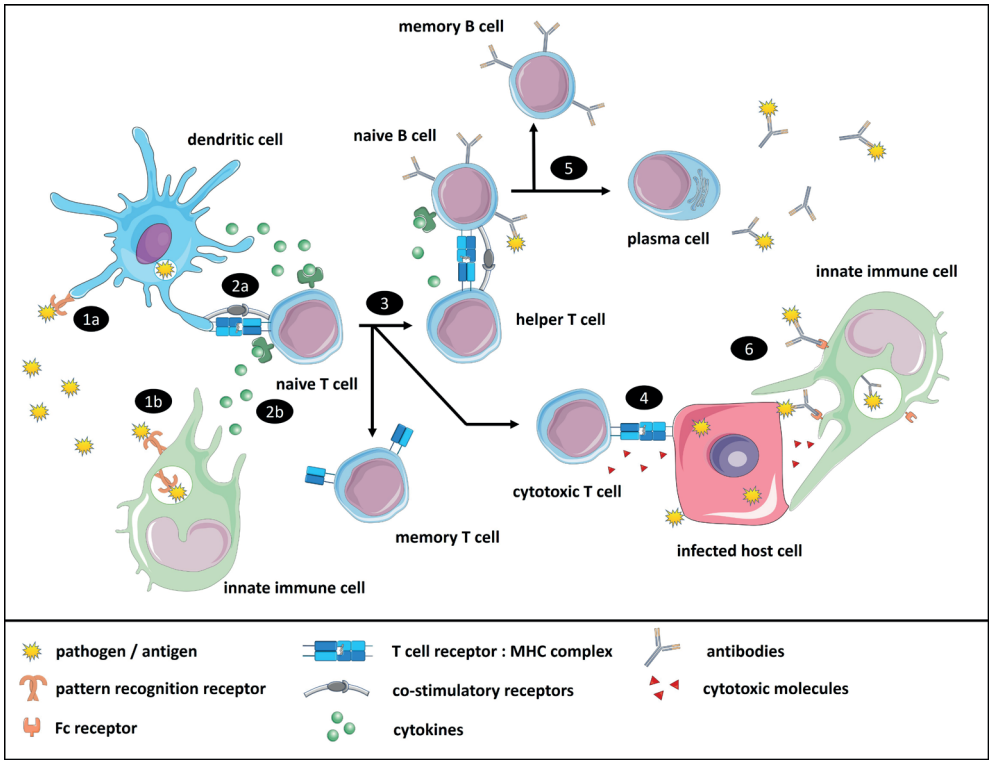


Figure 2. Simplified overview of a vaccine-induced immune response. (**1**) Innate immune cells recognize pathogen-associated molecular patterns (PAMPs), *e.g.* bacterial cell wall components or double-stranded RNA that are part of vaccine antigens, through pattern recognition receptors (PRRs). (**2**) Upon recognition, vaccine antigens are taken up by innate immune cells, and processed for antigen presentation on major histocompatibility complexes (MHC) class I and II to (naive) antigen-specific T cells. Innate immune cells orchestrate the adaptive immune response by releasing cytokines that affect the differentiation of T cells. (**3**) As a result, $CD4^+$ T cells differentiate into various types of $CD4^+$ helper T cells (T_H1, T_H2, T_H17, T_{REG} etc.) or memory T cells, whereas $CD8^+$ T cells differentiate into $CD8^+$ cytotoxic T cells, or memory T cells. (**4**) Cytotoxic T cells recognize antigen-derived peptides presented on MHC class I molecules of infected host cells that are subsequently killed. (**5**) Antigen-specific naive B cells that bind antigens through their B cell receptor will endocytose and process the antigens, and present these on MHC class II molecules to helper T cells. Upon recognition helper T cells will produce cytokines that facilitate B cell proliferation and differentiation into plasma cells or memory B cells. (**6**) Plasma cells produce antigen-specific antibodies that can neutralize or opsonize pathogens. Opsonized pathogens can be bound by Fc receptors on innate immune cells, resulting in the uptake and destruction of the pathogens. Furthermore, antibodies can bind to antigens exposed on the surface of infected host cells and stimulate antibody-dependent cellular cytotoxicity (ADCC) by innate immune cells. The icons used in this figure are adaptation from icons retrieved from the Servier Medical Art collection, which are licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

After the innate immune response is initiated by a vaccine, antigen-presenting cells will activate the adaptive immune system (**Figure 2**; step 3-5). An important aspect of the adaptive immune response for vaccination is the differentiation of B and T cells into effector and memory cells, of which the latter will quickly induce a secondary immune response upon

Table 1. Myeloid cell lines that have been described for use in potency testing of human and veterinary vaccines.

Name	Representative cell type	Responds to	Activation markers	Does not respond to	Ref.
MUTZ-3	human dendritic cells	*N. meningitidis* outer membrane protein	CD80, IL6, IL-8, TNF	LPS, *H. influenzae* type B polyribosyl ribitol phosphate	76
				Whole-cell *B. pertussis* vaccine	37
				LPS, R848, whole inactivated /	75
MM6	human monocyte	LPS, Pam3CSK	CD80, CD86, IL-1β, IL-6, IL-12p40, IL-10	subunit influenza virus	37
		whole-cell *B. pertussis* vaccine	IL-1β, IL-6, IL-12p40		37, 78
		N. meningitidis outer membrane protein	IL-1β, IL-6, IL-10, CXCL10		80
		LPS, FSL-1, Pam3CSK4, flagellin, R848	IL-1β, IL-6, IL-8, PGE2, TNF		81
THP-1	human monocyte	PMA+alum	IL-1β, CD80	Alum, MF59	82
		MDP+MPLA+alum	IL-1β, IL-8, TNF		83
HD11	chicken macrophage	Inactivated vaccines for infectious bronchitis virus, Newcastle disease virus	Phagocytosis		84
		Inactivated vaccine for *Av. paragallinarum*	Nitric oxide, IL-1β, CXCLi1, CXCLi2, IL-10		85

future encounter with the same pathogen. Batches of whole cell pertussis vaccines [89] and rabies vaccines [90] of different potencies were found to differ in their ability to induce T cell responses in splenocytes from vaccinated mice, suggesting that T cell assays can be used to test for vaccine potency when these T cell responses are known correlates-of-protection. The activation of naive T cells or the re-activation of memory T cells by vaccines has been mimicked *in vitro* using respectively autologous dendritic cell-T cell co-cultures or PBMCs to evaluate the potency of vaccines against yellow fever [91] and influenza [92] for use in humans, and for vaccines against blue tongue and rabies for use in cattle [93]. However, T cells are highly heterogenous and T cell assays have only been explored for use in pre-clinical development of vaccines. In addition, T cell assays with primary cells require blood collection from vaccinated animals, similar to serological assays. The use of a combination of several epitope-specific T hybridoma cell lines might be more promising for potency testing of vaccine batches [94], but requires further development.

The contribution of assays with innate immune cells to batch testing is product-specific and depends on the presence of PAMPs, adjuvants and other potentially immunostimulatory vaccine components. T cell assays may be useful when T cell responses are known correlates-of-protection. Due to their accessibility, infinitive lifespan, high numbers and low variability, (reporter) cell lines are particularly suitable for cell-based assays to test vaccines. Furthermore, cell-based assays can be useful to investigate whether there are any functional synergistic or antagonistic interactions between the adjuvant and other components in the vaccine, as will be discussed further below. Finally, the need for cell-based assays depends on whether the vaccine will be applied for priming or boosting the immune response. For example, booster vaccines may stimulate the reactivation of memory T cells independently of dendritic cells through memory B cells [93] and are therefore less dependent on stimulating innate immune cells than primer vaccines.

