TRAINED INNATE IMMUNITY AND TRANSGENERATIONAL EFFECTS IN CHICKENS

A conceptual approach of non-genetic inheritability

MICHEL B. VERWOOLDE

Propositions

1. Parental immune challenges determine the immune response in the next generation.
   (this thesis)

2. The role of IL-4 in inflammation is underestimated.
   (this thesis)

3. Speculative reasoning is needed to discover the unexpected unknown.

4. The unawareness of ignorance causes a biased and overestimated assumption with unfit confidence.

5. The best way to brainstorm is during an interactive walk.

6. Becoming a parent catalyzes the completion of a PhD dissertation.

Propositions belonging to the thesis, entitled

Trained innate immunity and transgenerational effects in chickens: A conceptual approach of non-genetic inheritability

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Trained innate immunity and transgenerational effects in chickens

A conceptual approach of non-genetic inheritability

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Trained innate immunity and transgenerational effects in chickens

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Thesis

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Table of contents

SUMMARY

Within the poultry husbandry sector, the containment of infectious diseases has always been a significant focus area and needs new sustainable and alternative strategies to control and minimize the vulnerability for increased incidences of infectious diseases and related health problems. An important factor within the poultry husbandry sector is the impact of the hens on the well-being and fitness of their offspring. It now becomes clear that immunological transgenerational effects have long lasting effects on the physiology of an organism. Especially the impact of the mother on the next generation via non-genetic manners, and within this, the role of the innate immune system is poorly understood.

The innate immune system has the potential to protect chickens against infections in a non-pathogen specific manner, especially at a young age when the adaptive immune system has not yet fully developed and the newly hatched chicks are depending on the innate immune system and maternal antibodies. A mechanism within monocytes and macrophages of the innate immunity has been described, where one innate antigenic stimulus can lead to an amplified response against other unrelated stimuli. This state of activated macrophages indicates a memory like mechanism and this protective effect was developed without the presence of T and B lymphocytes. This concept is known in mammals as trained innate immunity and is characterized by a crosstalk between immune, epigenetic and metabolic pathways in monocytes, macrophages and NK cells, featuring three core mechanisms, namely: 1) immune adaptations, 2) metabolic reprogramming and 3) epigenetic DNA modifications. The main research goal of this thesis was to study if trained innate immunity and transgenerational effects occurs in chickens. In my thesis I tried to answer the following research questions:

1. Does trained innate immunity exist in chickens?

2. Can transgenerational effects be induced by the innate immune system in chickens and is trained immunity involved in this process?

In **chapter 2**, I investigated effects of maternal immunization of laying hens on specific antibody production in the next generation and found that maternal immune activation with LPS increased HuSA-specific IgY responses in the offspring. Furthermore, maternal immune activation with LPS inhibited the stimulating effect of dietary β-glucan on the specific IgY anti-HuSA response in the offspring. The observations of both experiments in **chapter 2** suggest a functional link between the maternal innate immune system and the immune system of the offspring. A proposed mechanism for this functional link was trained innate immunity. In the following three chapters I investigated whether innate training is present in chickens (**Chapter 3 and 4**) and whether transgenerational effects of maternal activation of innate immunity could influence the response against a pathogenic challenge and performance in broiler chickens (**Chapter 5**).

Chapter 3 describes the development and optimization of a model to study trained innate immunity *in vitro* in chickens. This model was adapted from studies in mammalian species. Primary blood derived monocytes were stimulated with β-glucan or LPS. Next, the cells were secondary stimulated with LPS and differences in pro-inflammatory responses were analysed as a read-out for trained innate immunity. In addition, possible effects

of the cytokines IL-4 and IFNγ on trained innate immunity were investigated to study some molecular and/or cellular pathways in more detail. Nitric oxide (NO) production levels were measured as an indicator of pro-inflammatory activity. In addition, the cells were analyzed by flow cytometry to characterize the population of trained cells and to investigate the expression of surface markers associated with activation. I observed that after the secondary LPS stimulation, surface expression of colony stimulating factor 1 receptor (CSF1R) and the activation markers CD40 and major histocompatibility complex class II (MHC-II) were higher on macrophages that were trained with a combination of β-glucan and IL-4 compared to unstimulated cells. The positive effects on gene expression were paralleled by enhanced NO production. These results provided the first indications that innate immune training is also present in chicken.

In **chapter 4**, I further compared trained innate immunity of monocytes in different chicken breeds. To this end, I investigated whether isolated primary monocytes from layers and broilers differ in their innate training capacity. Monocytes of both breeds isolated from blood were trained *in vitro* with β-glucan, recombinant chicken IL-4 or a combination of both, and restimulated with lipopolysaccharide (LPS). I demonstrated again trained innate immunity in chicken but now based on parameters associated with immune function (i.e. IL1-β, MHCII, CD40, iNOS, NO) and metabolism (HIF-1α, PPARγ, lactate). Training of laying and broiler hen monocytes resulted in increased mRNA levels of IL-1β, iNOS and HIF-1α, but enhanced surface expression of CD40 and NO production was only observed in layers. This *in vitro* study demonstrates that monocytes from different genetic backgrounds can be trained. However, the observed differences suggest a differential effect on immune functionality associated with innate training. Trained macrophages of both breeds showed an increase in mRNA levels of the pro-inflammatory parameters (e.g. IL1-β) and also in in the metabolic associated parameters (e.g. HIF-1α). Since I did not perform measurements on epigenetic DNA modifications in my studies, the role of core mechanism 3) "epigenetic DNA modifications" in chickens remains to be elucidated. Based on analogy with mammalian species I expect a role for epigenetic programming in innate training in chickens. In mammals, the Dectin-1–Akt–mTOR–HIF-1α pathway plays a role in the interplay between epigenetics and metabolism. Interestingly, I found increased HIF-1α gene expression levels in trained macrophages which suggests a role for the Dectin-1–Akt–mTOR–HIF-1α pathway in trained innate immunity of chickens as well.

Next, I investigated whether transgenerational effects of maternal activation of innate immunity could influence the response against a pathogenic challenge and performance in broiler chickens (**Chapter 5**). This final proof of principle study combined previous gathered knowledge in an *in vivo* transgenerational animal experiment to investigate if activation of the maternal innate immune system with MAMPs affects performance, innate immune responsiveness and disease resistance of the offspring. Therefore, I investigated whether activation of the innate immune system in broiler breeder hens via β-glucan in the feed and LPS intratracheally had effects on growth performance and immune responsiveness in the neonates. Immunological parameters previously selected (e.g. IL1β, iNOS, NO) (**Chapter 2, 3 and 4**) were used. To investigate changes in immune responsiveness and growth performance I used a *necrotic enteritis* challenge. I showed that maternal stimulation with LPS and β-glucan resulted in decreased gene expression

levels of IL-1β in blood-derived monocytes in the offspring. This effect indicated again transgenerational effects of the innate immune system. Next to that, maternal stimulation with LPS resulted in an improved feed efficiency in the offspring in the first week after a *necrotic enteritis* pathogenic challenge (**Chapter 5**). The data are a first indication that broiler breeder hens can affect immune responsiveness and feeding efficiency of their offspring in a transgenerational manner.

The research of this thesis is the first describing trained innate immunity and non-genetic transgenerational effects of the innate immune system in chickens. I found evidence of the modulating effects of innate immunity in a transgenerational fashion indicating transgenerational trained innate immunity in chicken (**Chapter 2, 3, 4 and 5**). Including DNA modifications parameters in future studies on trained innate immunity in chicken is highly recommended, because epigenetic inheritance is probably the mechanism for non-genetic transgenerational trained innate immunity. Furthermore, it is important to investigate variability and plasticity of the immune system at early and later life physiology as immune modulating effects also have consequences on whole body metabolism (performance) and behaviour (neuronal and endocrine function) and vice versa. What the optimal circumstances are awaits further research wherein this thesis provided valuable information and insights on this.

My overall conclusion is that maternal immune experiences, such as infections or vaccinations, influence the immune system in the offspring. This also influence the effects of dietary interventions with feed additives on the immune system in the offspring. Within this, trained innate immunity has great potentials to be used in adapting current vaccination and feeding strategies to further improve disease resistance, animal performance and animal welfare in the poultry husbandry industry. Knowledge about transgenerational effects of maternal immunization or infection will contribute to a better understanding of the variation in immune phenotypes, disease resistance and metabolic disorders in the next generation(s).

CHAPTER 1

General introduction

The poultry animal husbandry sector is an important supply chain of high-quality protein sources for human consumption. The increasing demand by the growing human population will result in a shortage of these high-quality protein sources [1]. Furthermore, within the conventional animal husbandry sector, poultry is also the most sustainable source of proteins. Poultry has, compared to other animal protein sources (e.g. beef, pork, sheep, rabbits), a more efficient feed to growth conversion (FCR) with relative low costs and impact on environment [2]. Therefore, poultry meat became the most important profitable protein source and its consumption is expected to grow even further for the next coming years [3]. With this growth, the poultry husbandry sector is facing new problems related to animal welfare, performance and sustainability [4-7]. For instance, this growth results in a higher number of chickens housed together, which makes potentially the impact of infectious diseases on the industry substantial. Also the change from battery cages to free roaming systems makes the industry also very vulnerable to infectious diseases. At the same time, providing antibiotics for preventive purposes or as antimicrobial growth promotors in broiler chickens has been totally banned in the European Union [8]. Altogether, the poultry husbandry sector needs new and alternative strategies to control and minimize the vulnerability for increased incidences of infectious diseases and related health problems. Within that, new sustainable solutions are needed to improve disease resistance, maintain animal performance and increase animal welfare.

One of the strategies to improve disease resistance is to strengthen the chicken immune system. Immune related changes directly in early life of newly hatched chicks will have effects later in life [9]. Measures that optimize protective immune functions are therefore considered as very effective. There are in fact three aspects within the life of a chicken that can be considered as a strategic moment to improve immune mediated protection. The first aspect is directly post hatch, when the immune system is not fully developed yet and newly hatched chicks are depending on the innate immune system and maternal antibodies [10-13]. For instance, changes in microbiota, housing conditions and feeding approaches are previous investigated parameters showing that early life immune modulation has effects later in life of the chicken on immune mediated protection [9, 14-17]. Next to that, most vaccines are administered directly post-hatch to improve resistance to specific infectious diseases [18]. The second aspect within the life of a chicken is *in ovo* vaccination to improve disease resistance in hatchlings [19]. For the third aspect the focus is on parental measures that have beneficial effects on the offspring. With respect to the latter, this includes research on genetic heritability of beneficial traits in the DNA such as the selection for slower-growing breeds [20]. However, research on non-genetic transgenerational approaches (e.g. histones and DNA modifications, imprinting, posttranslational gene silencing) is a growing area of interest with increasing numbers of new insights. It is important to investigate the non-genetic effect of the maternal physiology, including the immune system, on her offspring. Research on how to use this knowledge is needed to improve disease resistance and animal performance already from fertilization until the next generation(s). For instance, studies in wildlife (e.g. birds and fish) suggested that a mother prepares her offspring for her environment, through conveying maternal antibodies and microbiota composition [21-23]. A beneficial effect of these transgenerational mechanisms is that newly born offspring are already prepared for the environment in which they will grow up in and are geared up for exposure to

environmental pathogenic pressure. In poultry animal husbandry the management and environmental conditions of the mother and their offspring are often different. Hatch and grow-out of the chicks are done in the absence of the parents, with different dietary conditions and different vaccination schedules [24, 25]. These different husbandry measures for mother animals and offspring may result in a mismatch between generations resulting in impaired disease resistance in the neonate [26]. To fix this mismatch and to improve immune competence, it is important to gain more knowledge on immunological transgenerational effects for chickens before adjustments to the current management strategies can be introduced.

The innate immune system of chickens likely contributes to transgenerational effects [27]. Supporting literature describes a mechanism dedicated to this innate immune system that underlies these transgenerational effects [28, 29]. This concept is known as trained innate immunity. In this thesis I will describe this concept in chickens, the contribution of macrophages in trained innate immunity and how I believe trained innate immunity can be applied to improve general disease resistance, performance and welfare within one generation but especially also in a transgenerational fashion.

Interaction of the innate and adaptive immune system

The immune system can be divided into two parts. The innate immune system is activated within hours upon entry of a pathogen, forms the first line of defense and is not specific for a certain pathogen [30-36]. The response of the adaptive immune system, on the other hand, starts after several days, but is, in contrast to the innate immune system, pathogen specific.

White blood cells (Leukocytes) are important players of both innate and adaptive immunity. Several types of leukocytes are subscribed to the innate immune system including natural killer (NK) cells, granulocytes and monocytes. NK-cells are effector lymphocytes specialized in the recognition of virus-infected cells and tumor cells, but also exert defensive activity against bacteria [37, 38]. Within birds, heterophils, eosinophils and basophils are found and belong to the granulocytes [39]. Monocyte-derived cells differentiate into dendritic cells (DCs) and macrophages [39]. Both the differentiated granulocytes and monocytes share the ability of phagocytosis. Phagocytosis is considered to be one of the most primitive defense systems, which is also present in invertebrates [40]. It is an important non-pathogen specific system since the cells can use it to clear harmful and foreign substances and particles. In mammals and birds phagocytosis is also necessary for subsequent antigen presentation [41]. Macrophages and DCs are the major antigen-presenting cells (APCs). After phagocytosis, these APCs are able to express antigen originating peptides on the surface via major histocompatibility complex class I (MHC-I) and major histocompatibility complex class II (MHC-II).

In lymph nodes, antigen presentation and cytokine production results in activation of antigen specific cytotoxic CD8+ T cells and helper CD4+ T cells CD8+ T cells recognize tumor cells, damaged cells or infected cells (particular with virus) via endogenous antigens

presented on MHC class I. Once recognized, the physical interaction of CD8+T cells with these infected cells leads to killing of the infected cell which results in prevention of further spread of the infection. CD4+ T cells recognize exogenous antigen presented on MHC class II receptors on the surface of APCs (**Figure 1**). In addition to MHCII binding to the TCR, also binding of CD40 to CD40L is required for T cell activation. This interaction between APCs and T-cells is under guidance of APC produced cytokines and these cytokines regulate the differentiation CD4+ T cells in different subsets like Th1, Th2, Th17 and regulatory T cells (Tregs). Th1 cells and the production of Th1 like cytokines (e.g. IFNγ and TNFα) will recruit and activate macrophages classical activated macrophages (M1) (**Figure 2**). Th2 cells and the production of Th2 like cytokines (e.g. il-4 and il-13) will recruit and activate alternatively activated macrophages (M2). In mammals, classically activated macrophages are considered as pro-inflammatory and alternatively activated macrophages are considered as anti-inflammatory [42]. However, this clear phenotypical separation is based on *in vitro* polarization studies in mice [42], but not so clear *in vivo* and should be considered as a theoretical guideline [43]. Helper CD4+ T cells function as a bridge between the cellular part of the innate immune system and the humoral part of the adaptive immune system (**Figure 1**). A higher expression level of surface markers such as CD40 and MHC-II on APCs could result in an enhanced adaptive immune response [44-48].

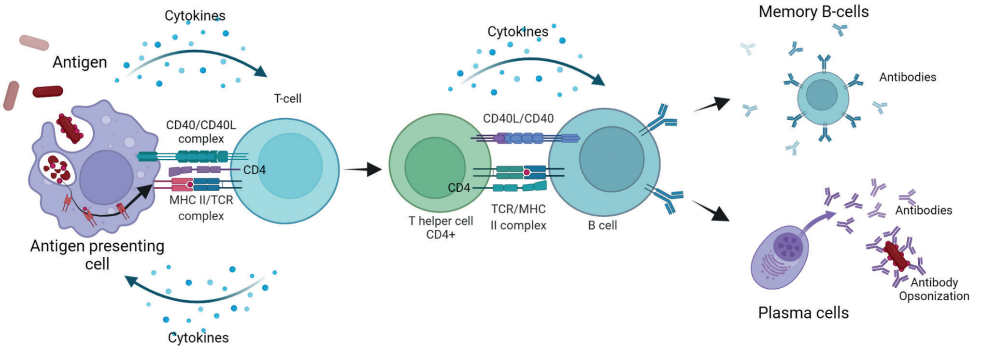

Figure 1. Interaction of the innate immune system and the humoral part of the adaptive immune system. Antigens are being phagocytosed by antigen presenting cells (APCs). APCs express antigen originating peptides on the surface via major histocompatibility complex class II (MHC-II). T-cells become active upon interaction with APCs via T cell receptor (TCR), the MHC-II and cytokines. These cytokines influence the differentiation of certain subsets of helper CD4+ T cells. The T cells will produce cytokines to recruit and activate macrophages. Helper CD4+ T cells activates the humoral part of the adaptive immune system by interacting with B cells. This interaction causes the B cell to multiply and differentiate into memory B cells and antibody producing plasma cells. Figure created with Biorender.com.

Humoral immunity includes the formation of antigen specific B cells. The interaction between CD4+ helper T cells and B cells results in B cell proliferation and differentiate into memory B cells and antibody producing plasma cells (**Figure 1**). Memory B cells may remain in the body up to life long and function as a memory system for specific antigens. Ones the body encounter again a similar antigenic trigger, a fast, efficient and specific

immune response will be activated by producing antigen specific antibodies. Antigen specific antibodies will subsequently opsonize and neutralize the antigen.

Monocytes differentiate into APCs (macrophages and DCs) and play a fundamental role in the classical immune memory that is mediated by T and B lymphocytes. Long term specific memory and the production of antigen-specific antibodies is mediated by T and B lymphocytes. However, in recent years studies in mammalian species show that innate immune cells also have memory-like functions. This type of memory is non-specific which is in contrast to the classical memory functions. This property is referred to as trained innate immunity [49].

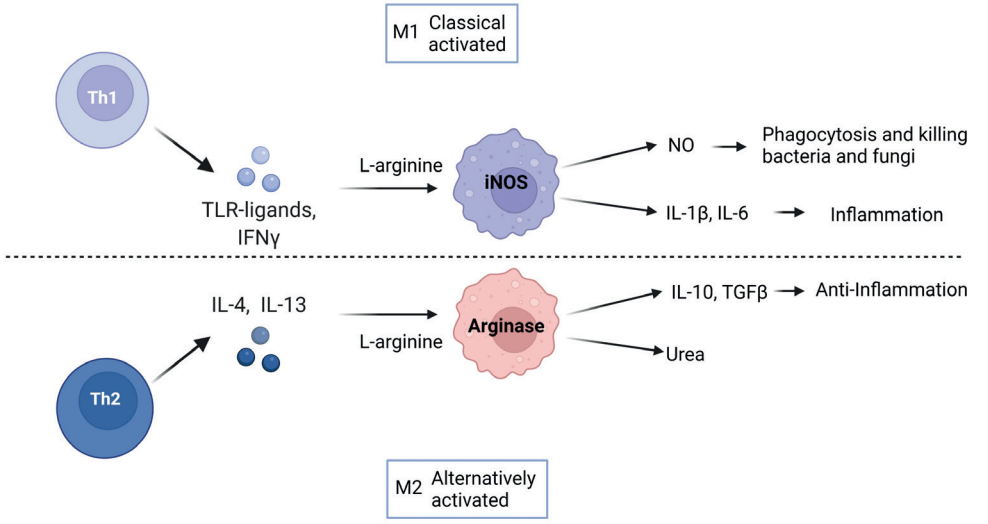

Figure 2. Classical activated macrophages that are associated with Th1 cells, produce pro-inflammatory cytokines (e.g. IL-1β, IL-6) and nitric oxide (NO). NO serves as a toxic agent towards infectious organisms and is therefore ascribed as one of the inflammation inducing component. The enzyme iNOS metabolizes L-arginine to nitric oxide (NO) and citrulline. Alternative activated macrophages that are associated with Th2 cells, produce anti-inflammatory cytokines (e.g. il-10, TGF-β) but have also an upregulation of arginase activity that is important for cell proliferation and wound healing. Side product of this synthesis is the release of urea. Figure created with Biorender.com.

Trained innate immunity as a model to improve disease resistance

Plants and invertebrates have the ability to develop increased protective mechanisms against pathogens after primary exposure, despite the lack of an adaptive immune system as seen in vertebrates [50, 51]. This phenomenon implies that memory formation in the immune system is not only a characteristic of the adaptive immune system, but also a property of the innate immune system. Humans vaccinated with *Bacillus Calmette–Guérin* (BCG) vaccine showed not only an enhanced pro-inflammatory response after a secondary

stimulation with *Mycobacterium tuberculosis,* but also to a secondary stimulation with non-related pathogens [52]. This so called trained innate immunity, is poly-specific, appeared to be initiated independent of T and B cells and remains up to 1 year after the initial activation [53]. Trained innate immunity is further characterized by an immuno-epigeno-metabolic cross-talk of cellular pathways in monocytes, macrophages and NK cells, featuring three core mechanisms, namely: 1) "immune adaptations", 2) "metabolic reprogramming", and 3) "epigenetic DNA modifications" (**Figure 3**). Although many studies have reported evidence for trained innate immunity in mammalian species, plants and invertebrates, knowledge on trained innate immunity in avian species is not reported [54-58]. Therefore, development of a chicken-model to investigate the three core elements (immune adaptations, metabolic reprogramming and epigenetic DNA modifications) is needed to gain more knowledge on trained innate immunity in chickens. Enhanced immune responses and metabolic reprogramming could lead to enhanced disease resistance and higher nutritional performance whereby epigenetic DNA modifications are likely crucial for long term and transgenerational effects.

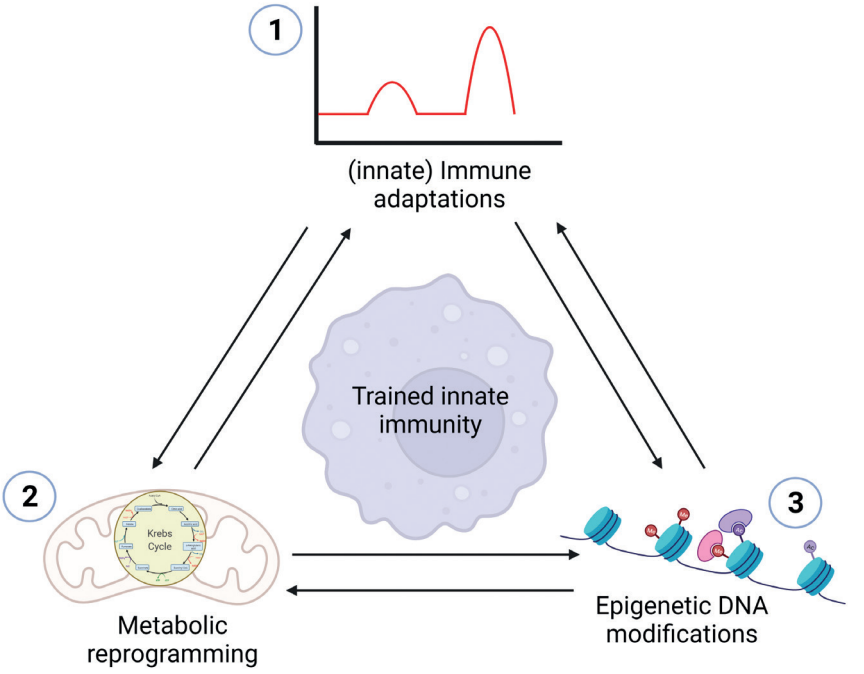

Figure 3. Trained innate immunity is characterized by an immuno-epigeno-metabolic cross-talk of cellular pathways with consist of three core elements: 1) immune adaptations, 2) metabolic reprogramming, and 3) epigenetic DNA modifications in monocytes, macrophages and NK cells Figure created with Biorender.com.

1) Immune adaptations

The first core element of the immuno-epigeno-metabolic cross-talk are immune adaptations. In mammals, *in vitro* primary stimulation of monocytes and macrophages with β-glucan from yeast *Candida albicans* cell walls, increased pro-inflammatory responses after a secondary stimulation with homologous as well as heterologous antigens [49, 59-62]. It was also found that an *in vivo* challenge in mice with a low amount of heat-killed *C. albicans* resulted in an enhanced production of tumor necrosis factor alpha (TNFα) and interleukin-6 (il-6) after a secondary lipopolysaccharide (LPS) stimulation 7 days later [61]. This state of activated macrophages indicates a memory like mechanism and this protective effect was developed without the presence of T and B lymphocytes. Within the trained innate immunity *in vitro* model it is therefore important to ensure that T and B lymphocytes are not present. Next to that, during *in vitro* and *in vivo* experiments it is important to have a wash out and/or resting phase between the primary stimulating component and the secondary aspecific and non-related stimulus (**Figure 4**). One of the most intensively investigated primary stimuli with respect to innate training is the glucose polymer β-glucan [55]. Therefore, I chose β-glucan as stimulatory component to investigate training capabilities of monocytes and macrophages in chicken. For mammalian species, an experimental *in vitro* model is described with respect to innate immune adaptations [55, 63]. This model is shown in **Figure 4**. A primary stimulus, such as β-glucan, is used to initiate training followed by a washing step to erase the primary stimulating elements and a resting period. After this resting period, a secondary stimulation is applied to measure the increased responsiveness of the macrophages that were stimulated with the primary stimulus. Since trained innate immunity is poly-specific, this secondary stimulus can theoretically be any Microbe-Associated Molecular Pattern (MAMP). A general applied innate stimulating component is LPS. LPS is a major component of the outer membrane of gram-negative bacteria, such as *Escherichia coli,* and is previously used in *in vitro* trained innate immunity studies [54, 55]. *E. coli* causes frequently infections in poultry and infect chickens mainly via the respiratory tract and intestines and is therefore relevant for chicken [64-66]. In mammals, it has been well documented that LPS and β-glucan is recognized by Toll-like receptor 4 and Dectin-1 receptor, respectively, which are both located on the cell-surface of innate immune cells including monocytes [35, 67-69]. Both receptors share the ability to target the NFκB mediated signaling in the nucleus of monocytes [70, 71]. NFκB is known as an important inflammatory gene inductor [72]. Interestingly, different MAMP have variable training effects in macrophages. Primary stimulation of β-glucan results in higher activity after secondary stimulation with LPS. Whereas, tolerance can be observed in monocytes primary stimulation with LPS followed by a second LPS stimulation in humans [54] and in birds [73, 74] (**Figure 4**).