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Adjuvanted vaccines

Many inactivated, toxoid and subunit vaccines require adjuvants to support activation of the immune system. For a long period of time, only aluminum salts and mineral oil emulsions were used as adjuvants. Aluminium salts are used as adjuvants for human vaccines such as diphtheria and tetanus vaccines [96] as well as veterinary vaccines such as the bluetongue vaccine for ruminants, some feline leukemia vaccines and the rabies vaccine for dogs, cats, ruminants and horses [97]. The first emulsion adjuvants were Freund's incomplete adjuvant, based on mineral oil emulsified in a water-in-oil formulation, and Freund's complete adjuvant, formulated with tubercle bacteria [98]. Mineral oil-based water-in-oil adjuvants are too reactogenic for use in humans [99,100], but are widely used in poultry, cattle and fish. The first emulsion adjuvant to be approved in humans was MF59, which is an oil-in-water adjuvant based on squalene [101].

Before batch release, adjuvanted vaccines are tested for identity, concentration, physical properties like viscosity and stability of the adjuvant (monographs 0062 Vaccines for Veterinary Use and 0153 Vaccines for Human Use; [17]). However, emulsion and aluminum salt adjuvants may interfere with *in vitro* methods to evaluate the antigens of final products [102]. The following section describes how this problem can be circumvented by testing intermediary products before blending of antigens and adjuvants [103], by extracting the antigenic fraction from final products or by using *in vitro* methods that are not affected by adjuvants.

Methods to extract antigens from final adjuvanted products have been developed for several vaccines. To enable the quantification of antigen by ELISAs, isopropyl myristate has been used to extract antigens from oil-adjuvanted poultry vaccines for Newcastle disease, infectious bronchitis and infectious bursal disease [27,104]. Similarly, antigens extracted from an oil-adjuvanted poultry vaccine against infectious coryza could be used to evaluate the presence of PAMPs by measuring their effects on nitric oxide production or cytokine expression in cell-based assays [85]. Isopropyl myristate could not be used to extract antigens from vaccines for food-and-mouth disease, which are formulated with Montanide ISA201 oil adjuvant, but an extraction with benzyl alcohol could be used instead for this vaccine [105], showing that different emulsion adjuvants require different extraction methods. For aluminum salt-adjuvanted vaccines, electrophoretic [106] or chemical competitive desorption methods [107] can be used to retrieve the antigens, although the latter methods may result in denaturation of antigens and affect their integrity. Such methods have been used by manufacturers for hepatitis A vaccines in order to enable antigen quantification by ELISA, but for competitive reasons the methods were not disclosed in the resulting publications [108,109].

Not all quantification methods require antigen extraction or desorption from adjuvanted vaccines. With respect to emulsion adjuvants, a multiplexed sandwich immunoassay for influenza vaccines against strains with pandemic potential was shown to be compatible with the squalene-based adjuvant MF59 [110]. An in situ method based on the fluorescent nucleic acid-reactive dye SYBR Green II has been developed to determine the stability of an inactivated vaccine for foot-and-mouth disease and was found compatible with aluminum salt, water-in-oil and oil-in-water adjuvanted vaccines [111]. However, to the best of our knowledge there are currently no *in vitro* antigen-specific methods to determine the quality of adjuvanted vaccines formulated as water-in-oil emulsions that do not require extraction of the antigen. Methods that non-specifically determine protein content are compatible with aluminum salt adjuvants, including fluorescence spectroscopy, based on intrinsic fluorescence of tyrosine and tryptophan amino acids [112] or fluorescent protein-reactive chemicals [113], and chemiluminescent nitrogen detection [114]. These methods are not antigen-specific and therefore not suitable for vaccines that contain antigens in complex media with other proteins, e.g. allantoic fluid present in poultry vaccines [84,115]. Some antigen-specific immunoassays have shown compatibility with aluminum salt-adjuvanted vaccines, which include sandwich [116] and competitive [117] ELISAs, Luminex [35], and immunofluorescent assays analyzed with a fluorometer [118,119] or flow cytometer [120]. Cell-based toxicity assays can be made compatible for aluminum salt-adjuvanted vaccines by using semi-permeable transwell inserts to prevent direct contact between the cells and aluminum salts, as demonstrated by the CHO cell-clustering assay used to test for residual toxicity of pertussis vaccines [44].

In summary, adjuvanted vaccines create additional challenges for the development of *in vitro* potency and safety tests, but adsorption and extraction methods, and the development of adjuvant-compatible methods offer possibilities to overcome these.

Altered batches to validate *in vitro* test methods

Non-compliant vaccine batches of substandard quality are necessary for the validation of new *in vitro* tests methods by confirming their capacity to discriminate between batches of different qualities [121]. However, modern well-controlled vaccine production processes that adhere to GMP guidelines hardly result in failed batches anymore [11,15] and there may be reluctance to share the remaining non-compliant batches. This has led to a paradox in which improved quality of vaccines makes it more difficult to validate new *in vitro* test methods. Another problem is the inherent variability of *in vivo* release tests used as gold standards [7–12]. These *in vivo* tests may show variation to an extent that some batches fail the test, while they are in fact compliant, i.e. their capacity to induce protective immunity in the target species is sufficient. Such batches can be expected to pass appropriate *in vitro* tests. Likewise, batches may pass *in vivo* tests, while they are in fact non-compliant, i.e. their capacity to induce protective immunity in the target species is insufficient.

To solve these problems, vaccine batches could be altered intentionally, for example by exposure to stresses that may decrease the stability of a batch during the vaccine production process (e.g. decreased or increased pH, osmolality, temperature) [11,31,121,122], by creating vaccines with reduced antigen content [11,122] or a different composition [38], by deviating from the standard inactivation method [51,122,123], or by changing the degree of adsorption to adjuvants. Importantly, altered batches should be representative for non-compliant batches that may realistically be produced as a result of disturbances in vaccine production processes. In the European Pharmacopoeia, heat treatment is given as an example to create a proper altered batch of the inactivated poliomyelitis vaccines , which is used for product-specific validation of the *in vitro* D-antigen assay, in order to replace the former *in vivo* potency test in chickens, guinea pigs or rats (Ph. Eur method 2.7.20, [17]). The altered batches need to be designed and produced already during the development phase of *in vitro* test methods to identify the sensitivity of these assays for detecting non-compliant batches.

Cooperation between academia, industry and regulatory institutions is crucial for the transition from *in vivo* to *in vitro* batch testing of vaccines

Two previously identified constraints for the transition to *in vitro* batch testing of vaccines were the lack of priority of research into *in vitro* test methods that are most needed, and the varying knowledge about vaccine production processes among scientists of different disciplines [15]. International, multi-stakeholder (academia, industry and regulators) collaborations were described as key to move the transition to *in vitro* vaccine testing forward. Over the last five years, this necessity was met by the VAC2VAC consortium (http://www.vac2vac.eu/), funded by the Innovative Medicines Initiative (IMI2) programme. In this consortium, vaccines and their intermediary products (e.g. antigens, adjuvants, excipients and additives), knowledge about production processes, and new technologies for vaccine quality control were shared between the different stakeholders. The aim of the consortium was to develop new tests and approaches that support the use of the consistency approach in batch testing of established vaccines.