Trained macrophages from mice and humans show after secondary stimulation enhanced production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα [54, 55]. MHC class II receptors and the receptor CD40 are known to be upregulate upon activation of innate immune cells (**Figure 1**). NO, as a product of iNOS activity, is an effector molecule of activated macrophages that kills microbes within macrophages through its reactivity with proteins, DNA, thiols and iron at the active site of many enzymes [75]. Increased NO production by trained monocytes may lead to increased killing capacity upon phagocytosis [76]. Increased phagocytosis results in enhanced antigen presentation [77]. Higher levels

of surface markers such as CD40 and MHC-II may lead to an enhanced adaptive immune response. Both CD40 and MHC-II play an important role in the interaction of APCs and T-cells and therefore indirect also affect B cell activation [44-48, 78, 79] (**Figure 1** and **Figure 5)**. Indeed, *in vivo* challenge of cows with heat-killed *Mycobacterium bovis* resulted in increased bacterial killing capacity (phagocytosis) of monocyte-derived macrophages [80]. The increased phagocytosis was gained without the presence of T or B cells interaction and was therefore referred as trained innate immunity. Based on studies on trained innate immunity in invertebrates and mammalian species, one may expect that innate training also occurs in chickens. Since trained innate immunity can stimulate innate as well as adaptive immune functions, it may play a role in improved disease resistance.

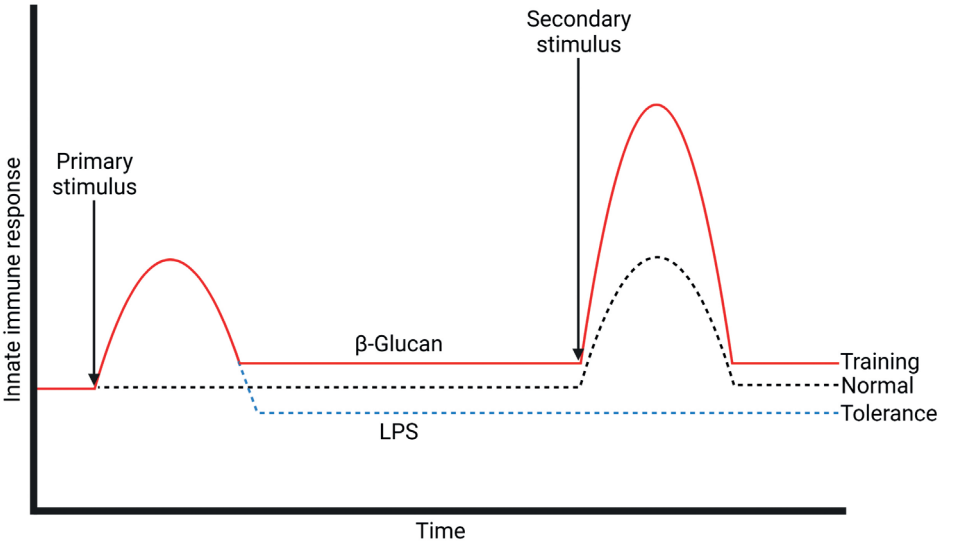

Figure 4 The concept of trained innate immunity. A primary stimulus with β-glucan or LPS will initiate an innate immune response in monocytes. After a resting period, primary stimulation of β-glucan result in higher activity after secondary stimulation with LPS. Whereas, tolerance can be observed in monocytes primary stimulation with LPS followed by a second LPS stimulation. Figure created with Biorender.com.

Important bio markers of innate immune responses that are applicable for chicken research are for instance cytokine (gene expression), monocyte surface markers, nitric oxide (NO) production and related gene expressions such as inducible nitric oxide synthase (iNOS). These parameters of innate immune responses are directly or indirectly related with each other (**Figure 5**)

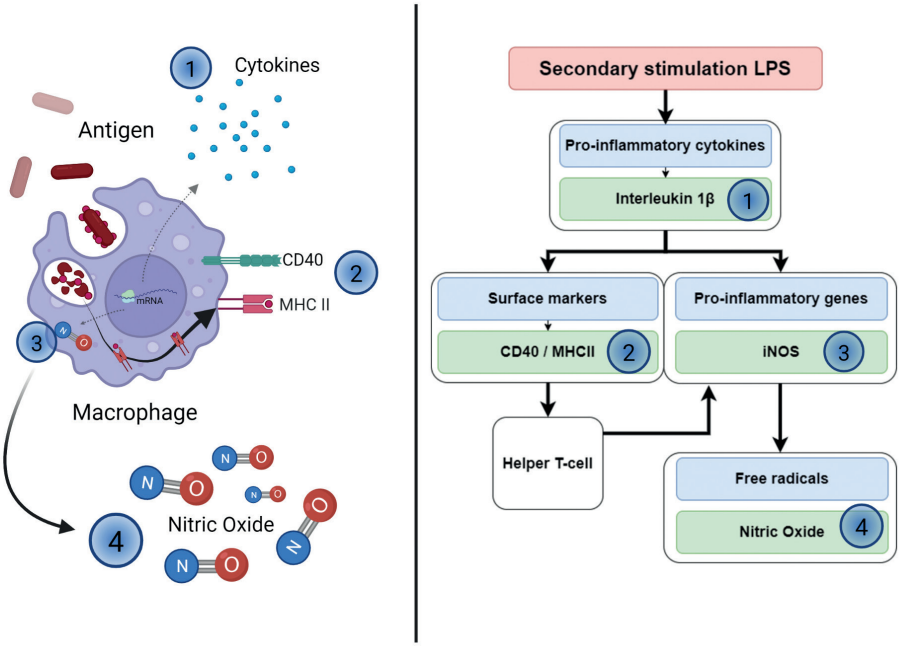

Figure 5. Important immune parameters for trained innate immunity. These parameters are expected be increased or decreased after the secondary stimulation with LPS (see Figure 3). Under pro-inflammatory circumstances macrophages produce cytokines like IL-1β (1), display surface activation markers MHCII and CD40 (2), show increased gene expression levels of iNOS (3) and consequently produce nitric oxide (4). Figure created with Biorender.com.

2) Metabolic reprogramming

A second core element of the immuno-epigeno-metabolic cross talk is metabolic reprogramming [81]. Apart from inflammatory regulating mechanisms, metabolic reprogramming takes place in trained immune cells [82, 83]. The Dectin-1–Akt–mTOR–HIF-1α pathway has been found as signal transduction pathway involved in trained innate immunity, whereby Dectin-1 is the receptor for β-glucan molecules [84]. Hypoxia-inducible factor 1-alpha (HIF-1α) acts as a master transcription factor not only controlling inflammatory responses, but also metabolic reprogramming [85-87]. Indeed, in trained innate immune cells, the metabolic state of mammalian macrophages shifts from the normal oxidative phosphorylation (OXHPOS) route of producing energy towards the more efficient aerobic glycolysis (**Figure 6**). This shift is known as the "Warburg Effect" [82, 83]. This metabolic shift results in increased lactate production [85, 88, 89].

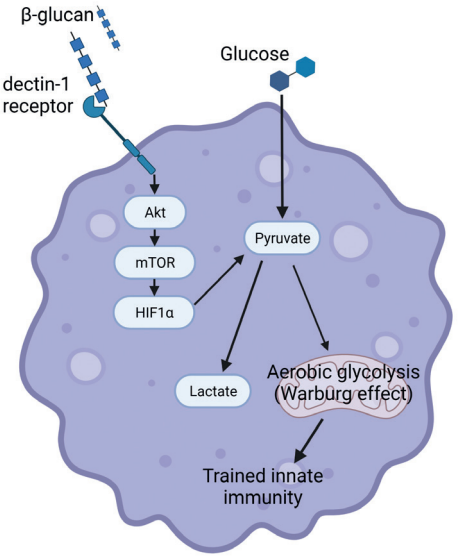

Figure 6 The Dectin-1–Akt–mTOR–HIF-1α pathway with β-glucan stimulated, showing metabolic activation of trained monocytes [84]. The oxidative phosphorylation shifts towards the aerobic glycolysis (Warburg effect) via glucose and pyruvate with the formation of lactate. Figure created with Biorender.com.

Studies in human and mice demonstrated that adaptations in cellular metabolism are key to trained innate immunity, whereby both IL-1β and HIF-1α play a pivotal role [84, 85, 90]. Tissues suffering from an injury are becoming hypoxic tissues (low oxygen level) and are potentially a site of pathogenic infiltration [91]. Probably under the tight control of HIF-1α, macrophages are apt to infiltrate hypoxic tissues whereby they switch their metabolic program to glycolysis [92]. Furthermore, it has been found that not only the glycolysis but also glutaminolysis and lipogenesis are involved in trained innate immunity [93].

3) Epigenetic programming

A third core element of the immuno-epigeno-metabolic cross-talk is epigenetic programming. Transcriptional and epigenetic reprogramming form the underlying cause for trained immunity [82, 93, 94]. Trained immunity, induced by β-glucan, promotes glycolysis, which is the result of increased histone 3 trimethylation of lysine 4 (H3K4me3). H3K4me3 is a hall mark of open chromatin and increased gene expression, at promoter sites of essential glycolytic genes [84]. Thus, besides histone modification, changes in posttranslational modification by TCA metabolites may also occur. Indications are found that accumulation of succinate affects lysine succinylation of enzymes of the glycolytic pathway and TCA cycle [95]. Several metabolites of the TCA cycle serve as cofactor for chromatin modification-catalyzing enzymes [96]. α-Ketoglutarate, for example, is a co-factor that can favor histone demethylation [97], and fumarate and succinate, contrariwise, are described as inhibitors of demethylation processes [93, 98, 99]. Therefore, epigenetic programming, metabolic reprogramming and, consequently, immune adaptive effects

are closely connected within the previous explained immuno-epigeno-metabolic cross talk, which is characterized as the cross talk behind trained innate immunity. With the appearance of epigenetic mechanisms that could be initiated by the innate immune system, it is thought that transgenerational effects could be initiated by the similar mechanism. Since epigenetic modifications of germline genes are heritable to next generations [29, 100, 101], trained innate immunity could have transgenerational effects as well in chicken. This is also known as transgenerational epigenetic inheritance as previously described by Berghof et al. [27].

Transgenerational effects in poultry and the innate immune system - Knowledge gap

An important factor within the poultry husbandry sector is the impact of the hens on the well-being and fitness of their offspring. Within poultry, the mode of action and potential benefits of these transgenerational effects, and within this, the role of the innate immune system has not been investigated extensively. However, it now becomes clear that these transgenerational effects have long lasting effects on the physiology of an organism [26, 102]. Especially the impact of the mother on the next generation via non-genetic manners is poorly understood. In the past years, more evidence is supporting the theory that transgenerational immunological effects may play a role [103-106]. However, little scientific evidence is available about *how* the maternal innate immune system affects the offspring. It has been suggested that stimulation of maternal innate immune cells may affect the immune system in the next generations via epigenetic inheritance [27]. It is proposed that transgenerational epigenetic effects could be initiated by trained innate immunity mechanisms and within that via the immuno-epigeno-metabolic cross-talk (**Figure 3**). β-glucan and LPS could therefore be potential components to influence the neonatal immune system in a transgenerational manner [107].

From mammalian species we know that metabolic-associated mechanisms and immune responsiveness are affected by information of the mother in a transgenerational fashion [108]. Especially interesting are *in vitro* studies in human and mice that showed changes in activation of innate immune cells which are transferred to next generations [53]. Furthermore, immune activation with LPS in pied flycatchers caused elevated antibody production in the offspring [103]. These transgenerational mechanisms demonstrate that the offspring is affected by information of the mother in a transgenerational fashion [109-111]. It is hypothesized that epigenetic mechanisms cause transgenerational effects on neonatal immunity in mice [111] and birds [102] and have long lasting effects on the physiology of an organism [26, 44, 102]. In poultry, however, little is known about these transgenerational mechanisms and their consequences on immune responsiveness, disease resistance and metabolic disorders in the neonates.

The aim and outline of this thesis

The research described in this thesis aimed to study transgenerational effects of the innate immune system in chicken and within this trained innate immunity. The innate immune system has the potential to improve disease resistance of poultry in a nonspecific manner, especially at a young age when the adaptive immune system has not yet fully developed and the newly hatched chicks are depending on the innate immune system and maternal antibodies [10-13]. I hypothesized that training this innate immune system can strengthen the first line for defense against pathogens and enhance the adaptive immune system on a later stage (**Figure 1**). My aim was not only to find evidence for trained innate immunity in one generation, but study effects in the next generation via a transgenerational approach. Thus, the main research objective was to study if transgenerational effects of innate immune activation occur in chickens. This research goal is subdivided into the following research questions:

1. Does trained innate immunity exist in chickens?

2. Can transgenerational effects be induced by the innate immune system in chickens and is trained immunity involved in this process?

The outline of the thesis is as follows. In the general introduction (**Chapter 1**) the relevance of innate immunity for poultry is highlighted. Also the link between innate and adaptive immunity is explained. Then the novel concept of trained innate immunity is explained as it is characterized extensively in mammalian species. Furthermore, the importance of monocytes and macrophages within this novel concept is discussed. Finally the possibility of transgenerational effects in chicken is addressed and the gaps in knowledge are identified.

Chapter 2: Transgenerational effects of maternal immune activation on specific antibody responses in layer chickens.
In **chapter 2**, a first indication is shown of transgenerational effects initiated by cells of the innate immune system. Here we found that maternal immune activation with LPS influenced the antigen specific antibody response in the next generation. Furthermore, it was found that maternal immune activation with LPS inhibited the effects of dietary treatment with β-glucan on antigen specific antibody responses. It was therefore reasonably to suggest that components that activate the maternal innate immune system, influence the neonatal immune system in a transgenerational manner and as a consequence the disease resistance of the neonate [107]. A proposed mechanism was trained innate immunity. In the following chapters of this thesis was investigated whether the concept of trained innate immunity is also valid for chicken (**Chapter 3 and 4**) and whether transgenerational effects of maternal activation of innate immunity could influence the response against a pathogenic challenge and performance in broiler chickens (**Chapter 5**).

Chapter 3: Evidence of trained innate immunity in chickens
Chapter 3 describes the development and optimization of an *in vitro* trained innate immunity model in chickens. This model was adapted from studies in mammalian species. Primary blood derived monocytes were stimulated with β-glucan or LPS. Next, the cells were secondary stimulated with LPS and differences in pro-inflammatory responses were analyzed as a read-out for trained innate immunity. In addition, possible effects of the cytokines IL-4 and IFNγ on trained innate immunity were investigated to study some molecular and/or cellular pathways in more detail (see **Figure 2**). The results provided the first indications that trained innate immunity is also present in chicken. Based on this analysis, NO production, surface expression marker MHCII and surface expression marker CD40 were selected as readout for future experiments (**Chapter 4 and chapter 5**).

Chapter 4: Innate immune training and metabolic reprogramming in primary monocytes of broiler and laying hens
Using the same *in vitro* model, **chapter 4** describes the variation in innate training between broiler chickens and laying hens and between chickens of different ages. It is known that innate immune responses vary between chickens that are genetically selected for different purposes like laying chickens and broiler chickens [12, 112, 113]. Trained innate immunity was observed in both layers and broilers, although variation was observed on NO production, surface expression markers MHCII and CD40. Differences and variations on how the innate immune system behave under variable circumstances is valuable to better understand the potentials and mechanisms of innate training in poultry.

Chapter 5: Transgenerational effects of innate immune activation in broiler breeders on growth performance and immune responsiveness.
In this chapter I investigated whether activation of the innate immune system in broiler breeder hens via β-glucan in the feed and LPS intratracheally, has effects on growth performance and immune responsiveness in the neonates. Immunological parameters previously selected (e.g. IL1β, iNOS, NO) (**Chapter 2, 3 and 4**) were used. Next to that, this study also included the investigation of changes in immune responsiveness and growth performance using a *necrotic enteritis* challenge. The data are a first indication that broiler breeder hens can affect immune responsiveness and feeding efficiency of their offspring in a transgenerational manner. This final study combined previous gathered knowledge in an *in vivo* transgenerational animal experiment. This proof of principle study was performed to investigate if activation of the maternal innate immune system with MAMPs affects performance, innate immune responsiveness and disease resistance of the offspring. Knowledge about transgenerational effects of maternal immunization or infection will contribute to a better understanding of the variation in immune phenotypes, disease resistance and metabolic disorders the next generation(s).

Chapter 6: General discussion
Finally all outcomes of the chapters were combined and used to answer the main research questions and fill up the knowledge gap. Furthermore, remaining knowledge gaps were formulated together with suggestions for future research.

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CHAPTER 2

2

Transgenerational effects of maternal immune activation on specific antibody responses in layer chickens

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Abstract

Activation of the maternal immune system may affect innate and adaptive immune responses in the next generation and may therefore have implications for vaccine efficacy and dietary immune modulation by feed additives. However, transgenerational effects on immune responses in chickens have been investigated to a limited extend. The present study investigated effects of intratracheal (i.t) specific and aspecific immune activation of laying hens on specific antibody production in the next generation. In two experiments laying hens received intratracheally an immune stimulus with human serum albumin (HuSA) or lipopolysaccharide (LPS). In experiment 1, hatchlings of the immune activated hens were at 4 weeks i.t. immunized with HuSA or HuSA+LPS. Maternal immune activation with LPS increased HuSA specific IgY and IgM responses in offspring. These results suggest a transgenerational effect of the maternal immune system on the specific antibody response in the next generation. In experiment 2 hatchlings received either β-glucan-enriched feed or control feed and were i.t. immunized with HuSA. Maternal immune activation with LPS decreased IgY anti-HuSA responses after HuSA immunization within hatchlings that received β-glucan enriched feed. The results of Experiment 2 suggest a transgenerational link between the innate immune system of mother and specific antibody responses in offspring. Despite variabilities in the outcomes of the two experiments, the observations of both suggest a link between the maternal innate immune system and the immune system of the offspring. Furthermore, our results may imply that maternal activation of the innate immune system can influence immune modulating dietary interventions and vaccine strategies in the next generation.

Keywords: Chicken, transgenerational, innate immunity, β-glucan, lipopolysaccharide, antibody response

Introduction

Within chicken husbandry, the containment of infectious diseases is very important. One of the factors that might be of influence is the impact of the maternal immune system on disease resistance in the neonate, whereby activation of the maternal immune system may influence the immune system of the neonate in a transgenerational fashion. An example of these transgenerational effects is the transfer of maternal antibodies in birds that are passively transmitted to the neonate [1, 2]. Another example is transgenerational inheritance, in which epigenetic mechanisms, rather than changes in the DNA code, are involved. It is hypothesized that these epigenetic mechanisms cause transgenerational effects on neonatal immunity in mice [3] and birds [4, 5]. Apart from transgenerational effects on neonatal immunity, studies in mammals demonstrate an increased risk of developing stress-related problems, fertility problems, reproductive problems and higher susceptibility to metabolic disorders such as diabetes and obesity in the F_1 and even F_2 generations due to environmental effects in the F_0 generation [6-8]. Besides negative effects, transgenerational mechanisms may also be beneficial to the offspring. For example, immune activation with lipopolysaccharide (LPS) in pied flycatchers caused elevated antibody production in the offspring [9]. These transgenerational mechanisms, may enable the mother to prepare her offspring for their future environment whereby the immune responsiveness of the offspring is affected by information of the mother in a transgenerational fashion [3, 10, 11]. So far, it has not been studied whether activation of the maternal immune system in chickens influence the specific antibody response in the next generation. Therefore, the present study in laying hens aims to investigate whether maternal activation of the innate or adaptive immune system will affect the specific antibody responses in the next generation.

Laying hens were either immune activated with LPS as a microbial-associated molecular pattern (MAMP) acting as a non-specific activator of the innate immune system, or human serum albumin (HuSA) acting as a specific stimulator of the adaptive immune system. We hypothesize that the innate immune system might play an important role in transgenerational effects, which was previously proposed by Berghof et al. [5]. Effects of the maternal immune activators LPS and HuSA on the antibody responses and their isotypes to HuSA in the next generation were measured in two independent experiments. Like LPS, β-glucan act via receptors present on antigen presenting cells and therefore both have the potential to influence specific antibody responses [12, 13]. Specific antibody response is in the current study defined as a significant increase in the specific antibody level after an immunization with an antigen. If maternal immune activation indeed affects the immune system in the next generation, immune modulation by dietary additives might also be influenced [14-17]. Experiment 2 was therefore performed to study whether maternal immune activation could also influence the effects of dietary treatment with β-glucan on antibody responses in the next generation.

Materials and Methods

Ethics statement

Both experiments were approved by the Animal Welfare Committee of Wageningen University and Research in accordance with Dutch laws and regulations on the execution of animal experiments (Experimental Codes: 2013076.d & 2014057).

Experimental design

Transgenerational effects of maternal immune activation were studied by immunizing the hens with either PBS, HuSA or LPS. For experiment 1, the effects on the offspring specific antibody response were evaluated by immunization with HuSA or HuSA+LPS followed by measuring HuSA-specific IgM and IgY antibody titers in blood. A schematic design of the experiment is shown in **Figure 1**. In experiment 1 hens were randomly divided into 3 maternal groups (PBS, HuSA or LPS) with 15 individually housed hens per maternal group. During egg collection a total of 150 eggs of each maternal group were collected, incubated and hatched. A total of 60 female chicks, with 20 chicks per maternal group, were randomly selected and randomly divided over 2 pens. Each pen represents one of the two immunization groups (HuSA or HuSA+LPS). For experiment 2, the effects on the offspring specific antibody response were evaluated by immunization with HuSA followed by measuring HuSA-specific IgM and IgY antibody titers in blood (**Figure 1**). Experiment 2 also includes a dietary treatment, which started directly post hatch until the end of the experiment. A schematic design of the experiment is shown in **Figure 1**. Until hatch, design of experiment 2 was similar to that of experiment 1. Hereafter, a total of 60 female chicks, with 20 chicks per maternal group, were divided into 2 diet challenge groups, with 5 replicates per group. Entire offspring received an immunization with HuSA.

Chickens, housing, management and treatments

Two independent experiments were performed using commercial purebred White Leghorn chickens (WA chicken line; Hendrix Genetics B.V., Boxmeer, the Netherlands). For both experiments the chickens were kept according to standard management guidelines of Hendrix Genetics, which was previously described by Van der Klein et al. [18].

Experiment 1

Thirty-week-old hens were individually housed in wired cages of 0.6 m^2. The hens were randomly divided in 3 groups of each 10 hens. After 3 weeks habituation the hens were intratracheally (i.t.) immunized with: 0.5 ml PBS (control; Group PBS), 1 mg HuSA (Sigma-Aldrich corporations, St. Louis, MO, USA) in 0.5 ml PBS (Group HuSA) or 1 mg LPS (*Escherichia coli* serotype O55:B5, L2880, Sigma-Aldrich) in 0.5 ml PBS (group LPS). The i.t immunizations were performed by placing a 1.2×60 mm blunted anal cannula (InstruVet, Cuijk, the Netherlands), on a 1-mL syringe, gently into the trachea of the chick [19]. At the day of immunization all hens were inseminated with pooled sperm and this insemination was repeated after 2 weeks. In the third week after priming, which is one week after the second insemination, collection of fertilized eggs was started and 10 eggs per hen were collected during a period of 2 weeks. All eggs were marked per individual hen and stored at room temperature until incubation. Eggs were incubated at research facility "Carus" of Wageningen University & Research according to standard production practices.

The newly hatched chicks were sexed visually by wing feather sexing and 20 hens per maternal immune activation group were randomly selected and equally divided over 2 pens. Both pens contained chicks of all maternal treatments. All chicks within a treatment group were from different mother hens. These treatment groups were at 4 weeks post hatch i.t. immunized, as described earlier, with either: 1 mg HuSA in 0.5 ml PBS (group HuSA) or 1 mg HuSA + 1 mg LPS in 0.5 ml PBS (group HuSA+LPS). At day 0, 1, 7, 14, 21 and 28 post immunization 1 ml heparinized blood was collected from the brachial wing vein. Blood was centrifuged (5250 x g, 10 min at room temperature). Plasma was collected and stored at -20 °C until use.

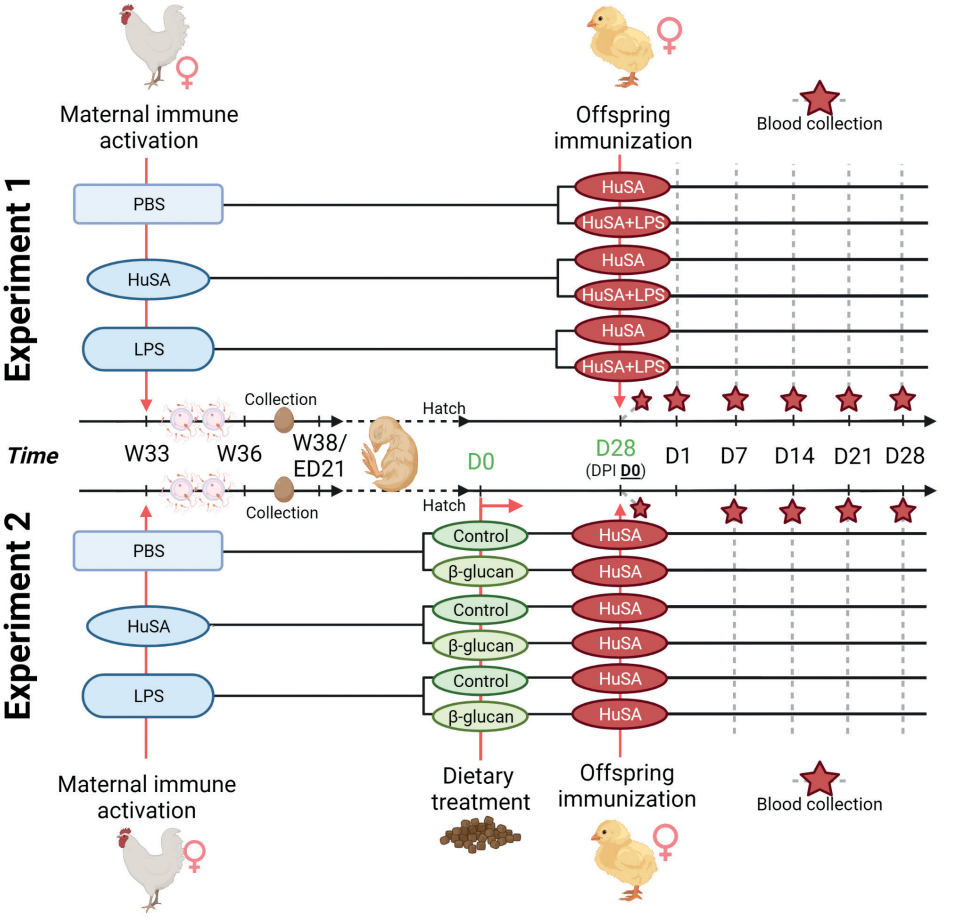

Figure 1. Timeline of the two transgenerational experiments. For both experiments, maternal immune activation with PBS, HuSA and LPS was performed on 33-week-old hens (W33). Hens were inseminated with pooled sperm at the day of immunization and repeated 2 weeks later. Eggs were collected when the hens reached the age of 36 weeks (W36) until 38 weeks of age (W38). For experiment 1, hatched chicks received at 28 days of age (DPI D0), the immunization with HuSA or HuSA+LPS. Blood was collected at DPI: D0, D1, D7, D14, D21 and D28 (marked with a red star). For experiment 2, hatched chicks received either a commercial starter diet (control) or a commercial diet enriched with a β-glucan additive (β-glucan). Chicks received at 28 days of age (DPI D0), the immunization with HuSA. Blood was collected at DPI: D0, D7, D14, D21 and D28 (marked with a red star). Figure created with BioRender.com

Experiment 2

Thirty-week-old hens were individually housed in wired cages of 0.6 m^2. These hens were divided in 3 groups of each 15 hens. After 3 weeks habituation the hens were intratracheally (i.t.) immunized, as described earlier, with: 0.5 ml PBS (control; Group PBS), 1 mg LPS (Sigma Aldrich) in 0.5 ml PBS (group LPS) or 1 mg HuSA (Sigma Aldrich) in 0.5 ml PBS (Group HuSA). At the day of immunization all hens were inseminated with pooled sperm and this insemination was repeated after 2 weeks. The collection of fertilized eggs was started one week after the second insemination and per hen 10 eggs were collected during a period of 2 weeks. All eggs were marked per individual hen and stored at room temperature until incubation. Newly hatched chicks were sexed and 20 hens per maternal immune activation group were selected. The in total 60 hens were allocated to 10 pens. These pens were randomly divided into 2 groups of 5 pens. Each group with 5 pens received either a commercial starter diet (control) or a commercial starter diet enriched with a β-glucan additive (250 ppm, Macrogard, Orffa, Werkendam, the Netherlands). All hens were at 4 weeks post hatch i.t. immunized, as described earlier, with 1 mg HuSA in 0.5 ml PBS. At day 0, 7, 14, 21 and 28 post immunization 1 ml heparinized blood was collected from the brachial wing vein. Blood was centrifuged (5250 x g, 10 min at room temperature) and plasma was collected and stored at -20 °C until use.

Table 1. Effect of maternal immune activation on HuSA specific IgM and IgY titers during 4 weeks after i.t. immunization of offspring at 4 weeks of age.

	Isotype	Maternal treatment[1]				Main effects (P-value)		
		PBS	HuSA	LPS	SEM	Time	Treatment	Time*Treatment[4]
anti-HuSA	IgM[2, 3]	3.2[ab]	3.0[b]	3.6[a]	0.2	<0.001	0.039	0.049
	IgY[2, 3]	5.1[b]	5.6[ab]	6.9[a]	0.7	<0.001	0.030	0.024

[1]Maternal treatment: i.t. immune activation of hens: 1mg HuSA or 1mg LPS in 0.5ml PBS.

[2]Least squares means of i.t. immunization HuSA and HuSA+LPS combined.

[3]Least square means within a row lacking a common superscript (a, b or ab) are significantly different ($P < 0.05$).

[4]Interacting variables are represented with an asterisk (*).

Detection of IgM and IgY antibodies binding HuSA

Titers of HuSA-specific IgM and IgY antibodies were determined in individual plasma samples by an indirect two-step ELISA as described previously [20, 21]. Briefly, flat bottomed 96-wells plates (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) were coated with 100 µL of 0.1M carbonate buffer (pH 9.6) containing 4 µg/mL HuSA (Sigma-Aldrich), incubated overnight at RT followed by a washing step. All washing steps during this ELISA assay were done with tap water containing 0.05% Tween ® 20 (Sigma-Aldrich). The washed plates were incubated for 90 min at RT with 4-step serial dilutions of serum samples in PBS containing 0.05% Tween ® 20, and 0.5% normal horse serum in duplicate. After washing, plates were incubated again for 90 min at RT with 100 µL of a 1:40.000 dilution of goat-anti-chicken IgY_{Fc} (Bethyl Laboratories Inc, Texas, USA) or a 100 µL 1:20.000 dilution of goat-

anti-chicken IgM (Bethyl Laboratories Inc) in PBS containing 0.05% Tween ® 20 and 0.5% normal horse serum. After washing, plates were incubated with tetramethylbenzidine and 0.05% H_2O_2 at RT and after 10 min the reaction was stopped with 1.25% H_2SO_4. The optical density was measured at 450 nm with a spectrophotometer (Multiscan™, Thermo Fisher Scientific, Waltham, MA, USA). Titers represents levels of antibodies relative to a standard positive control blood plasma sample. Titers were calculated as described by Berghof et al., (2018) [22].

Statistical analysis

Statistical analysis was done in SAS v 9.4 (SAS software by SAS institute INC.). All statistical analysis were performed with the PROC MIXED procedure. For both experiments the statistical model used for estimating maternal immune activation differences on antibody titers based on repeated observations in the offspring was as follows:

$$\boldsymbol{Y_{ijkl} = \mu + Treatment\ 1_i + Treatment\ 2_j + Day_k + (T1\ x\ D)_{ik} + Chicken_l + e_{ijkl}}$$

where Y_{ijkl} is the HuSA binding IgM or IgY titer of offspring chickens, Treatment 1_i is the fixed effect for maternal immune activation (i = HuSA, LPS or PBS), Treatment 2_j is the fixed effect for offspring treatments (j= HuSA, HuSA + LPS in experiment 1 and control diet or ß-glucan diet in experiment 2), Day_k is the fixed effect of day (post immunization) at which the blood was collected for antibody measurement (k= 0, 1, 7, 14, 21, 28), *(T1 x D)* $_{ik}$ is the fixed effect of the interaction between Treatment 1_i (T1) and Day_k (D), $Chicken_l$ is the random effect of the l_{th} chicken (l = 1-54) and e_{ijkl} is the residual term which was tested for approaching normality. The compound symmetry structure was used as covariance structure. Post hoc pair wise comparisons were corrected by Tukey-Kramer adjustment.

Results

Experiment 1: Effect of maternal immune activation on antibody production in offspring

Effects of maternal immune activation (PBS, HuSA, or LPS, respectively) on HuSA specific antibodies were measured in the offspring after immunization with either HuSA or HuSA+LPS at 4 weeks of age. Titers were measured at day 0, 1, 7, 14, 21 and 28 post immunization and the least-squares means (LSmeans) of the titers are presented in **Table 1**. Overall, the average antibody titers of both isotypes increased from day 0 to day 7 followed by a gradual decrease after day 7 onwards, indicating a specific antibody response to HuSA ($p<0.001$; **Table 1**). Comparison of the HuSA and HuSA+LPS responses in the offspring revealed no differences on HuSA titers and showed similar kinetics in time (**Figure 2 A-D**). Therefore, HuSA and HuSA+LPS treatments were combined in the further statistical analysis. Maternal immune activation with LPS enhanced IgM responses to HuSA in the offspring, compared to offspring of the HuSA immunized mother hens (titers 3.6 and 3.0; $P<0.05$) (**Table 1**). Maternal immune activation with LPS resulted in higher IgY anti-HuSA titers in the offspring compared to offspring of the PBS treated mother hens (titers 6.9 and 5.1; $P \leq 0.05$; **Table 1**).

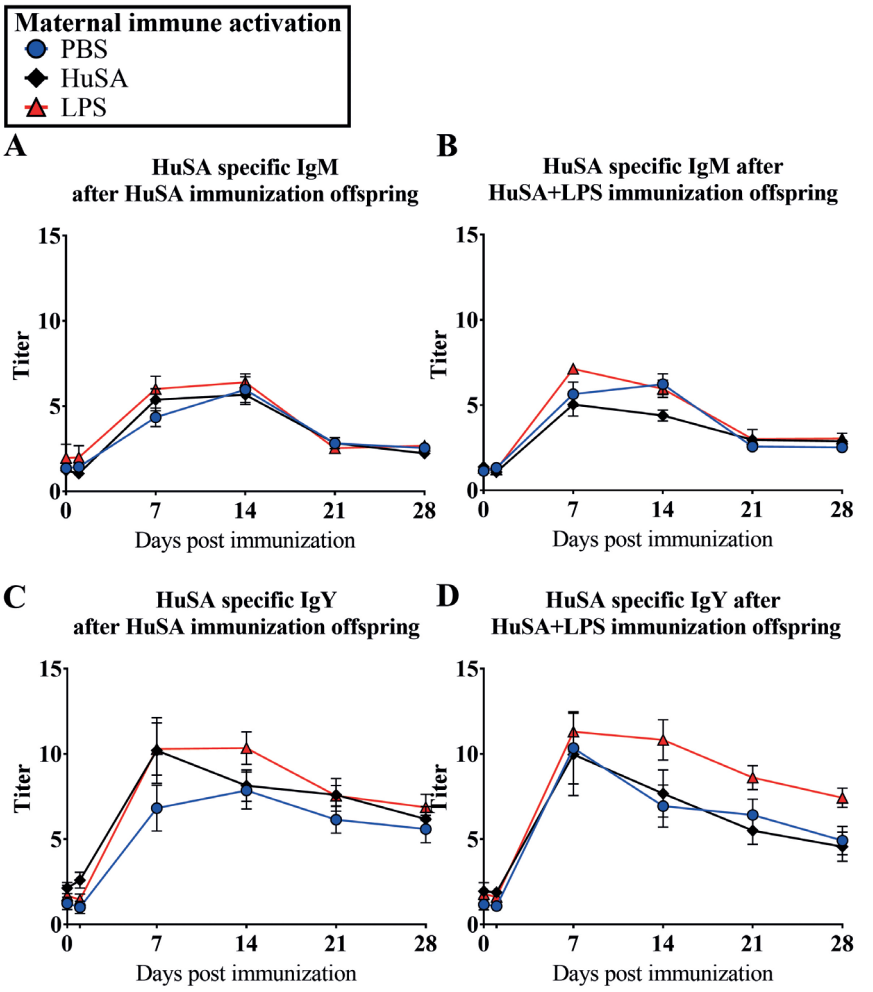

Figure 2. Results of experiment 1 with IgM and IgY anti-HuSA titers of chicks originating from immune activated hens with either PBS, HuSA or LPS. The chicks were immunized 4 weeks post hatch with either HuSA or HuSA+LPS. Titers were measured at 0, 1, 7, 14, 21 and 28 days post immunization. A) anti-HuSA IgM titers after HuSA immunization. B) anti-HuSA IgM titers after HuSA+LPS immunization C) anti-HuSA IgY titers after HuSA immunization. D) anti-HuSA IgY titers after HuSA+LPS immunization. Data are presented as means ± SEM, and N = 20 chickens per group.

Experiment 2: Effect of maternal immune activation on antibody production and dietary β-glucan modulation in offspring

Effects of maternal immune activation with PBS, HuSA or LPS on IgM and IgY anti-HuSA antibody titers were measured in the offspring after HuSA immunization with and without administration of dietary β-glucan. Titers of HuSA specific IgM and IgY antibodies were measured at day 0, 3, 7, 14, 21 and 28 and the LSmeans of the titers during this time period are shown in **Table 2**. Antibody titers of both isotypes increased in time, indicating a specific antibody response to HuSA ($p<0.001$; **Figure 3**). Both graphs in figure 2 show similar kinetics with highest titers measured 7 days post immunization whereafter the titers decline. In contrast to experiment 1, no differences in IgM and IgY anti-HuSA antibodies were observed upon maternal immune activation with PBS, HuSA, or LPS in offspring without dietary treatment (**Table 2**).

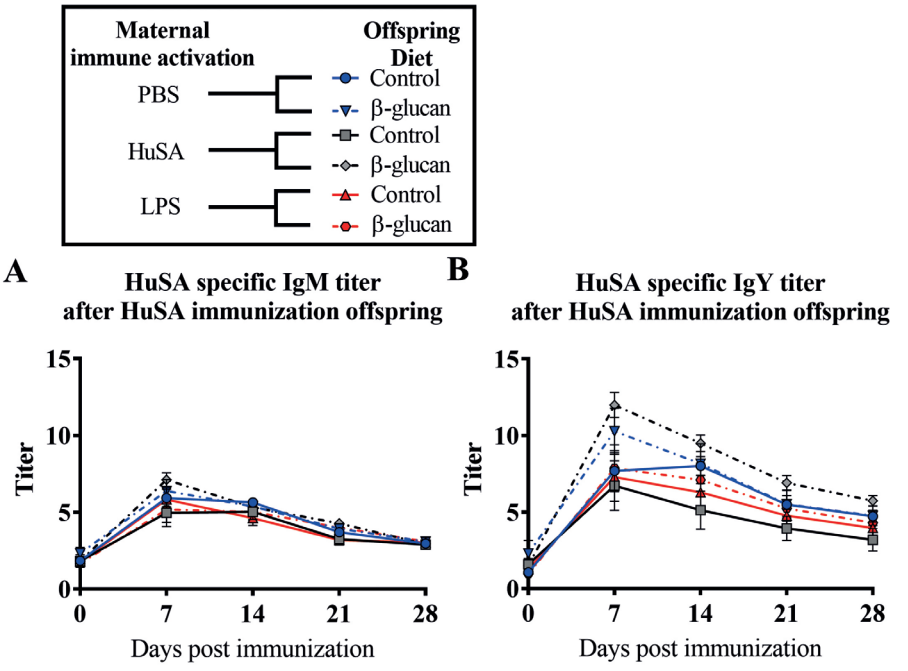

Figure 3. Results of experiment 2 with IgM and IgY anti-HuSA titers of chicks originating from hens immunized with either PBS, HuSA or LPS. The chicks were fed either the control diet or the diet enriched with β-glucan. Chicks were immunized 4 weeks post hatch with HuSA and titers were measured at 0, 3, 7, 14, 21 and 28 days post immunization. A) anti-HuSA IgM titers after HuSA immunization. B) anti-HuSA IgY titers after HuSA immunization. Data are presented as means ± SEM, and N = 10 chickens per group.

Table 2. Effect of dietary β-glucan on HuSA specific IgM and IgY titers in offspring after primary i.t. immunization with HuSA at 4 weeks of age.

	Isotype	PBS		HuSA		LPS			Main effects (P-value)[1,2]				
		Control	β-Glucan	Control	β-Glucan	Control	β-Glucan	SEM	Time	Treatment 1	Treatment 2	Treatment 1 * Treatment 2	Time * Treatment 1	Time * treatment 2[5]
anti-HuSA	IgM[3,4]	4.00	4.20	3.60	4.30	3.70	3.60	0.20	<0.001	0.228	0.206	0.351	0.870	0.345
	IgY[3,4]	5.40[ab]	6.21[ab]	4.12[b]	7.13[a]	4.72[ab]	4.80[ab]	0.60	<0.001	0.193	0.011	0.049	0.500	0.005

[1]Treatment 1: i.t. immune activation of mother hens: 1mg HuSA and 1mg LPS in 0.5ml PBS.

[2]Treatment 2: Dietary intervention with or without b-glucan enrichment in offspring; n = 5 pens per group.

[3]Least squares means.

[4]Least square means within a row lacking a common superscript (a, b or ab) are significantly different (P < 0.05).

[5]Interacting variables are represented with an asterisk (*).

Next, the effect of a dietary treatment with β-glucan was investigated. No main interaction effect between maternal immune activation and dietary treatment or main effects for maternal or dietary treatments on IgM were found. However, for IgY, an interaction was found between maternal immune activation and dietary treatment (P<0.05; **Table 2**). Maternal immune activation with HuSA followed by a β-glucan dietary treatment in the offspring resulted in a higher average IgY anti-HuSA antibody titer compared to chicks that obtained the standard diet (titers 7.13 and 4.12; P<0.05; **Figure 3** and **Table 2**). Maternal immune activation with PBS followed by a β-glucan dietary treatment in the offspring resulted numerically in a higher average IgY anti-HuSA antibody titer compared to chicks that obtained the standard diet (titers 6.21 and 5.40; n.s.; **Table 2**). In contrast, maternal immune activation with LPS followed by a β-glucan dietary treatment showed no difference compared to the same chicks that obtained the standard diet (titers 4.80 and 4.72; n.s.; **Table 2**).

Furthermore, an interaction was found between time and β-glucan dietary intervention for IgY anti-HuSA antibody titers (P<0.05; **Table 2**). **Figures 4A,B** show the IgM- and IgY-specific HuSA titers at day 7, the time point where the antibody levels reached their maximum. In accordance with the results shown in Table 2, the HuSA titers at time point day 7 show that maternal immune activation with HuSA followed by a β-glucan dietary treatment in the offspring resulted in a higher average IgY anti-HuSA antibody titer compared to the control group that obtained the standard diet (titers 11.99 and 6.73; P<0.05; **Figure 4B)**. Offspring originating from the LPS treated mother hens showed no difference with the control group that obtained the standard diet (titers 7.86 and 7.29; n.s.; **Figure 4B)**. Maternal immune activation with PBS followed by a β-glucan dietary treatment in the offspring resulted numerically in a higher average IgY anti-HuSA antibody titer compared

to the same chicks that obtained the standard diet (titers 10.28 and 7.69; n.s.; **Figure 4B)**. When comparing the three control offspring groups that obtained the standard diet, no differences in anti-HuSA titers were found (titers 7.69, 6.73 and 7.29; n.s.; **Figure 4B)**.

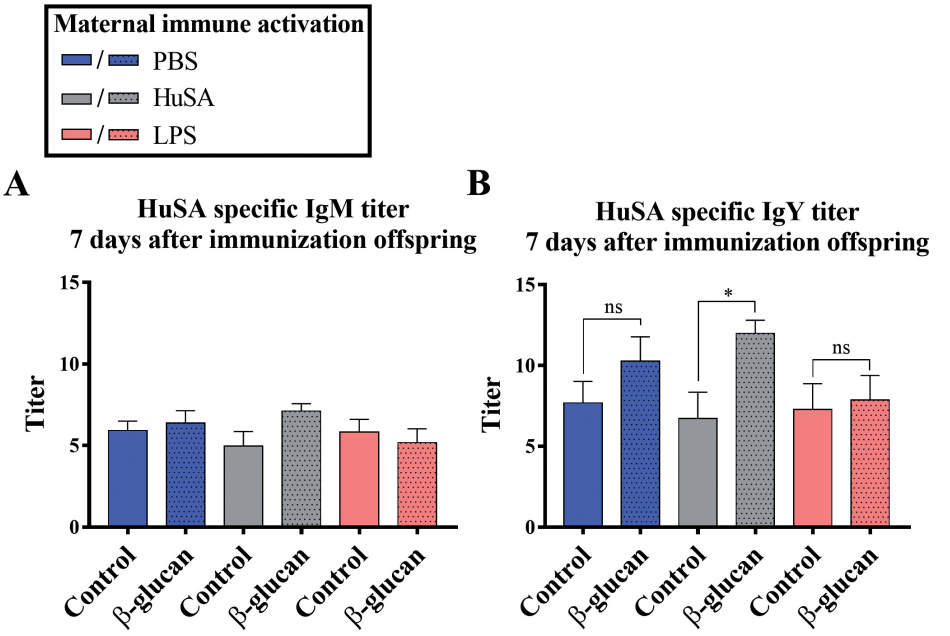

Figure 4. Results of experiment 2 with IgM and IgY anti-HuSA titers of chicks originating from hens immunized with either PBS, HuSA or LPS. The chicks were fed either the control diet or the diet enriched with β-glucan. Data of offspring 7 days post immunization are presented. A) IgM anti-HuSA titers 7 days after the HuSA immunization and B) IgY anti-HuSA titers 7 days after the HuSA immunization. Each bar represents means ± SEM, and N = 10 chickens per group. Effects are represented as * and were considered to be significant when $P \leq 0.05$. ns: not significant

Discussion

In this study, we investigated whether maternal innate and adaptive immune activation affected the specific antibody responses in the next generation. Two experiments were conducted to investigate whether specific and innate immunogenic stimulation of the hen, i.e. HuSA and LPS respectively, alter the offspring's specific antibody response. Furthermore, maternal innate immune activation by LPS seems to influence immune modulation by dietary β-glucans in the next generation. Although the results were not fully consistent, maternal immune activation with LPS appeared to have clear effects on the specific antibody responses in both experiments.

Within the current study, intratracheal immunization was used to mimic the practical circumstances of poultry in barns. Chickens inhale substantial amounts of endotoxins and

bacterial numbers present in dust particles in poultry barns, including the *E. coli* [23-25]. *E. coli* causes frequently infections in poultry and infect chickens mainly via the respiratory tract and intestines. LPS is a major component of the outer membrane of Gram-negative bacteria, such as *E. coli*. In the current study we used LPS, from the *E. coli* serotype O55:B5, intratracheally and is therefore a natural route to exposure of antigens, a good model to investigate effects on animal health and translatable to the practical circumstances. Intratracheal immunization with LPS and HuSA was previously successfully performed to initiate a substantial systemic immune response. Serological parameters, such as antibody titers, were affected and proven to be a good read out parameter [19, 24, 26]. Another study with chickens shows that intratracheally applied beads were found back in lungs, bursa, air sacs, humerus and radius [27], indicating that intratracheally administered antigens are easily absorbed.

In experiment 1, maternal immune activation with LPS leads to an increase in HuSA-specific IgY responses of the offspring. This observation with LPS suggests a transgenerational relation between the innate immune system of the hen and the immune system of the offspring. Our results are in accordance with a study in pied flycatchers, where maternal immune activation with LPS was found to have transgenerational effects resulting in elevated antibody production in the offspring [9].