Assay development is only the first step of the transition from *in vivo* to *in vitro* batch testing, which has to be complemented by a validation process [124], first in small-scale feasibility studies to test the transferability of the assay to other labs, later in large-scale multi-center validation studies that are preferably conducted at an international or even global level to standardize novel methods [18]. The latter validation studies can be performed as part of the Biological Standardisation Programme in Europe, in which Official Medicines Control Laboratories (OMCLs), manufacturers and other stakeholders can participate. Assays that have been successfully validated and are accepted by regulatory authorities can become incorporated into pharmacopoeias [124]. After incorporation, the methods still need to be implemented by individual manufacturers and OMCLs, which have to conduct assay validation before the conventional animal-based test can be fully replaced and eventually eliminated from pharmacopoeias. Engagement between academia, industry and regulators at an early stage of the development of *in vitro* alternatives is essential for getting all stakeholders acquainted with new methods, to facilitate validation studies and to achieve wide implementation of the *in vitro* alternatives [15,18,72].

Conclusions

This review describes scientific barriers that hampered the transition from *in vivo* to *in vitro* batch testing of vaccines, especially for those that include inactivated pathogens, toxoids or pathogen subunits, and the opportunities to overcome these. An increasing number of *in vitro* potency and safety tests have been developed to replace *in vivo* tests. Currently implemented *in vitro* potency tests largely comprise ELISAs or other immunochemical methods that assess antigen identity, quantity and integrity characteristics, while other properties may also be critical for vaccine quality. There is an increasing consensus that one-to-one replacement by *in vitro* alternatives is not always possible and that a combination of *in vitro* alternatives may be needed. Cell-based assays can provide functional information about the interaction between a vaccine and the immune system, which is impossible to capture with ELISAs only. Adjuvanted vaccines are notably hard to test for quality using *in vitro* alternatives due to their incompatibility with some of these methods. However, the current scientific literature provides many possibilities to overcome these difficulties, including desorption and extraction methods of adjuvants and strategies to study the potential interactions between different vaccine antigens. The use of purposely altered batches enables the validation of new *in vitro* test methods when there is a lack of non-compliant batches originating from disturbances in the routine vaccine production process due to quality systems and GMP that are in place at the manufacturer. Finally, it is recognized that cooperation between scientists in academia, industry and regulatory institutes is key to move the transition to *in vitro* vaccine testing forward by sharing of products, knowledge and technologies.

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Supplementary Materials

Supplementary Table S1 can be provided by the authors on request.

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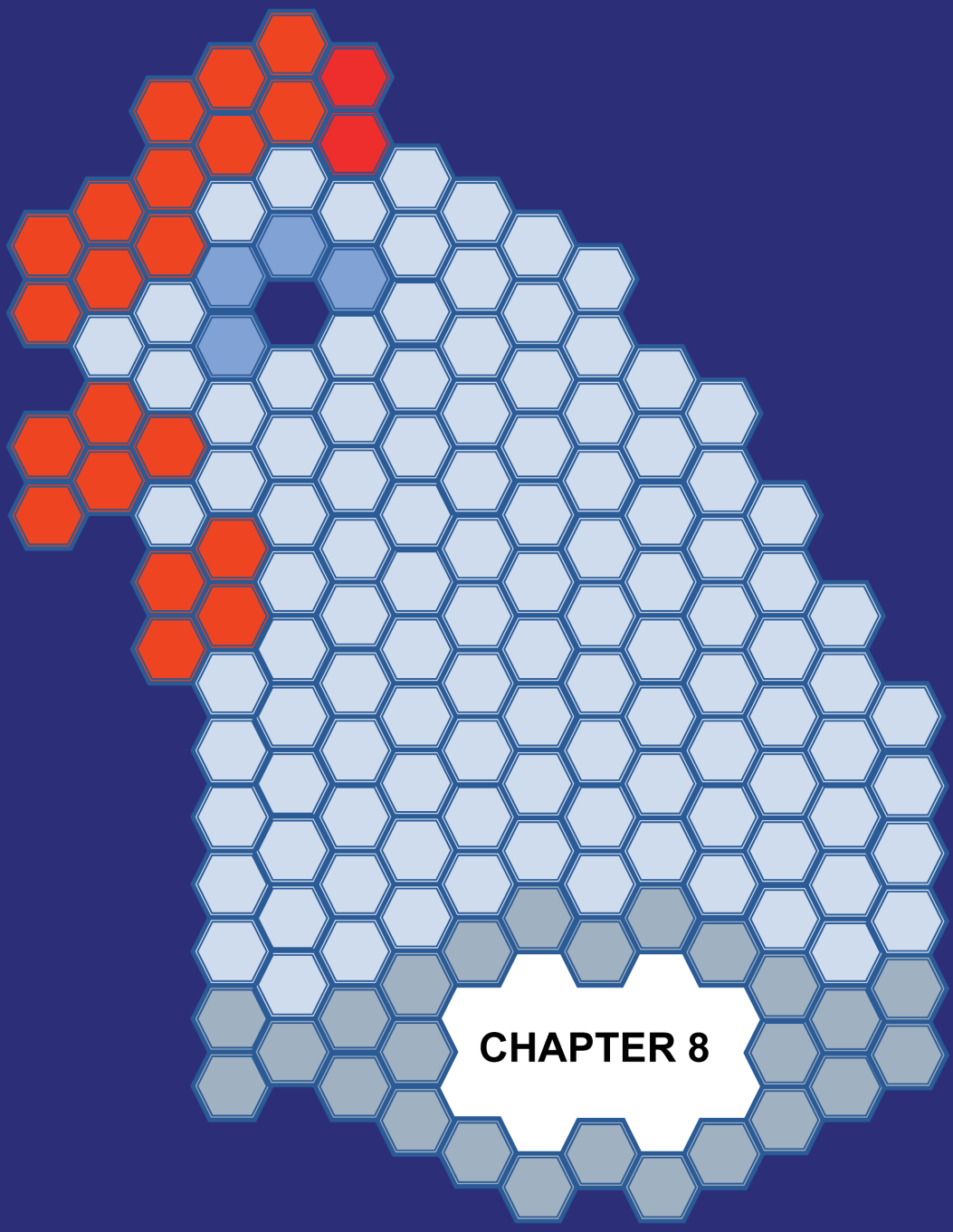
CHAPTER 8

Summarizing discussion

Summarizing discussion

More than a million animals are used annually in the European Union for batch release tests of medicinal products, predominantly for potency assessment [1]. A quarter of these tests are classified as causing severe pain and distress. With respect to inactivated poultry vaccines, most potency tests are still performed *in vivo* due to the lack of *in vitro* alternatives. It is important to move away from *in vivo* testing because of ethical concerns [2–5], variability, long duration and high costs of these tests [4,5], risks for personnel working with infected animals [4], and legal requirements to use *in vitro* methods instead of *in vivo* methods whenever possible [6]. The primary aim of this thesis was to set up *in vitro* cell-based assays to assess the immunostimulatory capacity of inactivated poultry vaccines and to determine their applicability for routine potency testing. For this purpose, we have set up novel cell-based assays using a macrophage cell line to assess the immunostimulatory capacity of inactivated poultry vaccines (**Chapters 2** and **3**). Secondly, we have used bone marrow-derived dendritic cells to search for novel biomarkers of immune activation by the vaccines (**Chapters 4** and **5**). Thirdly, we explored the applicability of a T cell proliferation assay in quality assessment of inactivated poultry vaccines (**Chapter 6**). Finally, we reviewed the recent progress made for the transition from *in vivo* to *in vitro* quality testing of veterinary and human vaccines (**Chapter 7**).

Cell-based assays to evaluate the capacity of inactivated poultry vaccines to stimulate innate immune cells

Innate immune cells sense pathogens with pattern recognition receptors

The extent to which innate immune cells sense and respond to immunostimulatory molecules of vaccines, including pathogen-associated molecular patterns (PAMPs) and adjuvants, depends on interactions with pattern-recognition receptors (PRRs). The chicken macrophage-like cell line HD11 [7] provides a suitable model to investigate innate immune responses to vaccines due to its broad expression of PRRs including *e.g.* toll-like receptors (TLRs). Our experiments showed that HD11 macrophages respond by increased phagocytosis (**Chapter 2**) and nitric oxide production (**Chapter 3**), two well-known effector functions of macrophages, to a range of bacterial, fungal and viral PAMPs or synthetic analogs. This demonstrated that the cell line is capable of detecting a diversity of immunostimulatory molecules potentially present in inactivated poultry vaccines. The only exception was poly(I:C), a synthetic analog of double-stranded RNA and representative for the genetic material of some viruses, to which HD11 cells did not respond [8–10] (**Chapter 3**). Other studies have demonstrated that transfection of poly(I:C) or double-stranded RNA to the cytosol may be required for recognition by HD11 cells [8,11].

Immune responsiveness of the HD11 cell line to inactivated infectious bronchitis virus, Newcastle disease virus and egg-drop syndrome virus vaccines *in vitro*

The HD11 cell line was stimulated with inactivated vaccines against the single-stranded RNA viruses IBV and NDV, and the double-stranded DNA virus EDSV (**Chapters 2** and **3**). Recognition of the single stranded RNA viruses occurs through TLR7 [12], while recognition of the double-stranded DNA virus EDSV is expected to occur through TLR21.

Previous studies have observed increased nitric oxide production and cytokine expression by HD11 cells in response to virulent NDV recognized, which was sensed through TLR7 [13]. We have also observed functionality of both TLR7 and TLR21 in HD11 cells after stimulation with the TLR agonists R848 (*i.e.* resiquimod) and oligodeoxyribonucleotides with CpG motifs, respectively (**Chapters 2** and **3**). Despite the presence of functional TLR7 and TLR21, our results showed that a trivalent vaccine comprising whole-inactivated IBV, NDV and EDSV did not induce nitric oxide production or enhanced cytokine expression (**Chapter 3**). Thus, there is a clear difference in the response of HD11 cells to virulent NDV [13] and the inactivated NDV vaccines used in this thesis. In contrast, the trivalent and other inactivated vaccines comprising IBV and NDV stimulated an increase in Fc receptor-mediated phagocytosis, which was caused by allantoic fluid present in the vaccine rather than inactivated viruses (**Chapter 2**).

It is possible that preservation of the virions present in the vaccine and recognition of the viral nucleic acids were affected by inactivation methods [14,15]. Alternatively, the inactivated viruses might not have ended up in the endosomal compartments where TLR7 and TLR21 are present, though vaccine uptake was confirmed microscopically by accumulation of droplets of mineral oil adjuvant (**Chapter 2**). Finally, although it has been shown that chicken immune cells respond to ligands known to activate mammalian TLR7 (*e.g.* R848) [12], activation of chicken TLR7 itself by either these ligands or single-stranded RNA through *e.g.* reporter gene assays has not yet been demonstrated [16,17]. Taken together, although the cell-based assays with the HD11 cell line showed effects of TLR ligands, we did not observe immune responsiveness associated with inactivated virions in vaccines for IBV, NDV and EDSV. The presented cell-based assays with the HD11 cell line may therefore be of limited use in potency testing of inactivated poultry vaccines of viral origin. Inactivated vaccines for IBV and NDV are often used in a prime-boost vaccination scheme and are preceded by live attenuated vaccines. Therefore, the working mechanism of these vaccines may rely predominantly on reactivation of memory B and T cells rather than activation of innate immune cells.

Immune responsiveness of the HD11 cell line to inactivated vaccine against *Avibacterium paragallinarum in vitro*

Studies on *Avibacterium* (*Av.*) *paragallinarum* bacteria and their interaction with immune cells are limited, despite the fact that the resulting disease, infectious coryza, leads to high mortality and drops in egg production [18]. The HD11 cell line was used to study the immunostimulatory capacity of an octavalent vaccine comprising five different *Av. paragallinarum* strains, as well as the three viruses IBV, NDV and EDSV (**Chapter 3**). The cell line showed increased nitric oxide production and gene expression of the cytokines IL-β, CXCLi1, CXCLi2, TNF, IL-10 and IL-12p40 after stimulation with inactivated *Av. paragallinarum* bacteria. The vaccine-induced responses were significantly reduced when the inactivated bacteria were pre-incubated with the LPS-binding antibiotic polymyxin B [19], indicating that activation occurred at least partially through the LPS-recognizing PRR TLR4. In addition to TLR4, the inactivated *Av. paragallinarum* vaccine may activate HD11 cells through stimulation of other TLRs such as TLR1/2 heterodimers or TLR21. Specific inhibitors for chicken TLR21 do not yet exist. Using the inhibitor CU-CPT22 of mammalian TLR1/2, we have tried to determine the contribution of chicken TLR1/2 to the activation of

HD11 cells by *Av. paragallinarum*. Unfortunately, CU-CPT22 did not inhibit the functionality of chicken TLR1/2, even after stimulation with the chicken TLR1/2 agonist Pam3CSK4 (*not shown*).

Taken together, our study has demonstrated that immunostimulatory molecules, at least LPS, are present in inactivated *Av. paragallinarum* vaccines and that their presence can be measured in cell-based assays with HD11 cells for potency testing. Future studies are needed to fully reveal whether immunostimulatory molecules other than LPS are present in inactivated *Av. paragallinarum* vaccines and need to be considered in potency testing.

Allantoic fluid

In order to produce vaccines, IBV, NDV and EDSV are propagated in the allantoic cavity of embryonated chicken eggs and obtained by collecting allantoic fluid. We observed that exposure of HD11 cells to allantoic fluid led to increased expression of chicken high-affinity IgY receptor CHIR-AB1 and enhanced Fc receptor-mediated phagocytosis (**Chapter 2**). Similarly, increased expression of CHIR-AB1 on natural killer cells in response to infectious bursal disease virus (IBDV) after propagation on embryonated chicken eggs was reported [20].

Further evidence for the immunostimulatory capacity of allantoic fluid was found when we stimulated chicken bone marrow-derived dendritic cells (chBMDCs) with an IBV antigen preparation resulting in upregulation of spleen tyrosine kinase (SYK) (**Chapter 5**). This protein is involved in both Fc receptor-mediated phagocytosis and inflammasome activation in response to monosodium urate crystals [21–23]. These crystals are damage-associated molecular patterns (DAMPs) that are the result of precipitation of uric acid at high concentrations as present in allantoic fluid [24,25]. Activation of inflammasomes by monosodium urate crystals is important for the conversion of pro-interleukin (IL)-1β, pro-IL-18 and pro-IL-33 into active pro-inflammatory cytokines [25,26].

An immunostimulatory function of allantoic fluid has not been studied before. Based on the presence of monosodium urate crystals, allantoic fluid is able to induce Fc receptor-mediated phagocytosis through upregulation of CHIR-AB1 and is likely to have a role in IL-1β secretion through activation of inflammasomes. The contribution of allantoic fluid present in inactivated poultry vaccines to immune activation will be interesting for future studies.