The results from experiment 1 were not completely confirmed by those from experiment 2. Maternal i.t. immune activation with either specific antigen HuSA or innate antigen LPS did not increase the antigen specific IgY response against HuSA in the offspring. However, for IgY, an interaction was found between maternal immune activation and dietary treatment ($P<0.05$; **Table 2**). This implies that maternal immune activation influenced the stimulating effect of dietary β-glucans on the specific antibody response in the offspring. Dietary β-glucans are known to enhance antibody responses in chicken, [13, 28, 29]. In the current study however, we show that chicks originating from the LPS immune activated hens were unresponsive to the dietary β-glucans. Thus, maternal immune experiences, such as infections or vaccinations, may influence the effects of dietary interventions with feed additives on the immune system in the offspring. This observation might indicate that maternal immune activation with LPS leads to tolerance of innate immune cells to β-glucans in the offspring, resulting in reduced HuSA specific IgY production. To prove this, future studies should therefore focus on the effects of maternal immune activation on antigen presentation and B cell activating coreceptors on antigen presenting cells.

Both independent experiments with laying hens suggest that, despite the variation in outcomes, immunological experiences of the mother influence specific antibody production in the offspring. Both experiments demonstrated a memory-like mechanism, influenced by the maternal immune system stimulated with LPS. The effect on specific IgY antibody responses in the offspring caused by innate immune stimulation suggests interaction between maternal innate and neonatal immune system. These results could indicate the presence of transgenerational trained innate immunity where innate stimulation has induced a poly-specific memory like function which is known as one of the main consequences of trained innate immunity.

In recent years much attention has been paid to this concept of trained innate immunity. Studies in a broad variety of animal species, including mammals, birds and fish, have shown that the innate immune system can be trained, indicating the conserved nature of this immune mechanism [30-32]. Trained innate immunity is defined as the activation of the innate immune system resulting in a memory-like enhanced responsiveness to subsequent triggers driven by epigenetic mechanisms and metabolic reprogramming. The training by innate antigens leads to an increased resistance to infections [33, 34]. This effect is long-lasting and is polyspecific, i.e., one innate antigenic stimulus can lead to an amplified response against other unrelated stimuli [35]. It was also described that there is a dynamic interaction between LPS and β-glucan by inducing and damping tolerance [36]. This was caused by DNA epigenetic changes of the H3K27ac and H3K4me markers and part of the trained innate immunity concept. LPS is an important activator of innate immune cells, such as monocytes, macrophages and dendritic cells in a wide variety of animal species, and plays an important role within trained innate immunity and related epigenetic effects [30, 37]. The transgenerational effects found in experiment 1 and experiment 2 could be explained by epigenetic effects of the innate immune system, which was already previously proposed by Berghof et al. [5]. Studies have shown that trained innate immunity is maintained by epigenetic mechanisms such as DNA modification [36]. These modifications influence the activity of immune-related genes in innate immune cells [37]. For example, *in vitro* trained chicken macrophages showed enhanced expression of B cell activating molecule CD40 [38]. Thus, trained macrophages may enhance B cell activation *in vivo* as well, resulting in enhanced antibody responses.

Transfer of epigenetic DNA modifications to next generations has indeed been observed in chickens [39]. The transgenerational epigenetic effects can be found in the F_1 and even F_2 generation [40]. It is therefore reasonably to suggest that components that influence the innate immune system of the mother animal, like LPS did in the current study, influence the maturation of the neonatal immune system in a transgenerational manner and as a consequence the disease resistance of the neonate [41]. The concept of trained innate immunity could fit well in the current study and is therefore proposed as an explanatory concept. However, epigenetic data supporting this theory is lacking in the current study. More research on this is needed investigate this new concept.

Especially the results of experiment 2 suggests that maternal immune activation with LPS leads to tolerance of innate immune cells to β-glucans in the offspring, resulting in a lack of HuSA specific IgY antibody production. On the other hand, anti-HuSA maternal antibodies, which may influence the embryonic development or may be present in the HuSA-group offspring of experiment 2, did not result in tolerance. This could have been expected, because tolerance formation against antigens is blocked when antigen specific maternal antibodies are present as described previously [42, 43]. Presence of substantial amounts of HuSA specific maternal antibodies is however unlikely. Because fertilized eggs were collected 3-5 weeks after HuSA immune activation. The level of HuSA specific maternal antibodies in the offspring is then likely negligible. Indeed, no differences of antibody titers at day 0 were observed for the different maternal treatment groups. However, we cannot completely rule out that HuSA specific maternal antibodies induced an imprinting effect before day 0 on development of the neonate. This should be addressed in future

studies and more research on this is needed. The main goal of this study was whether there are transgenerational effects of maternal immunization. Remains of LPS in the hen's body was therefore not measured in hens. However, it is very likely that LPS has been enzymatic degraded and removed out of the chicken's body before fertilization and egg collection took place. In mice it has been found that LPS has a half-life of 3-4 minutes in the blood and that enhanced innate pro-inflammatory responses were back to normal within 4 hours [44, 45]. Also a study with chickens show that the enhancing effect of LPS administration in the trachea on TLR4 expression were back to original state within 72 hours [46]. Fertilized eggs were collected 3-5 weeks after maternal immune activation, it is therefore highly plausible to expect that LPS was not present in the yolk.

The current study did not elucidate how the transgenerational transmission of this immunity takes place. A highly plausible possibility is that the activity of immune related genes in germline DNA of the embryo is influenced by DNA methylation, histone acetylation or formation of regulatory micro-RNA molecules [39, 40]. Especially this transfer of epigenetic modifications deserves the consideration for investigation to unravel the mechanisms that causes effects found in the present study. More read-out parameters which have already described to be associated in visualizing trained innate immunity and transgenerational epigenetics in mammals, including histone modification analyses or transcriptomics, may be worth considering [41, 47]. The inconsistency between the outcomes of both experiments could be explained by the biological variation and/or life history of the hens whereby environmental confounding factors such as temperature, hygienic circumstances and the effects of intestinal microbiota should not be ignored [48, 49]. This indicates the importance of unraveling the mechanisms that are influencing transgenerational inheritance. More knowledge about transgenerational effects of maternal immune activation or infection will contribute to a better understanding of the variation in immune phenotypes, disease resistance and metabolic disorders. Knowledge that may have consequences for the animal husbandry sector how to optimize their vaccination strategies and how to apply immunomodulatory dietary additives more effectively.

Conflict of Interest

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

Author Contributions

All authors contributed to conception and design of the study. MV, HP and AL organized the database. AL and HP performed the statistical analysis. MV and AL wrote the first draft of the manuscript. HP and AL contributed to the funding acquisition of the project. MV and AL contributed to the visualization of the data. JA, HP and AL contributed to the field work and primary measurements. All authors contributed to manuscript revision, read, and approved the submitted version.

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CHAPTER 3

3

Training of Primary Chicken Monocytes Results in Enhanced Pro-Inflammatory Responses

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Abstract

Beta-glucan-stimulated mammalian myeloid cells, such as macrophages, show an increased responsiveness to secondary stimulation in a nonspecific manner. This phenomenon is known as trained innate immunity and is important to prevent reinfections. Trained innate immunity seems to be an evolutionary conserved phenomenon among plants, invertebrates and mammalian species. Our study aimed to explore the training of primary chicken monocytes. We hypothesized that primary chicken monocytes, similar to their mammalian counterparts, can be trained with β-glucan resulting in increased responses of these cells to a secondary stimulus. Primary blood monocytes of white leghorn chickens were primary stimulated with β-glucan microparticulates (M-βG), lipopolysaccharide (LPS), recombinant chicken interleukin-4 (IL-4) or combinations of these components for 48 h. On day 6, the primary stimulated cells were secondary stimulated with LPS. Nitric oxide (NO) production levels were measured as an indicator of pro-inflammatory activity. In addition, the cells were analyzed by flow cytometry to characterize the population of trained cells and to investigate the expression of surface markers associated with activation. After the secondary LPS stimulation, surface expression of colony stimulating factor 1 receptor (CSF1R) and the activation markers CD40 and major histocompatibility complex class II (MHC-II) was higher on macrophages that were trained with a combination of M-βG and IL-4 compared to unstimulated cells. This increased expression was paralleled by enhanced NO production. In conclusion, this study showed that trained innate immunity can be induced in primary chicken monocytes with β-glucan, which is in line with previous experiments in mammalian species. Innate immune training may have the potential to improve health and vaccination strategies within the poultry sector.

Keywords: Innate immune memory; inflammatory response; β-glucan; flow cytometry; primary chicken monocytes; macrophages

1. Introduction

Vaccinations are important to control infectious diseases in poultry. Effective vaccines induce pathogen-specific protection by the formation of specific antibodies and T cells, which are part of the adaptive immune system. Moreover, pathogen-specific memory will develop due to the formation of memory T and B cells.

It has long been assumed that this immunological memory was a unique property of the adaptive immune system. However, it is now accepted that plants and invertebrates, which lack an adaptive immune system, still have the ability to develop increased protective mechanisms against pathogens after primary exposure [1,2]. This implies that memory formation is also a feature of the innate immune system.

In recent years, studies in mammalian species have shown that in vitro stimulation of monocytes and macrophages with β-glucan from yeast *Candida albicans* cell wall resulted in increased responses after a secondary unrelated stimulation [3-5]. Both increased surface expression of activation markers and elevated production of pro-inflammatory cytokines were observed. These enhanced responses are ascribed to epigenetic reprogramming, mediated by DNA histone modifications of the corresponding genes [3]. Furthermore, injecting mice with a low amount of heat-killed *C. albicans* resulted in an increased survival rate, and enhanced the production of tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) by monocytes after secondary lipopolysaccharide (LPS) stimulation 7 days later [3]. Humans vaccinated with bacillus Calmette–Guérin (BCG) showed an enhanced pro-inflammatory response after a secondary stimulation with *Mycobacterium tuberculosis* or other non-related pathogens [6]. This protective effect, which was independent of T and B cells, remained for up to a year after the initial activation [7]. The mentioned findings are referred to as trained innate immunity.

Although many studies have reported evidence for trained innate immunity in mammalian species, plants and invertebrates, knowledge on trained innate immunity in avian species is limited. In the present study, we investigated the effect of stimulation with β-glucan in primary chicken monocytes by determining surface expression of the lineage marker colony stimulating factor 1 receptor (CSF1R), and the activation markers major histocompatibility complex class II (MHC-II) and CD40. Nitric oxide (NO) production was measured to observe the pro-inflammatory responses of the cells.

More knowledge on the contribution of trained innate immunity in the induction of vaccine- and feed-mediated protection in poultry may improve the effectiveness of the current vaccination and feeding strategies. This study aims to explore the training of chicken primary monocytes. We hypothesized that primary chicken monocytes can be trained with β-glucan, similar to their mammalian counterparts. To this end, we primary stimulated chicken primary monocytes with β-glucan, β-glucan + IL-4 or LPS. A secondary stimulation with LPS was subsequently given to measure the increased responsiveness of the cells by determining the expression of cell surface markers and NO production as indicators for trained innate immunity.

2. Materials and Methods

2.1. Animals and Ethical Statement

Blood for cell isolation was derived from the high feather pecking line of the 18th and 19th generations of white leghorn chickens that were divergently selected for feather pecking behavior. These chickens were part of a study on feather pecking behavior (see [8,9]). The hens were housed in 2 m^2 pens with wood shavings on the floor under normal housing conditions matching the guidelines for laying hens. Birds received a standard rearing diet from hatching until 8 weeks of age, and another standard rearing diet from 8 until 10 weeks of age. Water and feed were provided ad libitum. Birds received vaccinations against Marek's disease (day 0), infectious bronchitis (day 0, week 2 and 8), Newcastle disease (week 1, 4and 10) and infectious bursal disease (week 4). Blood was collected from the wing vein from ten-week-old chickens. A heparinized syringe was used to prevent the blood from coagulating. This study was approved by the Animal Welfare Committee of Wageningen University and Research in accordance with Dutch laws and regulations on the execution of animal experiments (no: AVD104002015150 and no: AVD2015357).

2.2. Preparation of Microparticulate β-Glucan Suspension

Beta-glucan from the *Saccharomyces cerevisiae* cell wall (Macrogard, Orffa, Werkendam, the Netherlands) consists of non-soluble macroparticles and was therefore pre-treated to obtain a microparticulate suspension. The procedure was adapted from previously described methods [10,11]. The β-glucan was suspended in sterile DNase/RNase-Free distilled water (Invitrogen UltraPure, Carlsbad, California, USA) and shaken at room temperature using a laboratory platform rocker overnight. The next day, the suspension was diluted with NaOH to reach a final concentration of 10 mg/mL β-glucan in 0.03M NaOH (pH 12.4). The suspension was then heated at 70 °C for 2.5 h in a water bath with regular vortex shaking. A microparticulate suspension was created using a sterile syringe and needle (BD Microlance 27G ¾ nr 20) by drawing the suspension up and down 2 times. This treatment of β-glucan resulted in a homogeneous suspension of microparticulates (Figure A1). The suspension was aliquoted and stored at −20 °C until further use. Homogenization of the suspension was repeated every time just before the β-glucan was applied to the cells. This microparticulate β-glucan suspension is hereafter referred as 'M-βG'.

2.3. Stimulation of Primary Monocytes

The collected chicken blood was diluted 1:1 in Roswell Park Memorial Institute 1640 supplemented with 25 mM HEPES (RPMI 1640) (Gibco, Life Technologies Ltd., UK). The diluted blood was overlaid onto an equal volume of ficoll-paque (Histopaque-1119, density: 1.119 g/mL, Sigma-Aldrich corporations, St. Louis, MO, USA) to separate the leukocytes by density gradient centrifugation (700× *g*, 40 min at room temperature). The interphase containing the leukocytes was collected, washed 2 times with RPMI 1640 and re-suspended in culture medium. This culture medium contains RPMI 1640 supplemented with 25 mM HEPES, Glutamax ™, 10% heat inactivated chicken serum and 50 U/mL penicillin and 50 µg/mL streptomycin (all from Gibco).

The timeline of the ex vivo innate training experiment is shown in Figure 1. Leukocytes were seeded at a concentration of 1×10^6 cells per well in a 96-well flat bottom plate

(CELLSTAR, Greiner Bio-One, Alphen aan den Rijn, The Netherlands) in a total volume of 100 µL per well. The cells were incubated overnight at 41 °C in 5% CO_2 and 95% humidity. The next day, non-adherent cells were washed away with pre-warmed (41 °C) culture medium. Adherent cells from every individual chicken were stimulated in a volume of 200 µL per well with culture medium supplemented with M-βG (10 µg/mL), LPS from *E. coli* serotype O55:B5 (10 µg/mL, L2880, Sigma-Aldrich corporations, St. Louis, MO, USA), recombinant chicken IL-4 (100 ng/mL, Kingfisher Biotech Inc., Saint Paul, MN, USA) or a combination of M-βG and IL-4. As a control, cells were incubated in culture medium without additional stimuli during the stimulation period. Cells were collected for flow cytometry analysis 24 h after stimulation. From an identical experiment performed simultaneously, cell culture supernatant was collected 48 h post-stimulation to measure the production of NO. After these 48 h, all cells were washed two times with culture medium to remove the stimuli and cultured further in 200 µL culture medium per well at 41 °C in 5% CO_2 and 95% humidity. At D6, the cells were secondary stimulated with 200 µL LPS (10 µg/mL). Cells were collected 24 h after the secondary stimulation for flow cytometry analysis. From an identical experiment performed simultaneously, cell culture supernatant was collected after 48 h for subsequent analysis of NO production.

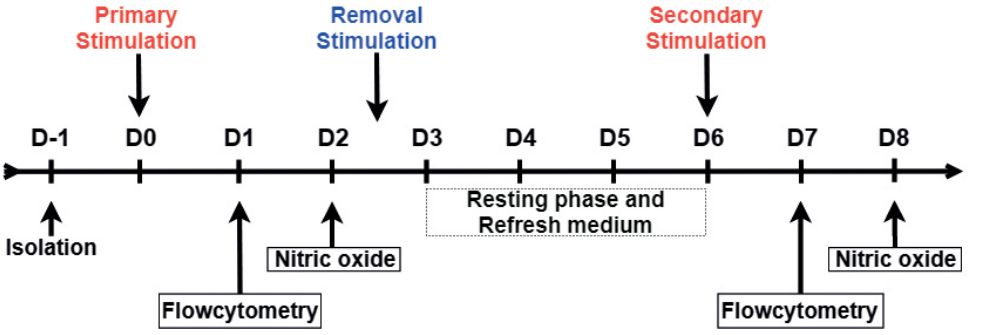

Figure 1. Schematic overview of the in vitro model for trained innate immunity. Adherent cells were primary stimulated with culture medium (UNSTIM), lipopolysaccharide (LPS), β-glucan microparticulates (M-βG), interleukin-4 (IL-4) and M-βG + IL-4 on day 0 (D0). On D1, cells were harvested for flow cytometry. On D2, cell culture supernatant was collected to measure the release of nitric oxide (NO), from an identical experiment performed simultaneously. Cells were subsequently washed to remove any stimuli and incubated in fresh culture medium until D6. On D6, all treatment groups were stimulated with LPS as secondary stimulation. The negative control (UNSTIM-UNSTIM) was not treated with LPS but only incubated with culture medium. Again, cells were subjected to flow cytometry analysis on D7 and NO release in the cell culture supernatant was measured on D8.

2.4. Nitric Oxide (NO) Production Assay

NO production was measured 48 h after the primary and secondary stimulation (Figure 1). NO was indirectly measured by quantifying the production of the more stable nitrite (NO_2^-), using the Griess reaction assay as previously described [12,13]. Briefly, 50 µl culture supernatant was transferred to a new 96-well flat-bottom plate (Greiner CELLSTAR®) and combined with 50 µl of Griess reagent consisting of a 1:1 mixture 2% Sulphanilamide in 5% H_3PO_4 and 0.2% N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O. The plate was incubated for 10 min at room temperature. The NO_2^- concentration was determined by measuring the optical density at 540 nm with a spectrophotometer (Thermo scientific,

Multiscan™). The results were interpolated on a standard curve made by serial diluting a sodium nitrite solution ($NaNO_2$) in the range from 100 µM to 0 µM.

2.5. Flow Cytometry

Flow cytometry was performed 24 h after the primary and secondary stimulation to phenotypically characterize the cell populations (Figure 1). Cells were washed with PBS w/o Ca^{2+} and Mg^{2+} (Gibco) and subsequently detached with 5 mM EDTA in PBS. The detached cells were transferred to a 96-well round-bottom plate (CELLSTAR, Greiner Bio-One, Alphen aan den Rijn, The Netherlands) and kept on ice. Staining and washing steps were performed in FACS buffer containing PBS w/o Ca^{2+} and Mg^{2+}, supplemented with 0.5% BSA and 0.005% NaN_3 (Sigma-Aldrich). The cells were stained with one of the following primary mouse monoclonal antibodies: anti-chicken CSF1R (clone ROS-AV170, IgG1; Bio-Rad), anti-chicken CD40 (clone LOB7/6, IgG2a; Bio-Rad, Hercules, CA, USA), or biotin-conjugated anti-chicken MHC class II (clone Ia, IgMκ, SouthernBiotech, Birmingham, AL, USA) at 4 °C in the dark for 20 min. After washing in FACS buffer, cells were incubated with the secondary antibodies: R-phycoerythrin (PE)-conjugated goat anti-mouse-IgG1 or allophycocyanin (APC)-conjugated goat-anti-mouse-IgG2a (both SouthernBiotech), together with Alexa Fluor 405-conjugated streptavidin (Invitrogen). Cells were again washed in FACS buffer and then stained with fluorescein (FITC)-conjugated mouse-anti-chicken KUL1-(IgG1) antibody (SouthernBiotech) for a period of 20 min at 4 °C protected from light. Finally, after washing the stained cells with FACS buffer, the 7-aminoactinomycin D (7-AAD; BD) was added to exclude nonviable cells. The samples were acquired on a FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo Software v. 10.5 (TreeStar Inc, San Carlo, CA, USA).

2.6. Statistical Analysis

Statistical analysis was performed using Prism version 7.04 software (GraphPad Software, San Diego, CA, USA). Differences in the mean among the experimental groups of the NO assay were analyzed using a one-way ANOVA with Tukey's multiple comparison tests. Flow cytometry data were expressed in geometric mean fluorescent intensity (gMFI) and fold change. A two-way ANOVA with Tukey's multiple comparison test was used for statistical analysis of the gMFI data. Fold change was calculated for each group with different primary stimulation conditions after secondary stimulation with LPS or unstimulated by $\frac{gMFI_{LPS}}{gMFI_{unstimulated}}$. A repeated measures one-way ANOVA with Tukey's multiple comparison test was used for statistical analysis of the fold change data. $p < 0.05$ was considered a significant difference. *p*-values between 0.05 and 0.1 were considered to indicate a tendency.

3. Results

3.1. In Vitro Culture Resulted in a Highly Homogeneous Macrophage Population after 7 Days of Culture.

Primary monocytes were isolated from chicken blood and characterized after 24 h (Figure 2A) and 7 days (Figure 2B) of culture by flow cytometry to get more insight into the composition of the cell population. The cells were gated for viability ($7\text{-}AAD^-$), high forward

and side light scatter (FSC vs. SSC), indicative of macrophages [14]. The macrophages expressed chicken macrophage markers KUL01 [14] and CSF1R [15], MHC-II and low levels of co-stimulatory molecule CD40 at both timepoints. On D7, the macrophage population was highly homogeneous, comprising >90% of the total cell population.

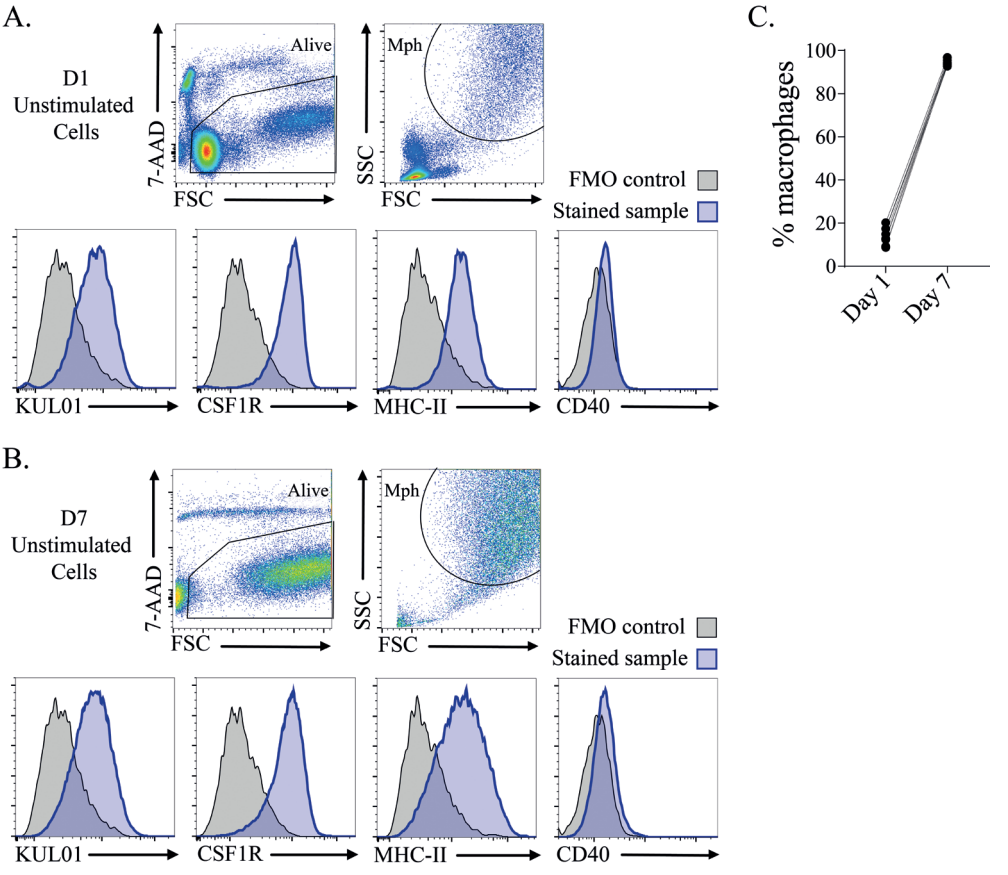

Figure 2. Adherent cells differentiated to KUL01$^+$ CSF1R$^+$ MHC-II$^+$ macrophages (Mph) after 7 days of culture. (**A**) Adherent cells were characterized after 24 h of culture by flow cytometry. (**B**) Adherent unstimulated cells were characterized after 7 days of culture by flow cytometry. The cells were selected for viability (7-AAD$^-$), forward and side light scatter (FSC vs. SSC), and assessed for the expression of KUL01, CSF1R, MHC-II and CD40. The histograms show expression of the macrophage markers in blue and fluorescent-minus-one (FMO) staining controls in grey. (**C**) The percentages of macrophages from day 1 and day 7.

3.2. Primary Stimulation with β-Glucan Microparticulates and IL-4 Enhanced NO Production after Secondary Stimulation with LPS.