Nitric oxide production by the HD11 cell line to evaluate the potency of inactivated poultry vaccines

Previous studies have demonstrated that HD11 cells show enhanced nitric oxide production and cytokine expression in the presence of replicating viruses [13,27,28], which we did not observe for poultry vaccines containing inactivated viruses (**Chapter 3**). In contrast to the inactivated viruses, inactivated *Av. paragallinarum* bacteria were found to activate the HD11 cell line resulting in nitric oxide production and enhanced cytokine expression. So, the capacity to stimulate innate immune cells *in vitro* was apparently lost for viral vaccines after inactivation, but remained intact for *Av. paragallinarum*.

Inactivated vaccines for *Av. paragallinarum* are currently tested for potency using *in vivo* vaccination-challenge studies, which inflict pain and distress to large groups of animals. The use of an alternative serological assay has been explored, but a correlation between vaccine potency and antibody titers was not found [29]. The nitric oxide production assay was fast and easy to perform, and seems a promising *in vitro* alternative. Using this assay, the potency of inactivated *Av. paragallinarum* bacteria can expressed as the half-maximum effective concentration (EC_{50}), allowing easy comparison between batches. The toxicity of some vaccine constituents (*e.g.* inactivation agent and preservatives) was overcome by extracting the bacterial contents from the adjuvanted vaccine using isopropyl myristate and methods similar to those used for antigen quantification of inactivated NDV vaccines [30].

Previously it was proposed to use the nitric oxide production assay for potency testing of whole-cell *Bordetella pertussis* vaccines [31]. The results of a nitric oxide production assay correlated with the results of the intracerebral mouse protection test that is currently used for these vaccines [32]. Furthermore, nitric oxide production was shown to be biologically relevant by protecting mice from severe infection during *Bordetella pertussis* infection *in vivo* [33,34].

Taken together, the nitric oxide production assay with HD11 cells may contribute to *in vitro* potency testing of inactivated vaccines against *Av. paragallinarum*. Future studies need to address whether there is a correlation between the results of this *in vitro* assay and the currently used *in vivo* vaccination-challenge test. Future studies may also address whether the nitric oxide production assay can be useful for potency testing of other inactivated bacterial poultry vaccines.

Alternatives for the use of the HD11 cell line in cell-based assessment of inactivated poultry vaccines

Chicken macrophage-like cell line MQ-NCSU

In addition to the HD11 cell line, the chicken macrophage-like cell line MQ-NCSU [35] may be a promising alternative for use in cell-based potency assays. The cells show similar functionality compared to HD11 cells including Fc receptor-mediated phagocytosis of opsonized antigens [35], and nitric oxide production and cytokine expression in response to bacteria [36,37]. The MQ-NCSU and HD11 cell lines differ in background (normal vs. specific-pathogen free; both are derived from White Leghorn hens), tissue of origin (spleen vs. bone marrow) and viruses used for immortalization (myelocytomatosis virus vs. Marek's disease virus) [7,35]. We have conducted a pilot experiment in which nitric oxide production and phagocytosis were compared between MQ-NCSU and HD11 cells in response to LPS and IBV antigen. We observed lower nitric oxide production by MQ-NCSU cells in response to LPS as compared to the HD11 cells, while nitric oxide was not produced in response to IBV antigen by any of the cell lines (*not shown*). Furthermore, we observed that phagocytosis was similarly enhanced in both cell lines in response to LPS or IBV antigen (*not shown*). Due to our observation that nitric oxide production was higher for the HD11 cell line we decided to focus on the HD11 cell line for use in cell-based assays to assess the immunostimulatory capacity of poultry vaccines.

HEK reporter cells

Although the chicken macrophage-like cell line HD11 is able to show the immunostimulatory properties of inactivated poultry vaccines, the contribution of specific PRRs cannot be determined. It is not known whether inhibitors of mammalian PRRs can be used to block chicken PRRs, although we were able to use the LPS-binding antibiotic polymyxin to discriminate between LPS/TLR4-mediated and other responses. As described in Chapter 7, an alternative approach to determine the immunostimulatory properties of inactivated poultry vaccines would be to use reporter cells transfected with expression vectors encoding for chicken PRRs. Human embryonic kidney (HEK)-Blue reporter cell lines stably co-express human or murine TLRs and a nuclear factor (NF)-κB-inducible reporter gene to demonstrate TLR activation. Similar HEK reporter cell lines have successfully been created to demonstrate the functionality of chicken TLRs including TLR1/2 [38], TLR3[16] TLR4 [39], TLR5 [40], TLR15 [41] and TLR21 [42]. For chicken TLR7 HEK reporter cell lines were created, but led to conflicting results on TLR7 functionality in chickens [16,17]. The use of reporter cells expressing chicken TLRs in vaccine potency testing might be interesting to explore in future studies.

The chicken bone marrow-derived dendritic cells as an *in vitro* tool in vaccine biomarker discovery

The potential of chicken bone marrow-derived dendritic cells in cell-based potency testing

Immortalization of primary cells deemed suitable to create stable cell lines may result in high numbers of cells, but partial loss of functionality may be a consequence. It has been proposed to use dendritic cells (DCs) in cell-based assays for vaccine immunogenicity testing due to their important role in the initiation of the adaptive immune response [43]. For this purpose, we have characterized chicken bone marrow-derived DCs (chBMDCs) generated by culturing bone marrow cells of 18-days-old chicken embryos for one week in the presence of granulocyte/macrophage colony stimulating factor (GM-CSF) (**Chapter 4**). A chBMDC culture was described before [44,45] and its heterogeneity has been recognized [44]. Nevertheless, the extent of this heterogeneity, including the potential presence of cell types other than DCs that may respond differently to stimuli [46,47], had not been addressed before for the chBMDC culture.

We observed that multiple cell types were present in the chBMDC culture including two populations with DC-like characteristics, namely MHC-II^{low} and MHC-II^{high} chBMDCs, which were different in morphology, phenotype and function. The MHC-II^{high} chBMDCs were found to be more mature than their MHC-II^{low} counterparts, both phenotypically and functionally. Furthermore, we observed that the mature phenotype of MHC-II^{high} chBMDCs was reversible and that reseeding the mature cells after sorting led to a transition towards the relatively immature phenotype of the MHC-II^{low} cells. Stimulation with LPS did not result in the full maturation of chBMDCs and instead led to an intermediate phenotype in which CD40 expression was increased, whereas CD80 and MHC-II expression levels were slightly decreased. Due to the presence of different cell types including DCs at different states of maturation, we believe that the chBMDCs culture is less suitable for cell-based potency testing of vaccines than stable cell lines.

Proteomic analysis of vaccine-stimulated dendritic cells to gain better understanding about vaccine-induced immune responses

Despite its limited potential in a cell-based assay for vaccine potency testing, the chBMDCs culture has moved research on chicken DCs forward and resulted in new reagents [48,49], as well as novel insights in DC responses to stimulation with immunostimulatory adjuvants [50–55], (pathogenic) microbes [56–60] and vaccines [51, 56,61–63]. Furthermore, the chBMDC culture has been used in studies using transcriptomic and proteomic analyses to investigate chicken DCs respond to adjuvants [55] and pathogens [60], respectively. In our study, we used chBMDCs to identify novel biomarkers of *in vitro* responses to inactivated poultry vaccines.

Proteomic analysis was conducted to investigate the responses of chBMDCs after stimulation with *Escherichia coli* LPS, inactivated IBV or an inactivated bivalent vaccine against IBV and NDV (**Chapter 5**). Similar to our studies with the HD11 cells line, we found a stronger response to LPS than to inactivated IBV or the vaccine. After stimulation with LPS we observed a decreased expression of differentially expressed proteins (DEPs) associated with phagolysosomal activity, which may lead to better preservation of exogenous antigens for (cross-)presentation [64–67]. Furthermore, LPS stimulation led to an increase in DEPs associated with proteasomal antigen processing and antimicrobial activity. Two significantly upregulated DEPs of LPS-stimulated chBMDCs were also found as upregulated differentially expressed genes (DEGs) using RT-qPCR. These included GBP4L, involved in the activation of innate immune cells by LPS and bacteria [68], and ACSL, which has a role in the synthesis of the inflammatory mediator prostaglandin E2 [69]. It would be interesting to further explore GBP4L and ACSL as candidate biomarkers of bacterial poultry vaccines in future studies.

Since overlap between inactivated IBV antigen- and vaccine-stimulated chBMDCs was not observed, we hypothesize that the DEPs of vaccine-stimulated chBMDCs may be induced by the mineral oil adjuvant rather than by the antigen. We observed an increase of DEPs associated with lipid metabolism and storage. A previous study proposed that part of the activity of emulsion adjuvants may depend on its ability to change lipid metabolism in target cells, thereby affecting endolysosomal acidification and antigen presentation [70]. Two significantly upregulated DEPs of vaccine-stimulated chBMDCs included PLIN2, involved in the storage of lipid droplets, and PSMB1, a proteasomal subunit, which were also found as upregulated DEGs using RT-qPCR. Future studies are needed to determine whether these proteins can be used as vaccine-associated biomarkers in cell-based quality assessment of inactivated poultry vaccines. Whereas the chBMDC culture may be difficult to use in routine vaccine quality control due to its heterogeneity, our results demonstrate that the culture could be used to identify novel markers of the immunostimulatory capacity of poultry vaccines.

T cell proliferation assay to assess cell-mediated immunity

The previous part has described the activation of innate immune cells by inactivated poultry vaccines. For protective immunity to be induced by vaccines, antigen-presenting cells of the innate immune system need to activate antigen-specific B and T cells of the adaptive immune system. The activation of B cells and subsequent antibody production is assessed

in serological assays for potency assessment of vaccines. In contrast, assays to assess the activation of T cells are not yet used for potency assessment of vaccines. In general, T cell assays cannot be used as animal-free alternative for vaccine potency testing, due to the need for blood collection from vaccinated animals to perform the assay, similar to currently used serological assays. However, animal-free T cell assays with T cell hybridoma cell lines might become promising *in vitro* alternatives in the future [71]. As a first step, we investigated whether induction of cell-mediated immunity (CMI) in response to an inactivated poultry vaccine against IBV and NDV could be measured using an *ex vivo* T cell proliferation assay (**Chapter 6**).

Peripheral blood mononuclear cells were collected from 33-weeks-old laying hens before and three weeks after vaccination with an inactivated poultry vaccine for IBV and NDV. These chickens were already exposed to a standard vaccination regiment including live attenuated vaccines for IBV and NDV before the onset of the study. In this way, the study reflected the field situation in which inactivated poultry vaccines are often used as booster vaccines and preceded by live attenuated vaccines. Upon *ex vivo* restimulation with inactivated IBV antigens, we observed proliferation of IBV-specific T cells when isolated before, but not after vaccination. In contrast, we did not observe T cell proliferation after *ex vivo* restimulation with inactivated NDV antigens or an inactivated vaccine for both IBV and NDV, both before and after vaccination. In this study, we deed not observe an increase in antigen-specific T cells after *in vivo* vaccination with the inactivated poultry vaccine for IBV and NDV. In contrast, some previous studies observed induction of CMI in chickens vaccinated with inactivated vaccines for IBV [72] and NDV [73,74], but to a lower level than found in chickens exposed to live attenuated vaccines [73,75] or virulent pathogens [76]. The T cell proliferation assay in its current form was not sensitive enough for routine quality control of inactivated poultry vaccines for IBV and NDV. Future studies are required to improve the assay and to investigate whether T cell responses correlate with protection after immunization with the inactivated vaccine for IBV and NDV.

Advancements in *in vitro* potency testing of inactivated poultry vaccines

The scientific constraints and potential solutions for replacement of *in vivo* batch tests of vaccines in general were discussed in a review (**Chapter 7**), many of which are applicable to inactivated poultry vaccines. Similar to many other vaccines, there is limited knowledge about the critical quality attributes of inactivated poultry vaccines. Vaccine properties other than antigen quantity or the capacity to induce humoral immunity have often been neglected. There is an increasing consensus that one-to-one replacement of *in vivo* tests may not always be achieved and a combination of *in vitro* alternatives to test for different vaccine properties may be needed. Other properties like the vaccine composition, susceptibility of the antigen to proteolytic degradation, the spatial organization of the antigen, and the presence of immunostimulatory molecules might require testing as well. Examples of the latter in inactivated poultry vaccines are allantoic fluid (**Chapter 2**) and *Av. paragallinarum* LPS (**Chapter 3**), which were not described before. In addition, there is still limited understanding about the mode-of-action of the mineral oil adjuvant used in many inactivated poultry vaccines. The function of this adjuvant has mostly been described as a depot that

releases antigen over a long period of time [77,78]. Results reported in **Chapter 5** suggest that the adjuvant of the inactivated poultry vaccines that were part of this study also interacts with immune cells and induces changes in cellular metabolism, proteasomal degradation and immune responses.

Another difficulty for the transition from *in vivo* to *in vitro* potency tested of poultry vaccines **Chapter 7** is the incompatibility of adjuvants or other vaccine constituents with some *in vitro* potency tests (**Chapter 7**). In **Chapter 3**, we observed that purification of inactivated *Av. paragallinarum* bacteria from an adjuvanted poultry vaccine reduced the toxicity for the HD11 cell line and led to increased nitric oxide production. The methods used to extract antigens from the adjuvanted vaccine, based on isopropyl myristate, were similar to those used for an enzyme-linked immunosorbent assay for NDV [79] and may be more widely applicable when adjuvants negatively affects the performance of *in vitro* assays. Of note, antigen extraction is undesirable when one also wants to determine the effects of adjuvants in cell-based assays.

Concluding remarks

The primary aim of this thesis was to set up *in vitro* cell-based assays to assess the immunostimulatory capacity of inactivated poultry vaccines and to determine their applicability for routine potency testing. Results of our investigations were clearly in favor of the use of cell-based potency assays for the inactivated *Av. paragallinarum* vaccine. Nitric oxide production and enhanced cytokine expression due to the presence of LPS was found. Hence, potency testing of this vaccine will most likely benefit by including a cell-based test to assess the immunostimulatory capacity of the vaccine. The applicability of cell-based assays for inactivated poultry vaccines of viral origin was less clear. A comparison between live attenuated and inactivated viral vaccines in future studies may indicate whether the inactivation process destroys the capacity of the viruses to stimulate innate immune cells. In the research presented in **Chapter 2**, we identified the immunostimulatory properties of allantoic fluid, a major constituent of inactivated poultry vaccines of viral origin, which led to enhanced Fc receptor-mediated phagocytosis through increased expression of CHIR-AB1. In addition, we found (**Chapter 5**) that chBMDCs exposed to an inactivated poultry vaccines of viral origin showed many changes in the expression of proteins that are potentially interesting candidate biomarkers to use in a cell-based potency assays, including proteins like PLIN2 and PSMB1. The use of stable cell lines rather than chBMDCs is preferable to assess these biomarkers in future studies, since stable cell lines can be expanded into a large number of cells. Moreover, future studies are needed to further validate the nitric oxide production assay as a potency test for inactivated *Av. paragallinarum* vaccines, which may facilitate the replacement of currently used vaccination-challenge studies. Finally, the contribution of allantoic fluid to vaccine potency may be addressed in future studies. Eventually, multiple *in vitro* assays comprising physicochemical, immunochemical and cell-based assays may be required to substitute the different *in vivo* vaccine batch tests that are currently used for inactivated poultry vaccines.