We investigated the pro-inflammatory responses in chicken monocytes by measuring NO production upon primary and secondary stimulation (Figures 3 and 4). The cytokine IL-4 was included in the study because IL-4 highly upregulated the expression of the major receptor for beta-glucan, dectin-1, in murine macrophages [16]. As indicated in Figure 3, NO production was increased after primary stimulation with LPS compared to the unstimulated cells (LPS: 17.23 ± 3.17, UNSTIM: 0.94 ± 0.13, $p < 0.001$). We did not observe NO production after primary stimulation with M-βG (0.88 ± 0.13), IL-4 (0.45 ± 0.12) or the combination of M-βG + IL-4 (1.01 ± 0.13) compared to the unstimulated cells. Six days later, cells were secondary stimulated with LPS.

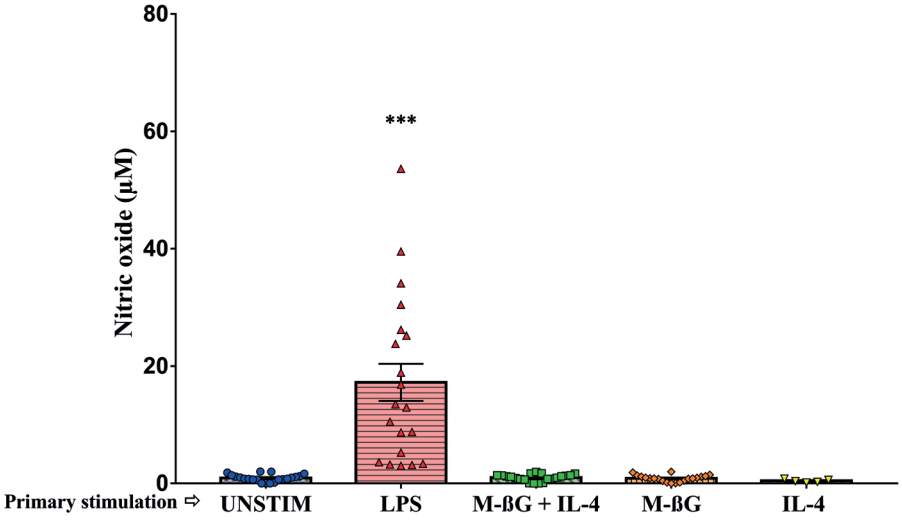

Figure 3. Primary stimulation with LPS resulted in enhanced nitric oxide production. Fresh isolated adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG + IL-4 (10 µg/mL + 100 ng/mL) or IL-4 (100 ng/mL). LPS induced NO production ($N = 21$ chickens; N IL-4 = 5 chickens). Each bar represents mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

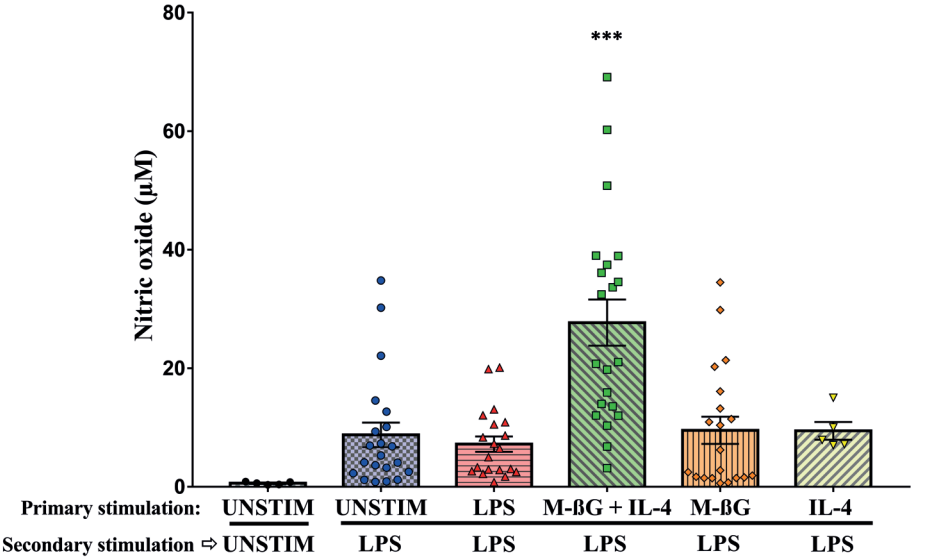

Figure 4. Primary stimulation with β-glucan microparticulates + IL-4 enhanced the NO production after secondary stimulation of LPS. Freshly isolated adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG + IL-4 (10 µg/mL + 100 ng/mL) or IL-4 (100 ng/mL) on D0. The cells were secondary stimulated with LPS (10 µg/mL), except for the negative control (UNSTIM-UNSTIM) on D6. NO production after the secondary stimulations are shown in this figure. Only cells primary stimulated with the combination M-βG + IL-4 showed increased NO production after a secondary stimulation with LPS compared to primary unstimulated cells (N = 21 chickens). Each bar represents mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

As indicated in Figure 4, this resulted in NO production which was higher in cells which were primary stimulated with M-βG + IL-4 compared to primary unstimulated cells (UNSTIM-LPS) (M-βG + IL-4: 27.70 ± 3.89, UNSTIM-LPS: 8.76 ± 2.08, $p < 0.001$). No differences were found in NO production after secondary stimulation with LPS for the cells that were primary stimulated with M-βG (9.51 ± 2.29), IL-4 (9.44 ± 1.50) or LPS (7.19 ± 1.29) compared to primary unstimulated cells (UNSTIM-LPS). Taken together, we found an increased NO production, which is indicative of a pro-inflammatory response, after secondary stimulation with LPS in cells which were primary stimulated with M-βG in combination with IL-4.

3.3. Primary Stimulation with β-Glucan Microparticulates and IL-4 Influenced CD40, MHC-II and CSF1R Surface Expression after Secondary Stimulation with LPS.

In addition to NO production, we investigated the expression of the surface markers KUL01, CSF1R, MHC-II and CD40. KUL01 and CSF1R are two well-known myeloid markers and were primarily used in this study to phenotypically characterize the macrophages in the cell population [14,15]. Surface markers CD40 and MHC-II are associated with activation of myeloid cells [17-21]. After 7 days, primary activation of the cells had gone down to baseline with respect to activation markers CD40 and MHC-II, although primary

stimulation with LPS resulted in a lasting increase in KUL01 expression and primary activation with beta-glucan + IL-4 resulted in a lasting decrease in CSF1R expression (Figure 5B). Secondary stimulation with LPS resulted in increased surface expression of CD40 compared to secondary unstimulated cells (Figure 5A,C). This increase was larger for macrophages primary stimulated with M-βG + IL-4 compared to macrophages primary stimulated with LPS or to primary unstimulated cells (fold change with M-βG + IL-4: 3.67 ± 0.27, fold change with LPS: 2.36 ± 0.15, $p < 0.01$; fold change with UNSTIM: 2.65 ± 0.19, $p < 0.05$). In contrast to CD40, surface expression of MHC-II and CSF1R was lower after the secondary LPS stimulation in macrophages primary stimulated with LPS or in primary unstimulated cells (Figure 5A,C). Interestingly, the lower expression of MHC-II and CSF1R was largely prevented by M-βG + IL-4 primary stimulation (MHC-II fold change with M-βG + IL-4: 0.89 ± 0.06, fold change with UNSTIM: 0.52 ± 0.05, $p < 0.001$; CSF1R fold change with M-βG + Il-4: 0.88 ± 0.10, fold change with UNSTIM: 0.61 ± 0.05, $p < 0.05$). KUL01 expression was lower in M-βG + IL-4 primary stimulated macrophages compared to primary unstimulated cells after secondary stimulation with LPS (fold change with M-βG + Il-4: 0.83 ± 0.04; fold change with UNSTIM: 0.98 ± 0.04, $p < 0.05$, Figure 5C).

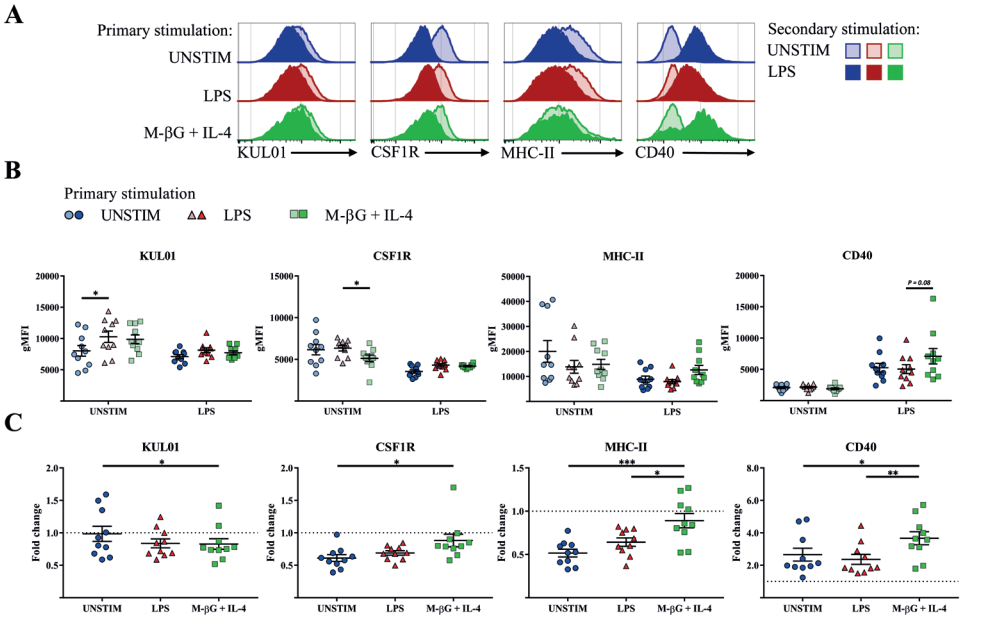

Figure 5. Primary stimulation with β-glucan microparticulates + IL-4 influenced the expression level of cell surface markers on macrophages after secondary stimulation with LPS. Surface expression of KUL01, CSF1R, MHC-II and CD40 was assessed after secondary stimulation with LPS (10 µg/mL). (**A**) Expression of the markers is shown by histograms for macrophages derived from one representative chicken upon secondary stimulation with LPS or secondary unstimulated cells (UNSTIM). (**B**) The effect of different primary stimulations after 7 days for secondary unstimulated cells (UNSTIM) and upon secondary stimulation with LPS. The expression of the markers KUL01, CSF1R, MHC-II and CD40 were expressed in geometric mean fluorescent intensity (gMFI). (**C**) The effect of different primary stimulations on secondary stimulated macrophages for surface expression of KUL01, CSF1R, MHC-II and CD40. The expression of the markers upon secondary stimulation with LPS was compared to secondary unstimulated cells (UNSTIM) and changes were expressed as a fold change in geometric mean fluorescent intensity (gMFI). For (B) and (C), each bar represents mean ± SEM ($N = 10$ chickens). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4. No Evidence of Training in Chicken Bone Marrow-Derived Adherent Cells.

Trained innate immunity is not conserved to blood monocytes only (e.g., Natural killer cells) [22]. Therefore, in parallel to the training of blood-derived primary monocytes, we tried to train chicken bone marrow-derived monocytes and myeloid progenitors using the same approach. NO production was again determined as a pro-inflammatory measure for trained innate immunity. Both primary stimulation and secondary stimulation are shown (Figures B1 and B2). The responses to the primary stimulations were similar to the blood-derived primary monocytes. NO production after the primary stimulation with LPS was increased compared to the unstimulated cells (LPS: 4.35 ± 0.23, UNSTIM: 2.37 ± 0.09, $p < 0.001$, Figure A2). However, the responses to the secondary stimulation with LPS did not result in enhanced NO production for M-βG + IL-4 primary stimulated cells compared to primary unstimulated cells (M-βG + IL-4: 4.12 ± 0.38, UNSTIM-LPS: 3.80 ± 0.50, Figure A3).

4. Discussion

The present study is to our knowledge the first study describing trained innate immunity in primary chicken monocytes. In this study, we measured NO production and analyzed the surface expression of the markers CD40 and MHC-II which are associated with monocyte activation, indicative of a pro-inflammatory response [17,18,20,21]. Primary stimulation with M-βG in combination with IL-4 resulted in an increased immune responsiveness to LPS, reflected by increased NO production and increased surface expression of CD40, MHC-II and CSF1R. Our results are in line with previous observations on trained innate immunity in mammalian species. Hence, we confirmed our hypothesis that primary chicken monocytes are trainable with β-glucan in combination with IL-4.

Trained macrophages from mice produced more NO compared to untrained cells after secondary LPS stimulation [23]. Furthermore, trained macrophages from mice and humans showed enhanced production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα [3,24]. Indeed, within our chicken study, training with M-βG + IL-4 resulted in increased cell surface expression of CD40 and MHC-II and elevated NO production. This association with CD40 expression fits earlier observations. In mice, it has been described that an increase in CD40 ligation was found to stimulate the expression of nitric oxide synthase (iNOS) [17]. NO, as a product of iNOS activity, is an effector molecule of activated macrophages that kills microbes within macrophages through its reactivity with proteins, DNA, thiols and iron at the active site of many enzymes [25]. Both the results on expression of the cell surface markers and NO production show that primary chicken monocytes can be trained, similarly to their mammalian counterparts.

NO production of trained monocytes may lead to increased killing capacity upon phagocytosis [26]. Indeed, a study in bovine monocyte-derived macrophages showed that increased bacterial killing capacity could be induced by macrophage training upon stimulation with heat-killed *Mycobacteria bovis* in vivo [27]. In that study, this was referred to as innate immune training.

It is known that increased phagocytosis results in enhanced antigen presentation [28]. A higher level of surface markers such as CD40 and MHC-II may lead to an enhanced adaptive immune response, since both CD40 and MHC-II play an important role in antigen presentation and the subsequent activation of the adaptive immune system [17,18,29,30]. The study with bovine monocyte-derived macrophages also indicates a relationship between trained innate immunity and increased antibody levels of the adaptive immune response [27].

Interestingly, training was only observed when monocytes were trained with M-βG in combination with IL-4. With the current read out parameters, we found no significant effects of training by M-βG and IL-4 separately. Beta-glucan is a known agonist of the pattern-recognition receptor dectin-1 found on mammalian phagocytes [31]. A positive correlation has been found between stimulation with IL-4 and/or IL-13 and surface expression of dectin-1 receptor in murine macrophages within 4 h [16]. In accordance with this observation, the addition of IL-4 to our cultures may have caused an upregulation of dectin-1 receptors, making the macrophages more responsive or accessible to M-βG. Although an intensive BLAST search in the latest *Gallus gallus* genome database (GRCg6a: build GCF_000002315.6) did not result in the identification of a dectin-1 chicken homologue, a dectin-l like β-glucan receptor is likely to be present on chicken heterophils and PBMCs (peripheral blood mononuclear cells), which have been found to respond to the dectin-1-specific agonist curdlan by an oxidative burst [32]. Whether dectin-1 or other pattern-recognition receptors for β-glucan, such as Toll-like receptor 2, Toll-like receptor 6 and Complement receptor 3, play a role in chicken innate immune training by β-glucan and IL-4 has to be elucidated and awaits further studies [31,33-36]. We did not find evidence for trained innate immunity in chicken bone marrow-derived cells, which is different from previous studies in murine bone marrow cells and may be interesting for further investigation to understand this discrepancy [37].

In humans, primary stimulation with LPS resulted in a tolerogenic state of the macrophages [24]. In the current study, no tolerance was observed, since no significant decrease was observed for LPS primary stimulated cells in the surface expression of the activation markers CD40 and MHC-II nor NO production upon secondary stimulation with LPS. The fact that we did not find evidence of LPS-induced tolerance in the current study contrasts with other in vivo studies in birds [38,39]. At this moment we are not able to clarify the absence of the tolerogenic state of the LPS primary stimulated cells. However, tolerance might be dependent on age, time of stimulation and dosage of the component, but the exact mechanism behind LPS tolerance is not fully known [40,41].

In our study, the surface markers CSF1R and KUL01 were not used as markers for training but were primarily used to phenotypically characterize the macrophages in the cell population. However, primary stimulation with M-βG + IL-4 resulted in higher CSF1R and lower KUL01 expression after secondary LPS stimulation (Figure 5C). This suggests that training affects the regulation of macrophage survival, differentiation and proliferation [15]. In line with our observation, reduced KUL01 expression was also found on bone marrow-derived monocytes after LPS stimulation [42,43].

We showed trained innate immunity by using a relatively large sample size of individual chickens (N = 21). Within this group of chickens, we observed substantial individual variation. Understanding these individual variations can be of great value in understanding the mechanism behind trained innate immunity. A possible explanation may be small differences in genetic background between these chickens. Another factor that may influence training are the DNA modifications that determine the activity of the genes, so called epigenetic changes [44]. These changes are independent of genetic background but are influenced by external factors such as feed, environment, age and even the parents.

In conclusion, we showed training of primary chicken monocytes. More research on, for example, cytokine production, metabolic mechanisms, and epigenetic changes will be of great value to understand the mechanisms behind trained innate immunity in chickens. Innate immune training may have potential to improve disease resistance of poultry in a nonspecific manner, especially at a young age when the adaptive immune system has not yet fully developed [13,45-47]. Dietary additives or vaccinations based on β-glucan could potentially be applied in vivo to train innate immune cells and improve resistance to a variety of pathogens. An in vivo experimental infection, investigating different pathogenic organisms, should assess whether in vivo training has cross protective effects and whether increased pro-inflammatory responses will not damage the host. Possible interactions of enhanced innate immunity with metabolic and/or behavioral physiology should be considered [48,49].

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Appendix A: Microparticulate β-Glucan Suspension

Beta-glucan is a polymer of glucose that can be found in cereals, bacteria, fungi and yeast. Multiple studies have been done on β-1,3/β-1,6-glucan originated from the yeast cell wall (e.g., *C. albicans* and *S. cereviseae*). However, this β-glucan is known to be non-soluble and is therefore hard to use in an ex vivo stimulation assay. Beta-glucan forms aggregates of macroparticulates when present in an aquatic suspension (Figure A1A). In this study, we developed a method partly based on available literature to obtain a homogeneous preparation of microparticulates [10,11].

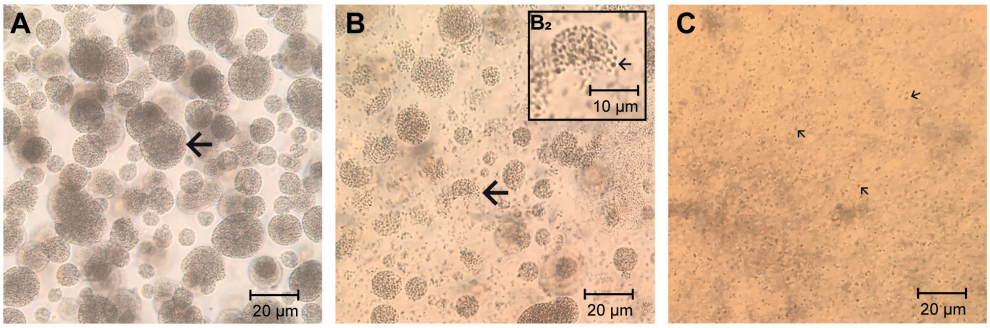


Figure A1. Microscopic pictures of β-glucan containing material from the *Saccharomyces cerevisiae* cell wall (Macrogard, Orffa). (**A**) In an aquatic suspension the β-glucan forms aggregates of macroparticulates (arrow) (**B**) β-glucan macroparticulates resuspended in 0.03M NaOH and heated at 70 °C for 2.5 h. The aggregates starting to fall apart (arrow) into microparticulates. (**B_2**) Closeup of a singular aggregate falling apart into individual microparticulates (arrow). (**C**) Homogeneous microparticulates suspension containing only individual β-glucan microparticulates (arrows). The suspension was created by using a sterile syringe by drawing the suspension of (B) up and down 2 times. Size bars are shown in the figures. Microscope magnification = 200 x, using an inverted microscope (Zeiss Primovert Inverted Microscope, Carl Zeiss Microscopy GmbH, Germany).

The aggregates of β-glucan macroparticulates in an aquatic suspension (Figure A1A) were resuspended in 0.03M NaOH and heated at 70 °C for 2.5 h. Aggregates starting to fall apart resulting in microparticulates (Figure A1B and A1B_2). A total homogeneous microparticulate suspension was created by using a sterile syringe (BD Microlance 27G ¾ nr 20) by drawing it up and down two times (Figure A1C).

Appendix B: NO Production of Bone Marrow-Derived Adherent Cells

Materials and Methods

Bone marrow-derived adherent cells were isolated from the femur and tibia bone of 8-day-old white leghorn chickens. All procedures were done under aseptic conditions. Bones were removed from the carcass, intensively cleaned with ice cold RPMI 1640 and kept on ice until use. Both ends of the bone were cut with scissors and the marrow was flushed with ice cold RPMI 1640 using a sterile syringe and needle (BD Microlance 21G nr 2). Bone marrow from the same bird was pooled and gently squeezed through a 70 µm cell strainer (Falcon™, Corning, Tewksbury, Massachusetts, USA) using a plunger and RPMI 1640. The cells were centrifuged (200× *g*, 10 min at room temperature), supernatant was discarded, and cells were re-suspended in culture medium. Bone marrow-derived adherent cells were seeded at a concentration of 1×10^6 cells per well in a Cell Culture Multiwell 24-well plate (CELLSTAR, Greiner Bio-One, The Netherlands) in a total volume of 1500 µL per well. The cells were incubated overnight at 41 °C in 5% CO_2 and 95% humidity. The next day, cells were stimulated in a volume of 2000 µL per well. The stimulation is the same as the previously described protocol for the primary monocytes in this report (Figure 1).

Results

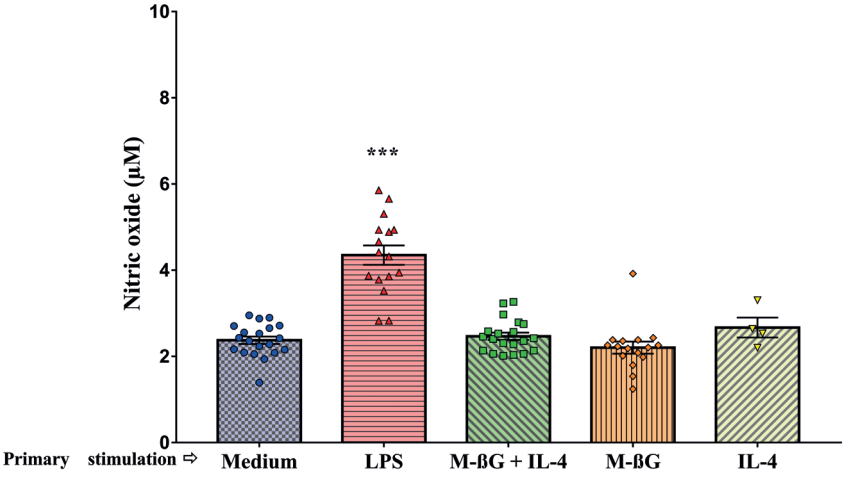


Figure A2. Bone marrow-derived adherent cells primary stimulation with LPS resulted in enhanced NO production. Fresh isolated chicken bone marrow-derived adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG + IL-4 (10 µg/mL + 100 ng/mL) or IL-4 (100 ng/mL). LPS induced NO production (N = 12–20 chickens; N for IL-4 = 4 chickens). Each bar represents mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

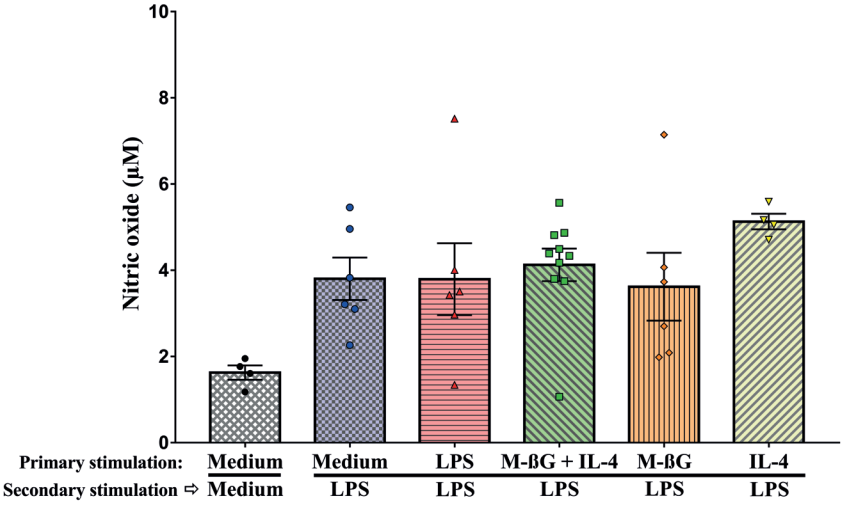
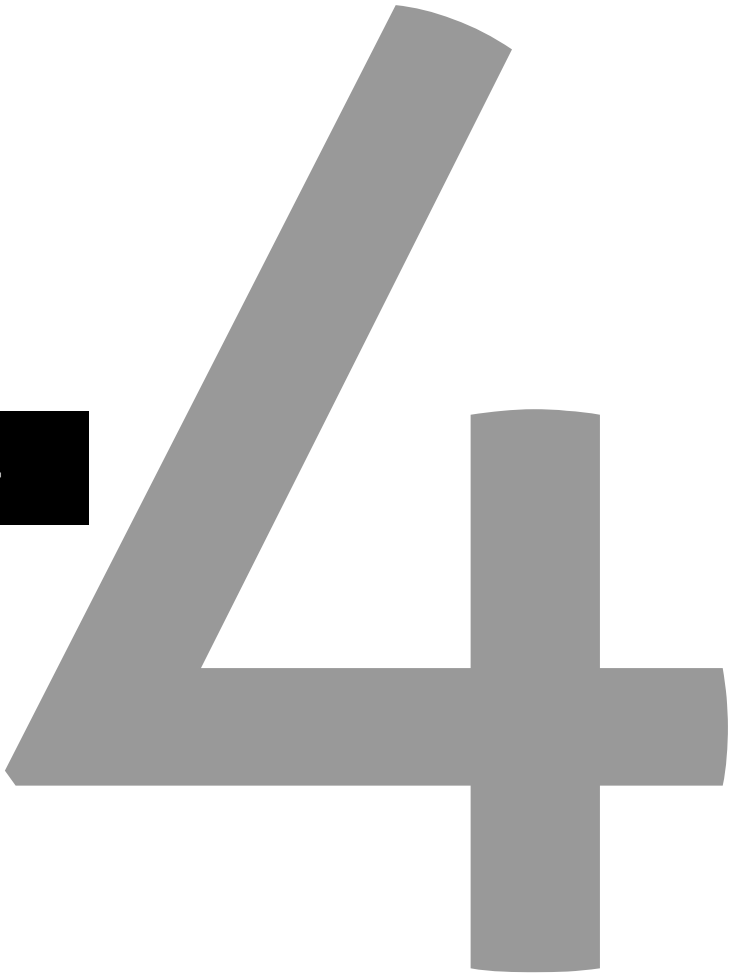


Figure A3. Bone marrow-derived adherent cells primary stimulated with M-βG + IL-4 failed to enhance NO production after secondary stimulation of LPS. Freshly isolated chicken bone marrow-derived adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG + IL-4 (10 µg/mL + 100 ng/mL) or IL-4 (100 ng/mL), on D0. The cells were secondary stimulated with LPS (10 µg/mL) except the negative control (UNSTIM-UNSTIM) on D6. Results after this LPS stimulation are shown in this figure. LPS induced NO production for all treatment groups. In contrast to blood-derived macrophages, a secondary stimulation with LPS did not affect the NO production in M-βG + IL-4 primary stimulated cells compared to the primary unstimulated cells (UNSTIM-LPS). (N = 6–10 chickens; N for UNSTIM-UNSTIM and IL-4 = 4 chickens). Each bar represents mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

CHAPTER 4



Innate immune training and metabolic reprogramming in primary monocytes of broiler and laying hens

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Abstract

Highlights

- Innate immune training is observed in monocytes from layers and broilers.
- Inflammation associated genes are influenced by training monocytes from layers and broilers.
- Training affects cellular metabolism associated genes in monocytes from layers and broilers.
- Training of primary monocytes is age-dependent.