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Appendix

Nederlandse samenvatting

Vaccinatie in pluimvee

Kippen worden al vroeg in hun leven gevaccineerd met levende maar sterk verzwakte virussen en bacteriën. Het afweersysteem herkent de ziekteverwekkers en ontwikkelt een afweerreactie waarbij ook geheugencellen gevormd worden. Deze geheugencellen zorgen ervoor dat het afweersysteem sneller kan reageren op een toekomstige infectie met dezelfde ziekteverwekkers. Het aantal geheugencellen dat een ziekteverwekker herkent kan in de loop van de tijd afnemen. Vandaar dat leghennen en broedkippen, die relatief lang leven, op latere leeftijd opnieuw gevaccineerd worden. Ditmaal worden echter vaker vaccins gebruikt bestaande uit geïnactiveerde (gedode) ziekteverwekkers. Dit leidt tot hernieuwde en vaak sterkere reactiviteit en dus nog betere bescherming.

De werking van het afweersysteem tijdens infectie of vaccinatie

Het afweersysteem is grofweg in te delen in een aangeboren en een aangeleerd afweersysteem. Het aangeboren afweersysteem bestaat uit lichaamsbarrières (bijvoorbeeld slijmvliezen), (witte bloed)cellen en moleculen die moeten voorkomen dat ziekteverwekkers het lichaam binnendringen en zich daarin vermeerderen. De onderdelen van het aangeboren afweersysteem zijn bij de geboorte al aanwezig en zorgen voor onmiddellijke bescherming tegen ziekteverwekkers. Wanneer vaccins worden toegediend zullen de cellen van het aangeboren afweersysteem als eerste reageren en ziekteverwekkers onschadelijk te maken. Een belangrijk onderdeel van het aangeboren afweersysteem zijn antigeen-presenterende cellen: witte bloedcellen die ziekteverwekkers opnemen en afbreken tot kleine fragmenten, genaamd antigenen. Deze antigenen worden vervolgens gepresenteerd aan de cellen van het aangeleerde afweersysteem samen met instructies voor het opwekken van een afweerreactie die de ziekteverwekker efficiënt kan bestrijden.

Het aangeleerde afweersysteem omvat twee typen witte bloedcellen, B- en T-cellen, die de antigenen van ziekteverwekkers specifiek herkennen en zich vervolgens gaan vermenigvuldigen. De B-cellen produceren antistoffen die aan ziekteverwekkers in lichaamsvloeistoffen kunnen binden om ze te neutraliseren. De T-cellen zorgen voor het opruimen van geïnfecteerde lichaamseigen cellen. Nadat de ziekteverwekkers zijn opgeruimd daalt in het lichaam het aantal B- en T-cellen dat de ziekteverwekker specifiek herkent. Een deel van de antigeen-specifieke B- en T-cellen die tijdens de vermenigvuldiging ontstaan, bestaande uit de eerder genoemde geheugencellen, blijft echter aanwezig als voorbereiding op een eventuele nieuwe infectie. Ook vaccins zorgen voor het ontstaan van beschermende B- en T-geheugencellen door op een veilige manier een infectie na bootsen.

Proefdiergebruik bij het testen van vaccins

Dierexperimenten zijn cruciaal voor het bepalen van de veiligheid en werkzaamheid tijdens de ontwikkelingsfase van vaccins. Ook na goedkeuring van vaccins blijven dierexperimenten vaak onderdeel van de kwaliteitscontrole die toegepast wordt op iedere nieuwe partij van geproduceerde vaccins. Proefdiergebruik voor kwaliteitscontrole van vaccins heeft een relatief groot aandeel in het proefdiergebruik in zowel Nederland als andere landen. Op allerlei gebieden zijn er ontwikkelingen gaande om het aantal proefdieren in experimenten te verminderen, ter vermindering van ernstig ongerief tijdens noodzakelijke dierexperimenten en om zoveel mogelijk dierexperimenten te vervangen door proefdiervrije alternatieven.

Echter, voor de kwaliteitscontrole van kippenvaccins worden nog steeds veel proefdieren gebruikt. Er zijn met name nog geen proefdiervrije alternatieven voor het bepalen van de werkzaamheid van geïnactiveerde pluimveevaccins.

Het doel van dit proefschrift

Er is behoefte aan alternatieve methoden voor kwaliteitscontroles van geïnactiveerde pluimveevaccins gezien het nog steeds hoge proefdiergebruik bij deze toepassing. Er zijn verschillende alternatieven mogelijk, waaronder het bepalen van de hoeveelheid ziekteverwekker in een vaccin of tests met celculturen in plaats van proefdieren. Dit proefschrift beschrijft studies naar tests met gekweekte afweercellen die kunnen bijdragen aan het bepalen van de werkzaamheid van geïnactiveerde pluimveevaccins.

Ontwikkeling van kwaliteitstests gebaseerd op cellijnen

In de studies van **hoofdstukken 2** en **3** worden tests beschreven die gebaseerd zijn op celculturen met een onsterfelijke cellijn van macrofagen, genaamd de HD11 cellijn. Macrofagen zijn een type antigeen-presenterende cellen die onderdeel uitmaken van het aangeboren immuunsysteem. Wij hebben verschillende afweerfuncties van macrofagen gemeten. Eén van deze functies is de productie van stikstofmonoxide dat kan bijdragen aan het doden van ziekteverwekkers. Daarnaast is de opname van deeltjes door HD11 cellen gemeten. Dit proces wordt fagocytose genoemd en is belangrijk voor het onschadelijk maken en doden van ziekteverwekkers. Ten slotte kunnen de HD11 cellen na interactie met de ziekteverwekkers zogenoemde cytokinen produceren. Dit zijn moleculen die andere (afweer)cellen in het lichaam activeren, waaronder de cellen van het aangeleerde afweersysteem.

De studies beschreven in **hoofdstukken 2** en **3** geven weer hoe HD11 cellen reageren na stimulatie met verschillende geïnactiveerde (gedode) kippenvaccins bestaande uit gedode virussen en bacteriën. Deze vaccins beschermen kippen tegen de ziekteverwekkers van infectieuze bronchitis, de ziekte van Newcastle, 'egg drop syndrome' en infectieuze coryza, of combinaties van deze ziekten. In **hoofdstuk 2** wordt beschreven dat één van de ingrediënten van kippenvaccins de opname van ziekteverwekkers (fagocytose) door HD11 cellen stimuleert. In **hoofdstuk 3** wordt beschreven dat kippenvaccins met geïnactiveerde bacteriën de productie van stikstofmonoxide en cytokinen stimuleren. De mate waarin de HD11 cellen geactiveerd worden en deze functies laten zien is indicatief voor de werkzaamheid van de verschillende vaccins. De beschreven methoden op basis van de HD11 cellijn zijn in staat om kippenvaccins op werkzaamheid te testen en kunnen daarmee bijdragen aan het vervangen van de huidige dierproeven.