Abstract

Recently, we have reported trained innate immunity in laying chicken monocytes. In the present study, we further investigated trained innate immunity of monocytes in layers and broilers. Monocytes of both breeds isolated from blood were trained *in vitro* with β-glucan, rec-chicken IL-4 or a combination of both, and restimulated with lipopolysaccharide (LPS), after which inflammation and metabolism-related responses were measured. Training of laying and broiler hen monocytes resulted in increased mRNA levels of IL-1β, iNOS and HIF-1α, but enhanced surface expression of CD40 and NO production was only observed in layers. Our *in vitro* study demonstrates that monocytes from different genetic backgrounds can be trained. However, the observed differences suggest a differential effect on immune functionality associated with innate training. Whether these differences in immune functions between layers and broilers have effect on disease resistance remains to be elucidated.

Keywords: Monocytes, chickens, immune training, metabolism, β-glucan, lipopolysaccharide

Abbreviations

HIF-1α, Hypoxia-inducible factor 1-alpha; PPARs, peroxisome proliferator-activated receptors; NO, nitric oxide; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthetase; M-βG, β-glucan microparticulates from Saccharomyces cerevisiae cell wall; NO, Nitric Oxide; RPMI 1640, Roswell Park Memorial Institute 1640 supplemented with 25 mM HEPES; Culture medium: RPMI 1640 supplemented with 25 mM HEPES, Glutamax™, 10% heat inactivated chicken serum and 50 U/mL penicillin and 50 µg/mL streptomycin; BCG, Bacillus Calmette–Guérin; FSC, forward scatter; SSC, side scatter; LDH, lactate dehydrogenase; lsmean, least square means; ARG2, Arginase 2; ACTB: Actin beta; IPO8: Importin 8; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; TNF: Tumour necrosis factor; PDK: pyruvate dehydrogenase kinase; H3K4me3: Histone 3 trimethylation of lysine 4.

1. Introduction

Containment of infectious diseases has always been an important focus in poultry husbandry. Various strategies including vaccinations and immunomodulating feed additives are used to increase the immune-mediated protection against infectious diseases [1-3]. Maternal antibodies and innate immune cells such as monocytes and macrophages play a major role in the defence of chicks against pathogens, because adaptive immune functions have not yet fully developed at young age. [4-6]. Trained innate immunity, whereby monocytes pre-exposed to for example β-glucan reach an activated state, may have potential to increase resistance to a wide variety of pathogens in the first weeks post hatch [7-9]. However, it is known that innate immune responses vary between chickens that are genetically selected for different purposes like laying chickens and broiler chickens [10-12]. Therefore, the aim of this study is to investigate whether isolated primary monocytes from layers and broilers differ in their innate training capacity.

In vitro and *in vivo* studies with mammalian blood monocytes showed that training enhanced levels of the pro-inflammatory cytokines IL-1β, TNF and IL-6 upon a restimulation, indicating a more activated cell state [7]. Apart from regulation of inflammation, metabolic reprogramming takes place in trained immune cells, which indicates crosstalk between signalling pathways controlling innate immunity and cellular metabolism [13, 14]. This is also referred to as the immuno-epigeno-metabolic crosstalk [15]. The Dectin-1/Akt/mTor/HIF-1α signalling pathway has been found to be involved in this phenomenon in response to β-glucan molecules [16]. Hypoxia-inducible factor 1-alpha (HIF-1α) acts as a master transcription factor, not only controlling inflammatory responses, but also metabolic reprogramming [17]. Indeed, in trained innate immune cells, the metabolic state of mammalian macrophages shifts from the normal oxidative phosphorylation route of producing energy towards the more efficient aerobic glycolysis. This shift is known as the "Warburg Effect" [13, 14]. This metabolic shift results in increased lactate production [17-19]. Furthermore, peroxisome proliferator-activated receptors (PPARs), such as PPARγ, regulate metabolism and inflammation and are found to be involved in trained innate immunity [20, 21]. So far, it is unknown whether expression of HIF-1α and PPARγ are also involved in training of chicken macrophages.

Recently, we demonstrated that training of primary layer chicken monocytes with β-glucan in combination with rec-chicken IL-4 increased both nitric oxide (NO) production and surface CD40 expression after restimulation with lipopolysaccharide (LPS) [22]. These findings reflect the activated state of trained innate immune cells. However, compared with layers, it is known that broilers display a reduced production of pro-inflammatory cytokines and increased production of immunosuppressive cytokines, resulting in a reduced inflammatory response [10]. Moreover, broiler macrophage effector functions, such as phagocytosis, are impaired in response to LPS [23]. Other studies describe variable inducible nitric oxide synthetase (iNOS) gene expression levels and NO production in chickens of several genotypes [23-25]. Furthermore, immunological differences between broilers and layers were found for antibody responses and ileal immune-related gene expression [11, 12, 26]. Based on these immunological differences between broilers and

layers, we hypothesise that blood monocytes of both breeds may differ in the response to *in vitro* training with β-glucan.

In this study, we provide evidence that monocytes from the two breeds of chickens can be trained. Although, increased levels of iNOS, IL-1β and HIF-1α were found after training in both breeds, enhanced surface expression of CD40 and NO production was only observed in layers. These observations suggest a differential effect on (innate) immune functionality associated with innate training between layers and broiler chickens which may contribute to the previously described immunological differences between broilers and layers.

2. Material and methods

2.1 Animals and ethical statement

Twelve one-day-old Ross 308 broiler hens (Kuikenbroederij Morren bv, Barneveld, the Netherlands) and twelve one-day-old White Leghorn H&N Super Nick laying hens (Agromix Broederij en Opfokintegratie bv, Lunteren, the Netherlands) were obtained from commercial hatcheries and placed in separate pens for each breed. The hens were housed in 4 m^2 floor pens enriched with wood shavings on the floor and low perches. The broiler hens and laying hens received a standard rearing diet matching the requirements of the different breeds. Water and feed were provided *ad libitum* and the birds had access to heath lamps during the entire experiment. The temperature was monitored during the entire experiment. At day 11, all birds received the Newcastle Disease clone 30 spray vaccination. Hygiene requirements were applied to minimize the risk of introducing pathogens. At all-time clean shoes/boots were used when entering the stable. Clean shoe-covers and hand gloves were used when entering the pens and were changed between pens. This study was approved by the Animal Welfare Committee of Wageningen University and Research in accordance with Dutch laws and regulations on the execution of animal experiments (no: AVD1040020185427).

2.2 Isolation and culture of primary monocytes

Heparinized blood was collected from the wing vein and its amount was based on bodyweight according to the guidelines of the Animal Welfare Committee. Blood (2.0 mL) from all individual chickens of both breeds was taken at day 42 and kept separately for further analysis. For NO production assays, blood of the same chickens was also individually harvested at preceding days, namely day 21 (for Layers: 0.7 ± 0.2 mL; for broilers: 1.2 ± 0.5 mL) and day 28 (for Layers: 1.5 ± 0.5 mL; for broilers 2.0 ± 0.1 mL).

Primary monocytes from blood were isolated as described previously [22]. Briefly, mononuclear cells were purified by density gradient centrifugation (700 x g, 40 minutes at room temperature) and seeded at a concentration of $1*10^6$ cells per well in a 96-well flat bottom plate (CELLSTAR, Greiner Bio-One, the Netherlands), followed by incubation at 41°C in 5% CO_2 and 95% humidity overnight. The next day, non-adherent cells were washed away with pre-warmed (41°C) complete cell culture medium (i.e. RPMI 1640 supplemented with 25 mM HEPES, Glutamax™, 10% heat-inactivated chicken serum and 50 U/mL penicillin and 50 µg/mL streptomycin; all from Gibco). Directly after the density

gradient centrifugation step, a fraction of the mononuclear cells was used for differential leukocyte count. Monolayers of mononuclear cells were made using a cytospin device (Thermo Shandon Cytospin 3, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cells were stained with the Hemocolor staining kit (Merck KGaA, Darmstadt, Germany). Percentages of lymphocytes, heterophils and monocytes per 100 counted cells were calculated to determine possible variations in cellular compositions between individual chickens and breeds **(Fig. S1)**.

2.3 Training of primary monocytes

Training of primary monocytes isolated from blood was performed as previously described [22]. Briefly, the adherent primary monocytes were stimulated with β-glucan microparticulates from *Saccharomyces cerevisiae* cell wall (M-βG) (10 µg/mL, Macrogard, Orffa, Werkendam, the Netherlands), LPS from *Escherichia coli* serotype O55:B5 (10 µg/mL, Sigma L2880) or a combination of M-βG and recombinant chicken IL-4 (IL-4) (100 ng/mL, Kingfisher Biotech Inc., USA) in complete cell culture medium for 48h. Subsequently, cells were washed two times with culture medium to remove the stimuli and cultured further in 200 µl complete cell culture medium per well at 41°C in 5% CO_2 and 95% humidity. At D6 the cells, consisting almost entirely of monocyte-derived macrophages as evidenced by flow cytometry analysis [22], were restimulated with 200 µl LPS (10 µg/mL) for 24h. We used for the NO production assay a 48h LPS restimulation. These monocyte-derived macrophages are further referred to as macrophages. Then, the trained cells were harvested with 5 mM EDTA in PBS and either directly subjected to flow cytometry analysis or lysed with RLT lysis buffer and stored at -80°C until further RT-qPCR analysis (Qiagen, Hilden, Germany).

2.4 Nitric oxide (NO) production

NO was indirectly measured by quantifying the production of the more stable nitrite (NO_2^-), using the Griess reaction assay as previously described [11, 22, 27, 28]. Briefly, cell culture medium was collected from trained macrophages and combined with Griess reagent. The NO_2^- concentration was determined by measuring the optical density at 540 nm with a spectrophotometer (Thermo scientific, Multiscan™). The results were interpolated on a standard curve made by serial diluting a sodium nitrite solution ($NaNO_2$) in the range from 0 to 100 µM.

2.5 Total RNA isolation and gene expression analysis

Total RNA was isolated with the QIAGEN RNeasy mini kit (Qiagen, Hilden, Germany) followed by a DNA and DNase removal treatment (DNA-free DNA Removal Kit, AM1906, Invitrogen, Carlsbad, California, USA) according manufacturer's instructions. RNA quantity and purity were measured with a NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies LCC, Thermo Fisher, Wilmington, Delaware, USA). RNA quality was determined using the Agilent 2100 Bioanalyzer according manufacturer's instructions (Agilent Technologies, Santa Clara, California, USA). A standardized amount of 50 ng/µl RNA was reverse transcribed into complementary DNA (cDNA) using random hexamer primers (Roche Diagnostics, the Netherlands) and the SuperScript III Reverse Transcriptase kit (Invitrogen, 18080044). The cDNA was diluted 50x in 10 mM Tris pH 8.0 w/o EDTA (Invitrogen, AM9855G) for qPCR analysis. The qPCR assay was based on a 20 µl volume design using the SensiFAST™ SYBR®

Lo-ROX Kit (Bioline, Meridian Bioscience Inc., Cincinnati, Ohio, USA) together with a 5 µM primer set combination (**Table 1**) and was performed with a QuantStudio® 5 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Corporation, Foster City, California, USA). Amplification conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s each. A final melting curve protocol of increasing temperature from 60°C to 95°C with 0.1°C/s was applied. The results were interpolated on a standard curve made by 10x serial dilution of a known amount of corresponding cDNA product. Quantities were normalized to the geometric mean of 3 housekeeping genes (**Table 1**), which were identified as being the most optimal normalization genes among a set of candidates as determined with Normfinder algorithm software [29].

Table 1. Primers used for RT-qPCR

Target[1]	Sequence[2]	Accession no.
Housekeeping genes		
ACTB	F: 5′-GCCCTGGCACCTAGCACAAT-3′ R: 5′-GCGGTGGACAATGGAGGGT-3′	NM_205518
IPO8	F: 5′-ACCTCCGAGCTAGATCCTGT-3′ R: 5′-GGCTCTTCTTCGCCAACTCT-3′	XM_015287054
GAPDH	F: 5′-ATCCCTGAGCTGAATGGGAAG-3′ R: 5′-AGCAGCCTTCACTACCCTCT-3′	NM_204305
Genes associated with inflammation		
IL-1β	F: 5′-GACATCTTCGACATCAACCAG-3′ R: 5′-CCGCTCATCACACACGACAT-3′	XM_015297469
TNF	F: 5′-CCGCCCAGTTCAGATGAGTT-3′ R: 5′-GCAACAACCAGCTATGCACC-3′	XM_015294124
iNOS	F: 5′-CTACCAGGTGGATGCATGGAA-3′ R: 5′-ATGACGCCAAGAGTACAGCC-3′	NM_204961
ARG2	F: 5′-TCTGGAAACCTCCATGGGCA-3′ R: 5′-CAGATGCTGAAAGACAGGGCT-3′	NM_001199704
IL-10	F: 5′-GCTGAGGGTGAAGTTTGAGGA-3′ R: 5′-TCTGTGTAGAAGCGCAGCAT-3′	NM_001004414
Genes associated with metabolism		
HIF-1α	F: 5′-ACGTGTAAAGGCGTGCAAAA-3′ R: 5′-CGTGAGTTGGGGTAGTCCAC-3′	XM_015287266
PPARγ	F: 5′-GGGCGATCTTGACAGGAA-3′ R: 5′-GCCTCCACAGAGCGAAAC-3′	XM_015292933

[1]ACTB: Actin beta; IPO8: Importin 8; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; TNF: Tumour necrosis factor; iNOS: Inducible nitric oxide synthetase; ARG2: Arginase 2; HIF-1α: Hypoxia-inducible factor 1-alpha; PPARγ: Peroxisome proliferator-activated receptor gamma.
[2]F: forward; R: Reverse

2.6 Lactate production

The production of lactate by macrophages was measured using an enzymatic UV test with lactate dehydrogenase (LDH) according to the manufacturer protocol (Lactate FS procedure, DiaSys Diagnostic Systems GmbH, Holzheim, Germany). The extracellular

lactate was determined 24 hours after restimulation in the collected cell culture medium from the trained macrophages. Briefly, 15 µl of culture medium was mixed with 1000 µl reagent mix containing a buffer (pH 9.0, 500 mmol/L) and LDH (≥ 25 kU/L), incubated for 5 minutes at 37°C and followed by measurement of the absorbance at 340 nm with a spectrophotometer (Evolution 201 UV-Visible, Thermo Scientific, Waltham, USA). Next, the sample was mixed with 250 µl of nicotinamide-adenine-dinucleotide (NAD, 20 mmol/L), incubated for 5 minutes at 37°C and followed by measurement of the absorbance at 340 nm after 30 minutes with the same device. The lactate concentrations were calculated using a calibrator-mix which was provided by the manufacturer.

2.7 Surface marker expression

Flow cytometry was performed 24 hours after restimulation to phenotypically characterize the cell populations [22]. Briefly, the cells were stained with one of the following primary mouse monoclonal antibodies: anti-chicken CSF1R (clone ROS-AV170, IgG1; Bio-Rad), anti-chicken CD40 (clone LOB7/6, IgG2a; Bio-Rad), or biotin-conjugated anti-chicken MHC class II (clone Ia, IgMκ, SouthernBiotech, Birmingham, AL) at 4°C in the dark for 20 min. After washing in FACS buffer, cells were incubated with the secondary antibodies: R-phycoerythrin (PE)-conjugated goat anti-mouse-IgG1 or allophycocyanin (APC)-conjugated goat-anti-mouse-IgG2a (both SouthernBiotech), together with Alexa Fluor 405-conjugated streptavidin (Invitrogen) at 4°C in the dark for 20 min. After the secondary antibody incubation, the cells were again washed in FACS buffer and then stained with fluorescein (FITC)-conjugated mouse-anti-chicken KUL1-(IgG1) antibody (SouthernBiotech) for a period of 20 min at 4°C protected from light and resuspended in FACS buffer. Finally, after washing the stained cells with FACS buffer, 7-Aminoactinomycin D (7-AAD; BD) was added to exclude nonviable cells. The samples were acquired on a CytoFLEX™ flow cytometer (Beckman Coulter, California, USA). Data analysis was performed using FlowJo Software v. 10.5 (TreeStar Inc, San Carlo, USA). Background staining was assessed by fluorescent-minus-one (FMO) controls (**Fig. S2**).

2.8 Statistical analysis

Statistical analysis for all data was performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). The generalized estimating equations (GEE) procedure was used to estimate parameters of generalized linear model with correction for random animal effect. The residuals of these models were tested for normality. Explanatory variables of NO production data with age effect were treatment, age and their interaction. Flow cytometry data were expressed in fold change, which was calculated for each group with different primary stimulation conditions after secondary stimulation with LPS or unstimulated by $\frac{gMFI_{LPS}}{gMFI_{unstimulated}}$. Results were presented as least square means (lsmean) from $N_{\text{all days}} = 12$ layers, $N_{\text{Day21 and Day 28}} = 12$ broilers or $N_{\text{Day 42}} = 11$ broilers. *P*-values of comparisons were Tukey-Kramer adjusted. *P*-values < 0.05 were considered to be significantly different. *P*-values between 0.05 and 0.1 were considered to indicate a tendency.

3. Results

3.1 Trained macrophages of layers, but not of broilers, showed elevated nitric oxide production.

Primary monocytes isolated from blood of 42 days old broilers and layers were trained *in vitro*. At day six, the resulting macrophages were restimulated with LPS for 24h and the amount of NO accumulated in the medium was measured for untrained, M-βG + IL-4-trained and LPS-trained macrophages (**Fig. 1**). The LPS-trained macrophages group was included for comparison purposes. In layer macrophages, the NO production was greater in cells trained with M-βG ($P < 0.01$; $N = 12$) or M-βG in combination with cytokine IL-4 ($P < 0.05$; $N = 12$), compared to the untrained cells (**Fig. 1A**). In contrast, broiler macrophages trained with M-βG or M-βG in combination with IL-4 did not show increased production of NO, compared to corresponding untrained cells (**Fig. 1B**). However, broiler macrophages trained with only M-βG had elevated ($P < 0.05$; $N = 11$) NO production levels compared to the LPS or M-βG + IL-4 treatment group. Overall, upon restimulation broiler macrophages produced less NO compared with layer macrophages.

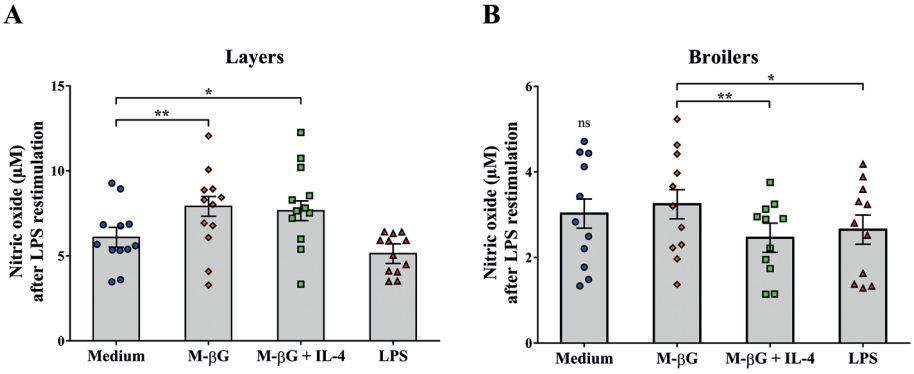
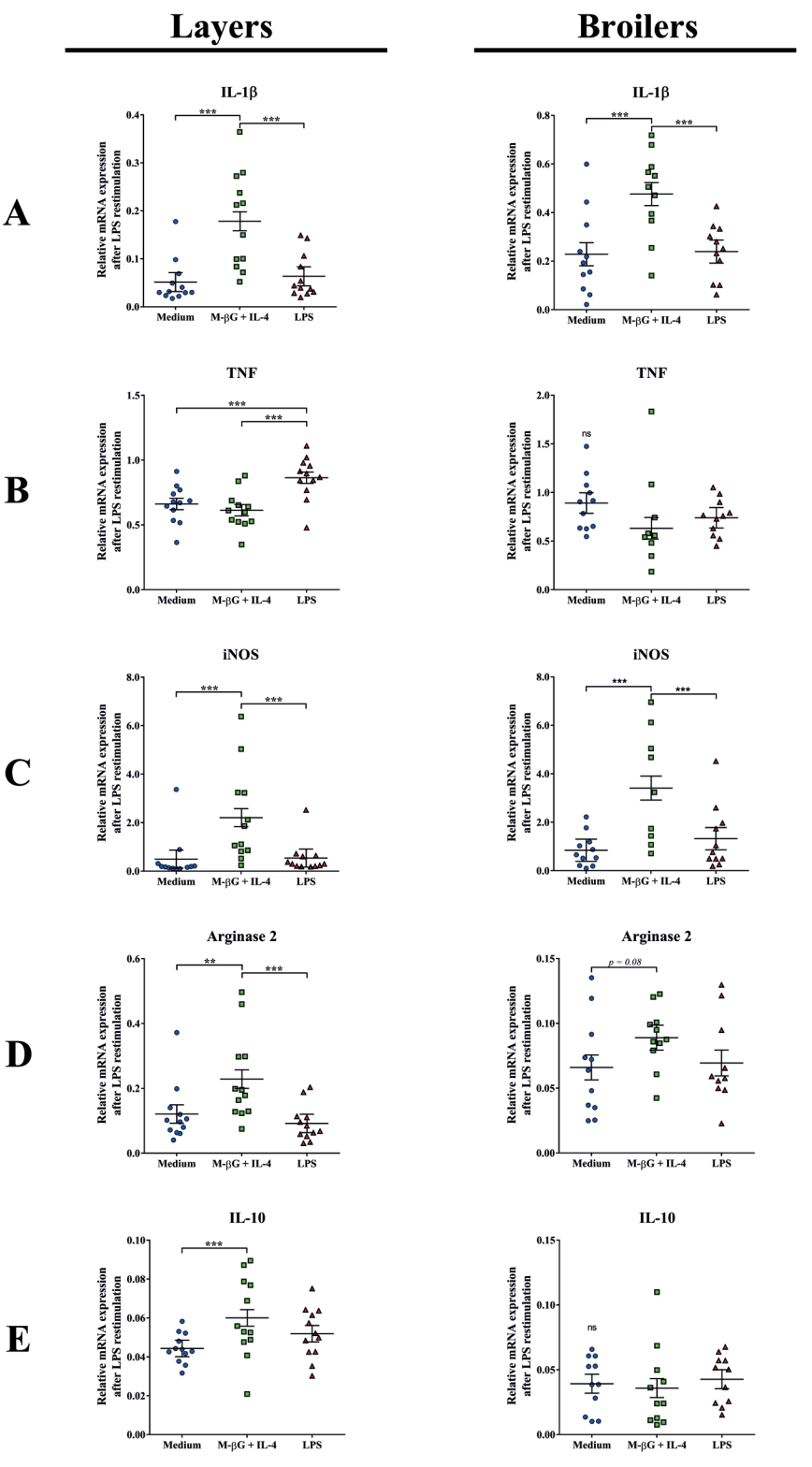


Figure 1. Innate immune training with microparticulate β-glucan in combination with or without interleukin-4 increased NO production in macrophages from layers but not broilers. Adherent primary blood monocytes harvested from 42 days old layers (A) or broilers (B) were trained with culture medium (Medium; untrained control group), M-βG (10 µg/mL), M-βG+IL-4 (10 µg/mL + 100 ng/mL) or LPS (10 µg/mL) for 48h on D0 and were restimulated with LPS (10 µg/mL) on D6. Presented is the NO production in the culture medium after a 48h lasting LPS restimulation. Each bar represents lsmeans ± SEM, and N = 12 layers and 11 broilers for corresponding groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

Figure 2. (opposite) Training effect on the expression levels of genes associated with inflammation in layer and broiler macrophages. Adherent blood monocytes from 42-days-old layers (A) and broilers (B) were trained with either culture medium (Medium; untrained control), LPS (10 µg/mL) or M-βG+IL-4 (10 µg/mL+100 ng/mL) for 48h on D0 and were restimulated (i.e. challenged) with LPS (10 µg/mL) on D6. Relative gene expression levels measured 24h after restimulation are presented. Each bar represents lsmeans ± SEM with N = 12 layers and 11 broilers. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.


Layers
Broilers
A
B
C
D
E
IL-1β
TNF
iNOS
Arginase 2
IL-10
Relative mRNA expression after LPS restimulation
Medium
M-βG + IL-4
LPS
***
**
ns
p = 0.08

3.2 Trained macrophages of both breeds showed increased expression of genes related to inflammation and cellular metabolism

Transcript levels of inflammation-associated genes were also measured in untrained, M-βG + IL-4-trained and LPS-trained macrophages from layers and broilers after restimulation with LPS (**Fig. 2**). For layers, training with M-βG + IL-4 resulted in greater ($P < 0.01$; $N = 12$) expression levels of IL-1β, iNOS, Arginase 2 (ARG2) and IL-10 after LPS restimulation compared to their corresponding untrained cells. Like layers, macrophages from broiler chickens trained with M-βG + IL-4 showed increased ($P < 0.001$; $N = 11$) transcript levels of IL-1β and iNOS, and a trend towards increased ($P = 0.08$; $N = 11$) ARG2 expression, compared to their corresponding untrained cells. Notably, the expression of IL-10 was affected by training layer macrophages with M-βG + IL-4, but broiler macrophages were not affected (**Fig. 2E**). Training with M-βG + IL-4 did not influence TNF mRNA levels, but training with LPS elevated ($P < 0.001$; $N = 12$) TNF-α expression levels in layer macrophages (**Fig. 2B**).

The influence of training on the transcript levels of metabolic-associated genes was also investigated (**Fig. 3)**. We selected the genes HIF-1α and PPARγ as indicators for changes in the glucose and lipid metabolic pathway, respectively. In both layer and broiler macrophages, training with M-βG + IL-4 resulted in elevated HIF-1α mRNA expression (**Fig. 3A**; $P < 0.001$; $N = 11$ - 12), but left PPARγ mRNA expression unaffected, after restimulation with LPS (**Fig. 3B**). The response on PPARγ expression was decreased ($P < 0.01$; $N = 12$) in layer macrophages trained with LPS. In broiler macrophages, this inhibitory effect was not found. However, the level of PPARγ expression after training with LPS was lower ($P < 0.05$; $N = 11$), compared to the M-βG + IL-4 trained group.

3.3 Trained macrophages did not show enhanced lactate production levels.

The production of lactate by trained macrophages from 42 days old layers and broilers was measured extracellularly **(Fig. 4)**. For layers, training with M-βG+IL-4 had no effect on lactate production levels after LPS restimulation compared to the untrained control group, but training with LPS reduced lactate levels ($P < 0.05$, $N = 12$) **(Fig. 4A)**. For broiler macrophages, we found a decrease in lactate production levels after training with M-βG+IL-4 ($P < 0.05$, $N = 11$) **(Fig. 4B)**, whereas no effect was found with LPS-trained macrophages compared to the untrained control group.

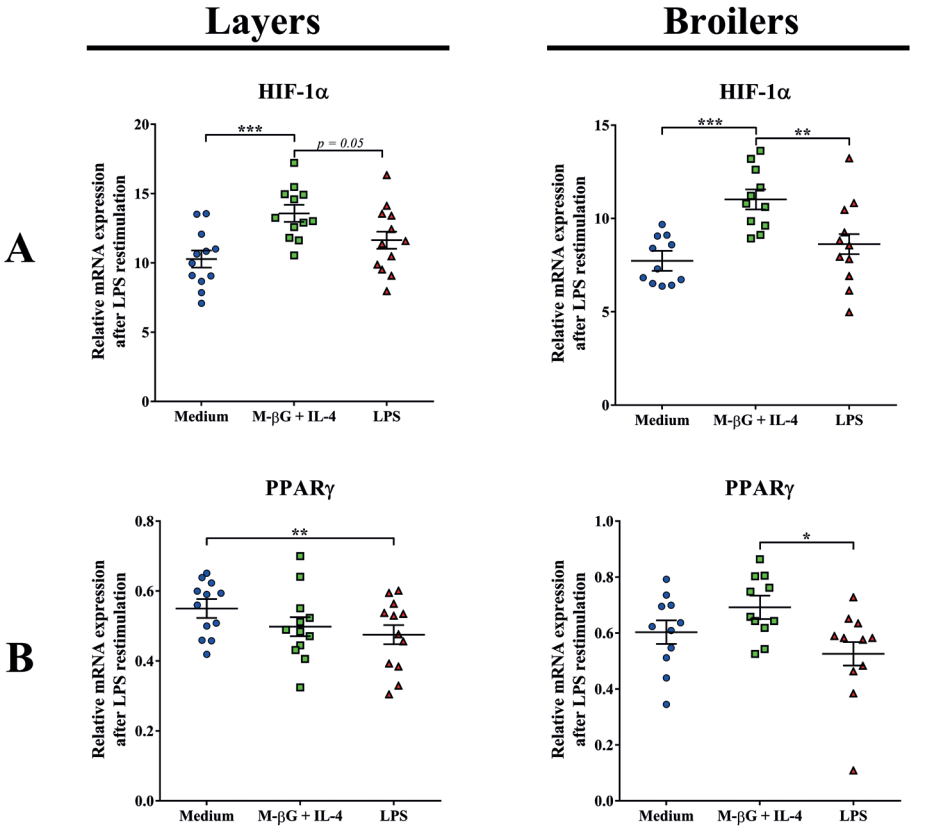


Figure 3. Training effect on the expression levels of genes associated with cellular metabolism in layer and broiler macrophages. Adherent blood-derived monocytes from layers and broilers were stimulated with culture medium (Medium; untrained control), LPS (10 µg/mL) or M-βG+IL-4 (10 µg/mL+100 ng/mL) for 48h on D0. The cells were restimulated with LPS (10 µg/mL) on D6. Relative gene expression levels of HIF-1α (A) and PPARγ (B) in the macrophages measured 24h after the restimulation are presented. Each bar represents lsmeans ± SEM, after normalization to the geometric mean of 3 stably expressed housekeeping genes (ACTB, IPO8 and GAPDH), with N = 12 layers, N = 11 broilers. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.4 Training increased surface expression of CD40 and MHCII on macrophages from layers but not broilers

Next, trained and untrained macrophages were subjected to flow cytometry analysis to characterize the cell populations (**Fig. 5**). The chicken macrophages of both breeds were gated for viability, forward scatter and side scatter (**Fig. 5A**). They comprised a cell population homogeneously positive for the surface proteins KUL01 and CSF1R, two well-known myeloid markers [30, 31] **(Fig. 5B)**.

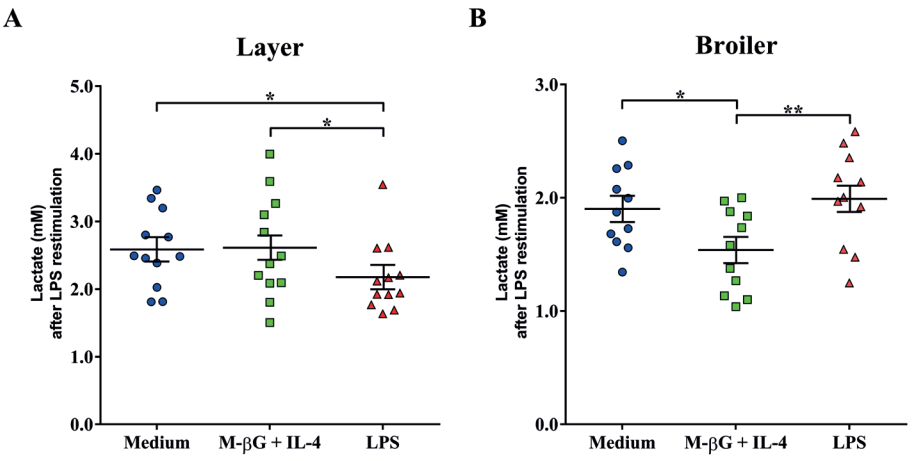


Figure 4. Extracellular lactate production of trained macrophages from layers and broilers. Monocytes collected from 42 days old layers (A) and broilers (B) were *in vitro* trained with either culture medium (Medium; untrained control), M-βG (10 µg/mL), M-βG+IL-4 (10 µg/mL+100 ng/mL) or LPS (10 µg/mL) for 48h on D0. The cells were restimulated with LPS (10 µg/mL) for 24h on D6. Concentrations of extracellular lactate in the medium after the LPS restimulation are presented. Each bar represents lsmeans ± SEM with N = 12 layers, N = 11 broilers. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

We determined the changes in CD40 and MHC-II protein surface expression in macrophages that were untrained (i.e. control), or trained with M-βG + IL-4 or LPS, after restimulation with LPS for 24h (**Fig. 5C** and **D**). Training with M-βG + IL-4 increased ($P < 0.001$, $N = 10$) the surface expression of CD40 on macrophages from layers (**Fig. 5C**), but not from broilers (**Fig. 5D**)**,** after restimulation with LPS. Notably, greater expression levels of CD40 were observed after LPS restimulation in untrained macrophages from broilers compared to layers. Expression of MHC-II was decreased ($P < 0.05$, $N = 10$) upon restimulation with LPS in macrophages from layers without training (**Fig. 5C**). This inhibitory action was less pronounced in macrophages trained with M-βG + IL-4 or LPS. Macrophages from broilers without training displayed an unaltered MHC-II expression after LPS restimulation **(Fig. 5D**). While training with M-βG + IL-4 did not affect MHC-II expression in macrophages from broilers, training with LPS decreased ($P < 0.05$, $N = 9$) MHC-II expression upon restimulation with LPS in these cells. This contrasted with layer macrophages (**Fig. 5C** and **D)**.

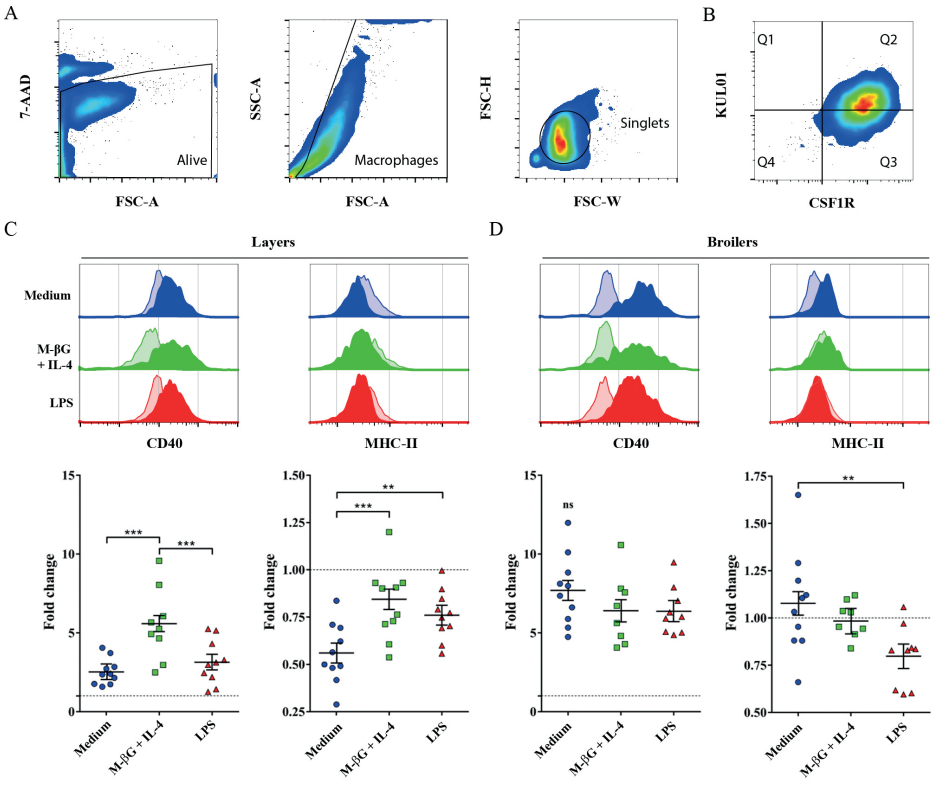


Figure 5. Surface expression of CD40 and MHC-II on macrophages from layers and broilers. Macrophages were stained and analysed by imaging flow cytometry. The cells were gated for viability using 7-AAD (left panel), by their scatter profile (middle panel), and for single cells (right panel) (A). The gated cells were evaluated for surface expression of KUL01 and CSF1R (B). Surface expression of CD40 and MHC-II was determined for macrophages from layers (C) and broilers (D) in which the upper panels show representative histograms of CD40 and MHC-II surface expression for the different treatments. Trained macrophages with or without 24h restimulation with LPS are shown by semi-transparent and filled histograms, respectively. The results are presented as fold change in surface expression upon LPS stimulation, which was calculated for each group with different training conditions. Each bar represents lsmeans ± SEM with N = 10 layers, N = 10 broilers. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.5 Trained macrophages originally collected from chickens with different ages, resulted in variable nitric oxide production levels

To investigate whether the training capacity of monocytes is dependent on age, we measured NO production after *in vitro* training of monocytes that were harvested also from younger, i.e. 21, 28 and 42-days-old, layers and broilers (**Fig. 6**). For layers, we found that training with M-βG increased the NO production in macrophages after restimulation with LPS irrespective of age, compared to untrained cells (**Fig. 6A**). Surprisingly, training with M-βG+IL-4 had no significant effect on NO production for 21-days-old layers compared to untrained cells. Macrophages from 28- and 42-days-old chickens showed a small increase in NO production after training with M-βG+IL-4, but this was not statistically significant. Furthermore, the NO production response increased gradually with age in M-βG+IL-4 trained layer macrophages, whereby the difference between day 21 and day 42 was significant ($P < 0.05$, $N = 12$) (**Table S1** and **Table S2**).

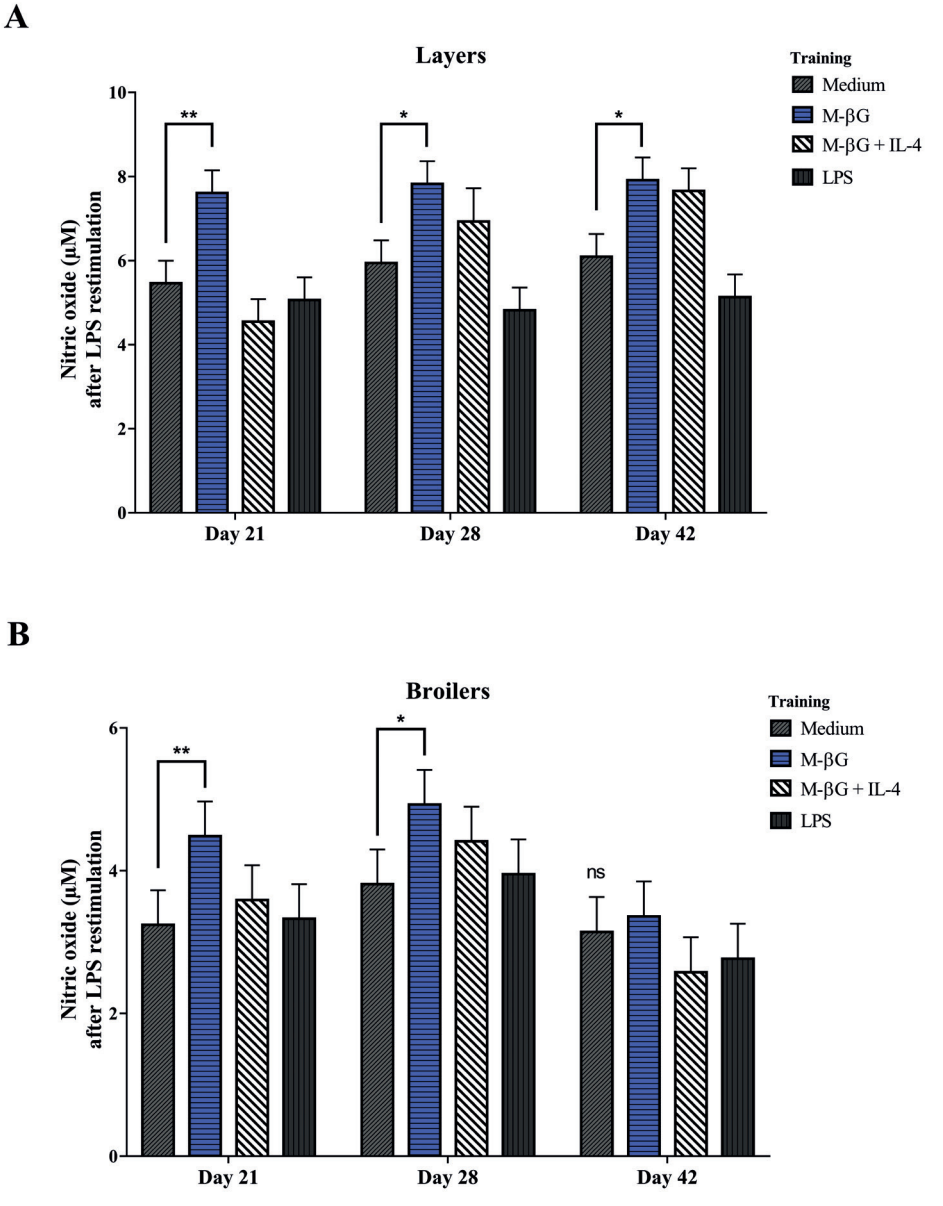


Figure 6. Training effects on monocytes from chickens with different ages on nitric oxide production. Primary monocytes collected from layers (A) and broilers (B) aging 21, 28 and 42 post-hatch were *in vitro* trained with either culture medium (Medium; untrained control), M-βG (10 µg/mL), M-βG+IL-4 (10 µg/mL+100 ng/mL) or LPS (10 µg/mL) for 48 h on D0. The cells were restimulated with LPS (10 µg/mL) for 48h on D6. The concentrations of NO_2^- in the medium after the LPS restimulation are presented. Each bar represents lsmeans ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $N_{\text{all days}} = 12$ layers, $N_{\text{Day21 and Day 28}} = 12$ broilers, $N_{\text{Day 42}} = 11$ broilers. Statistical output could be found in the supplementary material section (Table S1 and Table S2).

As for broilers, we also found an increase in NO production after training with M-βG in macrophages derived from 21 and 28, but not 42-days-old chickens when compared to untrained cells (**Fig. 6B**). Similarly to layers, no increase in NO production was seen in cells trained with M-βG+IL-4 compared to the untrained control group.

4. Discussion

Recently, we described that monocytes of laying hens could be trained with a combination of M-βG and IL-4, leading to an elevated inflammation-related response upon restimulation with LPS [22]. In the present study, we investigated whether isolated primary monocytes from layers and broilers differ in their innate training capacity by *in vitro* training with M-βG+IL-4. We explored inflammation-related pathways and indicators for metabolic reprogramming, because in mammals it was found that these signalling pathways are functionally linked with each other in trained immune cells [13, 14, 17, 32, 33].

In accordance with our previous study (Verwoolde et al, 2020), training of monocytes of laying hens by M-βG+IL-4 displayed increased NO production and enhanced surface expression of the activation marker CD40 after restimulation with LPS. In parallel, mRNA levels for the proinflammatory cytokine IL-1β, NO synthase iNOS, and anti-inflammatory cytokine IL-10 were increased. These findings are also in line with similar studies on trained innate immunity with mammals (including human, mice and bovine) and fish (teleost fish) [7, 19, 34-36]. Clearly, trained innate immunity is an evolutionary conserved phenomenon. Training of broiler hen monocytes also led to increased mRNA levels of IL-1β and iNOS, but, on the contrary, NO production and expression of IL-10 and CD40 were not elevated. Notably, when looking at the experiments in which age-dependency was assayed, the LPS-induced NO production was greater in M-βG-trained macrophages collected from 21- or 28-day-old chickens, irrespective of the breed of the chicken, but, in contrast to those from layers, training did not affect monocytes from 42-days-old broilers (**Figs. 1** and **6**). Albeit we observed LPS-enhanced expression levels of IL-1β in M-βG+IL-4-trained macrophages of both breeds, this training enhanced surface expression of CD40 in layer macrophages only. Remarkably, CD40 surface expression readily increased approximately 7 times in untrained broiler macrophages, compared with layer macrophages, upon LPS stimulation. This may explain why, in contrast to layer macrophages, training with M-βG+IL-4 did not further increase CD40 surface expression in broiler macrophages in response to LPS restimulation. As for the other macrophage activation marker, the inhibitory effect of LPS restimulation on surface expression of MHCII in untrained macrophages was blunted after training with M-βG+IL-4 or LPS; a training effect was only found for layers. All these observations together suggest that the differences in trained and untrained chicken macrophages may be due to differences in LPS-sensitivity [10]. This could be attributed to differential expression of TLR4 between genetical different chicken breeds [37].

We reported previously that training with IL-4 alone did not result in an enhanced innate immune response in layer macrophages, as demonstrated by NO production, indicating that M-βG is the active component that induces trained immunity (Verwoolde et al.,

2020), alike reported for macrophages of human [34], mice [7, 8] and carp [19]. Looking at the effects of IL-4, Galès et al. (2010) demonstrated the existence of a cytokine/PPARγ/Dectin-1 axis in mice by showing a stimulatory effect of cytokines IL-4 and IL-13 through the mediator PPARγ on the surface expression of β-glucan receptor Dectin-1. In the current study, however, we could not find an enhanced training effect of IL-4 on the LPS-triggered NO production when macrophages were trained with the combination of M-βG and IL-4 (**Figs. 1** and **6**). We proposed that the use of older laying hens (10 weeks-old) in our previous experiment was the reason for this discrepancy. Our current study indicates that the responsiveness to IL-4 in layer monocytes is indeed age dependent (**Fig. 6a**): an inhibitory effect of IL-4 on the training with M-βG was obtained in macrophages from younger layers which appeared to be alleviated in the older counterparts. In mice it is shown that the IL-4 signalling system desensitize with age in macrophages, microglia and T helper cells [38-40]. Therefore, our hypothesis that IL-4 has a positive effect on the expression of β-glucan receptor Dectin-1 in older layer monocytes, resulting in a more pronounced training response with β-glucan, as postulated in our previous study [22], needs to be verified in other studies.

Nitric oxide, produced by the enzyme iNOS, is a prominent mediator of inflammatory reactions [41]. Although we observed differences in NO production, chicken monocytes trained with M-βG+IL-4 showed, irrespective of the chicken breed, an approximate 7-fold elevation of iNOS expression. The failure of LPS to enhance NO production in M-βG-trained broiler macrophages compared to untrained cells and the relative low NO production on the whole in broiler macrophages must therefore be due to post-transcriptional mechanisms, such as interaction with other proteins, arginine supply, activity of the arginine membrane transporters and/or iNOS protein destabilization [41, 42].