Biologische markers die activatie van het afweersysteem aantonen na stimulatie met vaccins

Naast de doelgerichte aanpak die hierboven beschreven is hebben we ook gezocht naar nieuwe biologische markers die indicatief kunnen zijn voor de werkzaamheid van geïnactiveerde kippenvaccins. Naast macrofagen, waaronder de HD11 cellijn, zijn ook dendritische cellen belangrijke antigeen-presterende cellen. Er zijn geen cellijnen die dendritische cellen van kippen representeren, maar in **hoofdstuk 4** beschrijven wij een methode om dendritische cellen te kweken vanuit beenmerg afkomstig van kippenembryo's. De studie beschreven in **hoofdstuk 5** geeft weer welke veranderingen dendritische cellen ondergaan ten gevolge

van stimulatie met geïnactiveerde kippenvaccins. Op basis van deze studie hebben we verschillende veranderingen in de aanmaak en aanwezigheid van eiwitten geïdentificeerd die kunnen dienen als biologische markers voor de werkzaamheid van de vaccins.

De rol van T cellen in de afweerreactie na vaccinatie met geïnactiveerde kippenvaccins

Er zijn verschillende soorten dierproeven die gebruikt kunnen worden als kwaliteitstest voor de werkzaamheid van vaccins. Eén methode is het vaccineren en vervolgens infecteren van dieren, waarna gekeken wordt hoeveel dieren klachtenvrij blijven. Een veelgebruikte diervriendelijkere methode is het bepalen van de hoeveelheid antistoffen die het dier aanmaakt na vaccinatie. Zoals beschreven is het doel van vaccinatie ook om te zorgen dat het afweersysteem T-geheugencellen aanmaakt. Het is nog vrijwel onbekend of T-geheugencellen ook aangemaakt worden na vaccinatie met geïnactiveerde kippenvaccins en dit hebben we daarom onderzocht in de studie beschreven in **hoofdstuk 6**. Kippen werden gevaccineerd met een geïnactiveerd kippenvaccin voor bescherming tegen het infectieuze bronchitis virus (IBV) en een virus dat de ziekte van Newcastle veroorzaakt (NDV). Vervolgens zijn voor en na vaccinatie bloedmonsters genomen en zijn de witte bloedcellen opnieuw bloot gesteld aan de geïnactiveerde virussen om te bepalen of antigeen-specifieke T-geheugencellen aanwezig waren. Vooraf aan de vaccinatie vonden we celdeling van T-cellen na herhaalde blootstelling met IBV, wat duidde op de aanwezigheid van T-geheugencellen. Na de vaccinatie werd de celdeling van deze T-geheugencellen echter niet langer geobserveerd. Er kon dus niet worden vastgesteld dat de vaccinatie heeft geleid tot de aanmaak van antigeen-specifieke T-geheugencellen. De aanwezigheid van IBV-specifieke T-geheugencellen die we konden meten vooraf aan de vaccinatie was waarschijnlijk al eerder opgewekt door vaccinatie met een levend verzwakt vaccin tegen dit virus.

Recente ontwikkelingen in de overgang naar proefdiervrij testen van vaccins

De studies beschreven in dit proefschrift waren voornamelijk gericht op het ontwikkelen van proefdiervrije alternatieven voor het bepalen van de werkzaamheid van geïnactiveerde kippenvaccins. **Hoofdstuk 7** beschrijft recente ontwikkelingen in het proefdiervrij testen van vaccins voor zowel dieren als mensen. Proefdiergebruik is voornamelijk omvangrijk bij het testen van geïnactiveerde vaccins en vaccins op basis van onschadelijk gemaakte gifstoffen van bacteriën (toxoïden). Voor sommige van deze vaccins zijn wel proefdiervrije alternatieven ontwikkeld om de werkzaamheid te bepalen. De meeste proefdiervrije tests zijn gebaseerd op het bepalen van de hoeveelheid geïnactiveerde ziekteverwekker in het vaccin. Het is echter duidelijk geworden dat dit niet altijd voldoende is om de werkzaamheid van vaccins te garanderen. Andere belangrijke eigenschappen zijn de samenstelling van het vaccin, structuurbehoud van de geïnactiveerde ziekteverwekker en de capaciteit om antigeen-presenterende cellen te activeren. Het is duidelijk dat meestal meer dan één proefdiervrije test nodig zal zijn om de huidige dierproeven te vervangen.

Conclusies en blik op de toekomst

Het doel van de studies beschreven in dit proefschrift was te onderzoeken of tests op basis van celculturen gebruikt kunnen worden om de werkzaamheid van geïnactiveerde kippenvaccins aan te tonen. In de studies van **hoofdstukken 2** en **3** wordt aangetoond dat dergelijke tests inderdaad kunnen bijdragen aan het bepalen van de werkzaamheid. Daarnaast kunnen

op basis van de biologische markers gevonden in de studie van **hoofdstuk 5** nieuwe tests gebaseerd op celculturen ontwikkeld worden. Óf deze proefdiervrije tests daadwerkelijk dierexperimenten kunnen vervangen zal moeten blijken uit toekomstige studies waarin de proefdiervrije tests en dierexperimenten met elkaar vergeleken worden. Naar verwachting zal een combinatie van meerdere proefdiervrije tests nodig zijn om de huidige dierproeven voor geïnactiveerde kippenvaccins te kunnen vervangen. De tests die in dit proefschrift beschreven zijn kunnen hier een belangrijke bijdrage aan leveren.

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Curriculum Vitae

Robin Henricus Godefridus Antonius van den Biggelaar was born on July 5th, 1993 in Geldrop, the Netherlands. After graduating from the WereDi Gymnasium in Valkenswaard in 2011, he started his studies in Biomedical Sciences at Utrecht University. During this bachelor's program, he participated in an extra-curricular honors program. Furthermore, he conducted a short internship under supervision of dr. Helmi Pett and Prof. dr. Teun Bousema at the Department of Medical Microbiology at the Radboud University Medical Centre. Here, he also wrote his bachelor thesis about the use of primaquine against *Plasmodium falciparum*, the causative agent of malaria, in endemic countries where glucose 6-phosphate dehydrogenase (G6PD) deficiency is common. He obtained his bachelor's degree in 2014, after which he continued his studies with the master program Infection and Immunology at Utrecht University. Between 2014-2015, he conducted a 9-month internship at the Virology division of the Faculty of Veterinary Medicine at Utrecht University under supervision of dr. Mark Bakkers and dr. Raoul de Groot. Here, he participated in elucidating the importance of coronavirus hemagglutinin esterase in the dynamic interaction between the viral particle and host sialic acids during infection. Between 2015-2016, he did another 6-month internship at the Centre for Infectious Medicine of the Karolinska Institute, in Stockholm, Sweden under supervision of dr. Joana Dias, dr. Edwin Leeansyah and Prof. dr. Johan Sandberg. The research focused on elucidating the phenotypical and functional differences between $CD4^{-}$ $CD8^{+}$ and $CD4^{-}$ $CD8^{-}$ mucosa-associated invariant T cells. Robin obtained his master's degree in 2016.

In 2016, Robin started his PhD project the Immunology division of the Faculty of Veterinary Medicine at Utrecht University under supervision of Prof. dr. Willem van Eden, Prof. dr. Victor Rutten and dr. Christine Jansen. His research concerned the development of *in vitro* alternatives to the quality tests that are currently conducted *in vivo* for batch release of inactivated poultry vaccines, which resulted in this thesis. During his PhD project, Robin was an active member of the Veterinary PhD council, organizing lectures and workshops for fellow PhD students. In 2021, Robin continued his scientific career as a postdoctoral researcher at Leiden University and the Leiden University Medical Centre under supervision of Prof. dr. Annemarie Meijer, Prof. dr. Tom Ottenhoff and dr. Marielle Haks to work on host-directed therapies for the treatment of *Mycobacterium tuberculosis*.

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