In terms of metabolic regulation, we observed an increased HIF-1α expression for trained macrophages in both breeds after LPS restimulation (**Fig. 3**). This increased HIF-1α expression suggests that innate immune training by β-glucan facilitates the signal of the TLR4/MyD88-dependent pathway to increase HIF-1α expression in chicken macrophages, similarly to a model postulated for human [43]. Interestingly, when comparing with HIF-1α expression of non-restimulated macrophages, we observed that stimulation with LPS decreased HIF-1α mRNA expression levels. However, this decreased expression was partially rescued by training with M-βG+IL-4 **(Fig. S3)**. This suggests a possible stabilizing effect of M-βG+IL-4 training on HIF-1α mRNA levels, as shown before [13, 14]. Contrary to HIF-1α, innate training with M-βG+IL-4 did not alter the expression of PPARγ in LPS-restimulated macrophages of both breeds.

In the present study we found that IL-1β, iNOS, and HIF-1α expression levels were similarly affected in trained macrophages of both breeds. Previous studies in human and mice demonstrated that adaptations in cellular metabolism are key to trained innate immunity, whereby both IL-1β and HIF-1α play a pivotal role [17, 44, 45]. They not only orchestrate inflammation reactions, but also metabolic processes [17, 32]. Macrophages are apt to infiltrate hypoxic tissues whereby they switch their metabolic program to glycolysis, probably under the tight control of HIF-1α [46]. This tunes with the evidence that LPS increases the level of tricarboxylic acid (TCA) cycle intermediates succinate, malate,

fumarate, α-ketoglutarate and citrate [47-49]. For example, increased levels of cytosolic succinate during the LPS-mediated activation of macrophages, potentiates stabilization HIF-1α expression at the protein levels through the inhibition of the prolyl hydroxylase domain enzymes [50]. In turn, this inhibition specifically promotes the expression of HIF-1α-dependent genes, including IL-1β and those encoding enzymes in the glycolytic pathway, including pyruvate dehydrogenase kinase (PDK) [51, 52]. PDK inhibits pyruvate dehydrogenase, thereby preventing the formation of acetyl-CoA from pyruvate [49, 53]. Thus, activation of macrophages by LPS occurs through several parallel pathways that include the activation of NF-κB, and the development of a glycolytic pathway with hampered TCA cycle and reduced oxidative phosphorylation phenotype, leading to promoted production of NO, reactive oxygen species and expression of pro-inflammatory cytokines.

Transcriptional and epigenetic reprogramming are the basis for trained immunity [13, 47]. Trained immunity, induced by β-glucan, promotes glycolysis, which is the result of increased histone 3 trimethylation of lysine 4 (H3K4me3). H3K4me3 is a hall mark of open chromatin and increased gene expression, at promoter sites of essential glycolytic genes [44]. Several metabolites of the TCA cycle serve as cofactor for chromatin modification-catalyzing enzymes [54]. α-ketoglutarate, for example, is a co-factor that can favor histone demethylation [55], and fumarate and succinate, contrariwise, are described as inhibitors of demethylation processes [47, 56, 57]. Besides histone modification, changes in posttranslational modification by TCA metabolites may also occur; there are indications that accumulation of succinate affects lysine succinylation of enzymes of the glycolytic pathway and TCA cycle [58]. Unfortunately, in the present study we were not able to demonstrate an upregulation of glycolysis with concomitant lactate production in β-glucan-trained chicken macrophages, as reported for their mammalian and teleost counterparts [19, 44, 47]. How and which metabolic pathways in chicken monocytes are basically reprogrammed in the process of innate immune training therefore awaits further studies.

Based on results in mammalian studies of trained innate immunity, we expected that the enhanced HIF-1α mRNA expression was paralleled by a greater TNF expression level in β-glucan-trained macrophages after LPS restimulation [7, 8]. This was, however, not found in the present study. Furthermore, increased IL-10 mRNA levels were only found in β-glucan-trained layer macrophages, not in that of broilers. This anti-inflammatory cytokine will increase in amount as a response to elevated pro-inflammatory cytokine levels to eventually control the inflammatory response [59]. Indeed, for layers a comparable increase in mRNA expression was found for IL-10 and IL-1β. Broiler macrophages, on the contrary, did not show an increase in IL-10 expression, despite the increase in IL-1β expression. This observation indicates differential effects on immune functionality in layers and broilers. However, we cannot rule out the possibility that we took a suboptimal moment of read-out for broilers. Since IL-10 is an anti-inflammation-associated gene, we expected a similar mRNA expression profile for the anti-inflammation-associated gene ARG2. Two major isoforms, cytosolic ARG1, and mitochondrial ARG2 have been characterized in mammals. In chicken, only ARG2 expression has been found. Contrary to ARG1, the physiologic role of ARG2 isoform is poorly understood but it is thought to

play a role in nitric oxide and polyamine metabolism. In the present study, we found a significant and numerical increase in ARG2 transcript levels in trained macrophages from layers and broilers, respectively. Based on these findings, it is tempting to speculate that ARG2 performs the role of ARG1 in chicken to catalyse the hydrolysis of L-arginine into L-ornithine and urea, taking L-arginine away for iNOS to produce NO that may shift the macrophage from pro-inflammatory to anti-inflammatory phenotype [60, 61].

Besides training with M-βG+IL-4, we trained chicken monocytes with LPS in the supposition that this would evoke tolerance to LPS restimulation, as described for mice [7, 8]. However, we could find no evidence for such mechanism, although lactate production in LPS-trained layer macrophages was decreased upon LPS restimulation in parallel of an increased HIF-1α expression in these cells. This suggests that, in the context of innate immune training, LPS might exert another role in chicken monocytes.

In conclusion, we have provided additional evidence for trained innate immunity in blood monocytes of layer and broiler chickens. Our results are in accordance with previous studies on innate training in mammals. The observed differences in training parameters between the two chicken breeds might imply a differential effect on immune functionality associated with trained innate immunity. Furthermore, innate training with components such as β-glucan may improve the effectiveness of the current vaccination and feeding strategies in layers and broilers. Further studies are needed to explore the potential of innate training in relation to disease resistance in young chickens.

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Supplementary material

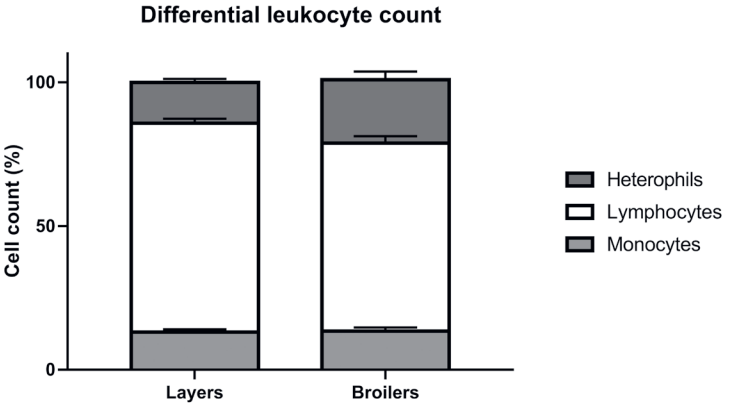

Figure S1. Differential leukocyte count of the isolated population. No differences in cell count were found in the monocyte population between the breeds. N_{layer} = 12 chickens, $N_{broiler}$ = 11 chickens.

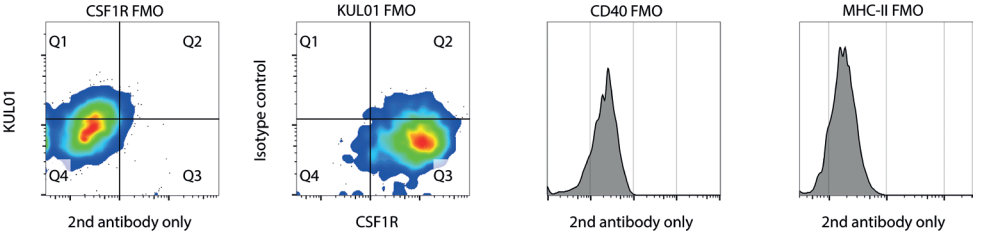

Figure S2. Flow cytometry staining controls. Background staining was assessed by fluorescent-minus-one (FMO) controls, in which macrophages were stained with all antibodies, except one. The missing antibodies were replaced by R-PE-conjugated goat anti-mouse-IgG1, FITC-conjugated mouse isotype control, APC-conjugated goat-anti-mouse-IgG2a or Alexa Fluor 405-conjugated streptavidin (shown from left to right).

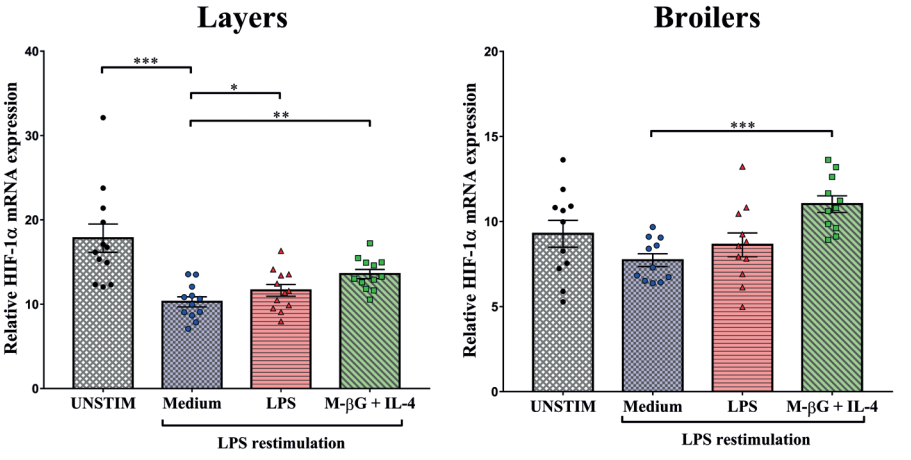

Figure S3. Stabilization of HIF-1α mRNA gene expression. Training with M-βG +IL-4 stabilizes the gene expression of HIF-1α by rescuing the decrease after a restimulation with LPS. Adherent blood-derived monocytes from layers and broilers were stimulated with culture medium (Medium; untrained control), LPS (10 µg/mL) or M-βG+IL-4 (10 µg/mL+100 ng/mL) for 48h on D0. Trained cells were restimulated with 10 µg/mL LPS on D6 for 24h. As a control, cells incubated in medium during the training period were restimulated in the absence of LPS on D6 for 24h (UNSTIM). Each bar represents means ± SEM, after normalization to the geometric mean of 3 stably expressed housekeeping genes (ACTB, IPO8 and GAPDH), with N = 12 layers, N = 11 broilers. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table S1. Selection of the statistical analysis output of NO production of macrophages isolated from layers with different ages. Age 0 = 21 days; Age 1 = 28 days; Age 2 = 42 days.

Differences of Least Squares Means Layers							
Effect	**Treatment**	**Age**	**Treatment**	**Age**	**Estimate**	**Standard Error**	**Adj P**
Treatment	MEDIUM		LPS		0.8266	0.3141	0.0476
Treatment	MEDIUM		M-βG		-1.9513	0.3141	<.0001
Treatment	MEDIUM		M-βG + IL-4		-0.5459	0.3683	0.4519
Treatment	LPS		M-βG		-2.7779	0.3141	<.0001
Treatment	LPS		M-βG + IL-4		-1.3725	0.3683	0.0018
Treatment	M-βG		M-βG + IL-4		1.4055	0.3683	0.0013
Age		0		1	-0.7102	0.6152	0.4832
Age		0		2	-1.0291	0.5981	0.2025
Age		1		2	-0.3189	0.3079	0.5561
Treatment*Age	MEDIUM	0	MEDIUM	1	-0.4818	0.7614	1.0000
Treatment*Age	MEDIUM	0	MEDIUM	2	-0.6328	0.7614	0.9995
Treatment*Age	MEDIUM	0	LPS	0	0.3969	0.5440	0.9999
Treatment*Age	MEDIUM	0	LPS	1	0.6399	0.7614	0.9995
Treatment*Age	MEDIUM	0	LPS	2	0.3283	0.7614	1.0000
Treatment*Age	MEDIUM	0	M-βG	0	-2.1492	0.5440	0.0076
Treatment*Age	MEDIUM	0	M-βG	1	-2.3653	0.7614	0.0950
Treatment*Age	MEDIUM	0	M-βG	2	-2.4541	0.7614	0.0700
Treatment*Age	MEDIUM	0	M-βG + IL-4	0	0.9144	0.5440	0.8727
Treatment*Age	MEDIUM	0	M-βG + IL-4	1	-1.4713	0.9552	0.9254
Treatment*Age	MEDIUM	0	M-βG + IL-4	2	-2.1954	0.7614	0.1628
Treatment*Age	MEDIUM	1	MEDIUM	2	-0.1511	0.5440	1.0000
Treatment*Age	MEDIUM	1	LPS	0	0.8787	0.7614	0.9912
Treatment*Age	MEDIUM	1	LPS	1	1.1217	0.5440	0.6504
Treatment*Age	MEDIUM	1	LPS	2	0.8101	0.5440	0.9403
Treatment*Age	MEDIUM	1	M-βG	0	-1.6675	0.7614	0.5606
Treatment*Age	MEDIUM	1	M-βG	1	-1.8835	0.5440	0.0357
Treatment*Age	MEDIUM	1	M-βG	2	-1.9723	0.5440	0.0218
Treatment*Age	MEDIUM	1	M-βG + IL-4	0	1.3962	0.7614	0.7955
Treatment*Age	MEDIUM	1	M-βG + IL-4	1	-0.9896	0.7929	0.9835
Treatment*Age	MEDIUM	1	M-βG + IL-4	2	-1.7136	0.5440	0.0850
Treatment*Age	MEDIUM	2	LPS	0	1.0298	0.7614	0.9696
Treatment*Age	MEDIUM	2	LPS	1	1.2728	0.5440	0.4563
Treatment*Age	MEDIUM	2	LPS	2	0.9611	0.5440	0.8318
Treatment*Age	MEDIUM	2	M-βG	0	-1.5164	0.7614	0.6979
Treatment*Age	MEDIUM	2	M-βG	1	-1.7325	0.5440	0.0776
Treatment*Age	MEDIUM	2	M-βG	2	-1.8213	0.5440	0.0497
Treatment*Age	MEDIUM	2	M-βG + IL-4	0	1.5473	0.7614	0.6706
Treatment*Age	MEDIUM	2	M-βG + IL-4	1	-0.8385	0.7929	0.9958
Treatment*Age	MEDIUM	2	M-βG + IL-4	2	-1.5625	0.5440	0.1670
Treatment*Age	LPS	0	LPS	1	0.2430	0.7614	1.0000
Treatment*Age	LPS	0	LPS	2	-0.06866	0.7614	1.0000
Treatment*Age	LPS	0	M-βG	0	-2.5462	0.5440	0.0005
Treatment*Age	LPS	0	M-βG	1	-2.7623	0.7614	0.0216
Treatment*Age	LPS	0	M-βG	2	-2.8511	0.7614	0.0150

Differences of Least Squares Means Layers							
Effect	**Treatment**	**Age**	**Treatment**	**Age**	**Estimate**	**Standard Error**	**Adj P**
Treatment*Age	LPS	0	M-βG + IL-4	0	0.5175	0.5440	0.9983
Treatment*Age	LPS	0	M-βG + IL-4	1	-1.8683	0.9552	0.7213
Treatment*Age	LPS	0	M-βG + IL-4	2	-2.5923	0.7614	0.0422
Treatment*Age	LPS	1	C	2	-0.3117	0.5440	1.0000
Treatment*Age	LPS	1	M-βG	0	-2.7892	0.7614	0.0194
Treatment*Age	LPS	1	M-βG	1	-3.0052	0.5440	<.0001
Treatment*Age	LPS	1	M-βG	2	-3.0940	0.5440	<.0001
Treatment*Age	LPS	1	M-βG + IL-4	0	0.2745	0.7614	1.0000
Treatment*Age	LPS	1	M-βG + IL-4	1	-2.1113	0.7929	0.2603
Treatment*Age	LPS	1	M-βG + IL-4	2	-2.8353	0.5440	<.0001
Treatment*Age	LPS	2	M-βG	0	-2.4775	0.7614	0.0644
Treatment*Age	LPS	2	M-βG	1	-2.6936	0.5440	0.0002
Treatment*Age	LPS	2	M-βG	2	-2.7824	0.5440	<.0001
Treatment*Age	LPS	2	M-βG + IL-4	0	0.5862	0.7614	0.9998
Treatment*Age	LPS	2	M-βG + IL-4	1	-1.7996	0.7929	0.5046
Treatment*Age	LPS	2	M-βG + IL-4	2	-2.5237	0.5440	0.0006
Treatment*Age	M-βG	0	M-βG	1	-0.2161	0.7614	1.0000
Treatment*Age	M-βG	0	M-βG	2	-0.3049	0.7614	1.0000
Treatment*Age	M-βG	0	M-βG + IL-4	0	3.0637	0.5440	<.0001
Treatment*Age	M-βG	0	M-βG + IL-4	1	0.6779	0.9552	0.9999
Treatment*Age	M-βG	0	M-βG + IL-4	2	-0.04615	0.7614	1.0000
Treatment*Age	M-βG	1	M-βG	2	-0.08880	0.5440	1.0000
Treatment*Age	M-βG	1	M-βG + IL-4	0	3.2798	0.7614	0.0022
Treatment*Age	M-βG	1	M-βG + IL-4	1	0.8940	0.7929	0.9927
Treatment*Age	M-βG	1	M-βG + IL-4	2	0.1699	0.5440	1.0000
Treatment*Age	M-βG	2	M-βG + IL-4	0	3.3686	0.7614	0.0014
Treatment*Age	M-βG	2	M-βG + IL-4	1	0.9828	0.7929	0.9843
Treatment*Age	M-βG	2	M-βG + IL-4	2	0.2587	0.5440	1.0000
Treatment*Age	M-βG + IL-4	0	M-βG + IL-4	1	-2.3858	0.9552	0.3535
Treatment*Age	M-βG + IL-4	0	M-βG + IL-4	2	-3.1098	0.7614	0.0048
Treatment*Age	M-βG + IL-4	1	M-βG + IL-4	2	-0.7240	0.7929	0.9989

Table S2. Selection of the statistical analysis output of NO production of macrophages isolated from broilers with different ages. Age 0 = 21 days; Age 1 = 28 days; Age 2 = 42 days.

Differences of Least Squares Means Broilers							
Effect	**Treatment**	**Age**	**Treatment**	**Age**	**Estimate**	**Standard Error**	**Adj P**
Treatment	MEDIUM		LPS		0.04932	0.1847	0.9933
Treatment	MEDIUM		M-βG		-0.8590	0.1847	<.0001
Treatment	MEDIUM		M-βG + IL-4		-0.1284	0.1847	0.8989
Treatment	LPS		M-βG		-0.9083	0.1847	<.0001
Treatment	LPS		M-βG + IL-4		-0.1777	0.1847	0.7714
Treatment	M-βG		M-βG + IL-4		0.7306	0.1847	0.0008
Age		0		1	-0.6155	0.6298	0.5928
Age		0		2	0.6993	0.6315	0.5117
Age		1		2	1.3147	0.1644	<.0001
Treatment*Age	MEDIUM	0	MEDIUM	1	-0.5713	0.6864	0.9995
Treatment*Age	MEDIUM	0	MEDIUM	2	0.09919	0.6905	1.0000
Treatment*Age	MEDIUM	0	LPS	0	-0.08629	0.3152	1.0000
Treatment*Age	MEDIUM	0	LPS	1	-0.7119	0.6864	0.9964
Treatment*Age	MEDIUM	0	LPS	2	0.4740	0.6905	0.9999
Treatment*Age	MEDIUM	0	M-βG	0	-1.2437	0.3152	0.0076
Treatment*Age	MEDIUM	0	M-βG	1	-1.6868	0.6864	0.3782
Treatment*Age	MEDIUM	0	M-βG	2	-0.1187	0.6905	1.0000
Treatment*Age	MEDIUM	0	M-βG + IL-4	0	-0.3494	0.3152	0.9937
Treatment*Age	MEDIUM	0	M-βG + IL-4	1	-1.1711	0.6864	0.8615
Treatment*Age	MEDIUM	0	M-βG + IL-4	2	0.6632	0.6905	0.9982
Treatment*Age	MEDIUM	1	MEDIUM	2	0.6705	0.3240	0.6449
Treatment*Age	MEDIUM	1	LPS	0	0.4851	0.6864	0.9999
Treatment*Age	MEDIUM	1	LPS	1	-0.1406	0.3152	1.0000
Treatment*Age	MEDIUM	1	LPS	2	1.0454	0.3240	0.0688
Treatment*Age	MEDIUM	1	M-βG	0	-0.6723	0.6864	0.9978
Treatment*Age	MEDIUM	1	M-βG	1	-1.1154	0.3152	0.0281
Treatment*Age	MEDIUM	1	M-βG	2	0.4527	0.3240	0.9616
Treatment*Age	MEDIUM	1	M-βG + IL-4	0	0.2220	0.6864	1.0000
Treatment*Age	MEDIUM	1	M-βG + IL-4	1	-0.5997	0.3152	0.7550
Treatment*Age	MEDIUM	1	M-βG + IL-4	2	1.2345	0.3240	0.0119
Treatment*Age	MEDIUM	2	LPS	0	-0.1855	0.6905	1.0000
Treatment*Age	MEDIUM	2	LPS	1	-0.8111	0.3240	0.3493
Treatment*Age	MEDIUM	2	LPS	2	0.3748	0.3293	0.9921
Treatment*Age	MEDIUM	2	M-βG	0	-1.3429	0.6905	0.7284
Treatment*Age	MEDIUM	2	M-βG	1	-1.7860	0.3240	<.0001
Treatment*Age	MEDIUM	2	M-βG	2	-0.2179	0.3293	0.9999
Treatment*Age	MEDIUM	2	M-βG + IL-4	0	-0.4485	0.6905	1.0000
Treatment*Age	MEDIUM	2	M-βG + IL-4	1	-1.2703	0.3240	0.0082
Treatment*Age	MEDIUM	2	M-βG + IL-4	2	0.5640	0.3293	0.8583
Treatment*Age	LPS	0	LPS	1	-0.6256	0.6864	0.9989
Treatment*Age	LPS	0	LPS	2	0.5603	0.6905	0.9996
Treatment*Age	LPS	0	M-βG	0	-1.1574	0.3152	0.0186
Treatment*Age	LPS	0	M-βG	1	-1.6005	0.6864	0.4614
Treatment*Age	LPS	0	M-βG	2	-0.03237	0.6905	1.0000

Differences of Least Squares Means Broilers							
Effect	**Treatment**	**Age**	**Treatment**	**Age**	**Estimate**	**Standard Error**	**Adj P**
Treatment*Age	LPS	0	M-βG + IL-4	0	-0.2631	0.3152	0.9995
Treatment*Age	LPS	0	M-βG + IL-4	1	-1.0848	0.6864	0.9124
Treatment*Age	LPS	0	M-βG + IL-4	2	0.7495	0.6905	0.9947
Treatment*Age	LPS	1	C	2	1.1859	0.3240	0.0193
Treatment*Age	LPS	1	M-βG	0	-0.5317	0.6864	0.9998
Treatment*Age	LPS	1	M-βG	1	-0.9749	0.3152	0.0979
Treatment*Age	LPS	1	M-βG	2	0.5933	0.3240	0.7971
Treatment*Age	LPS	1	M-βG + IL-4	0	0.3626	0.6864	1.0000
Treatment*Age	LPS	1	M-βG + IL-4	1	-0.4592	0.3152	0.9487
Treatment*Age	LPS	1	M-βG + IL-4	2	1.3751	0.3240	0.0026
Treatment*Age	LPS	2	M-βG	0	-1.7177	0.6905	0.3592
Treatment*Age	LPS	2	M-βG	1	-2.1608	0.3240	<.0001
Treatment*Age	LPS	2	M-βG	2	-0.5927	0.3293	0.8144
Treatment*Age	LPS	2	M-βG + IL-4	0	-0.8234	0.6905	0.9885
Treatment*Age	LPS	2	M-βG + IL-4	1	-1.6451	0.3240	0.0001
Treatment*Age	LPS	2	M-βG + IL-4	2	0.1892	0.3293	1.0000
Treatment*Age	M-βG	0	M-βG	1	-0.4431	0.6864	1.0000
Treatment*Age	M-βG	0	M-βG	2	1.1250	0.6905	0.8943
Treatment*Age	M-βG	0	M-βG + IL-4	0	0.8943	0.3152	0.1801
Treatment*Age	M-βG	0	M-βG + IL-4	1	0.07257	0.6864	1.0000
Treatment*Age	M-βG	0	M-βG + IL-4	2	1.9069	0.6905	0.2120
Treatment*Age	M-βG	1	M-βG	2	1.5681	0.3240	0.0003
Treatment*Age	M-βG	1	M-βG + IL-4	0	1.3374	0.6864	0.7262
Treatment*Age	M-βG	1	M-βG + IL-4	1	0.5157	0.3152	0.8917
Treatment*Age	M-βG	1	M-βG + IL-4	2	2.3500	0.3240	<.0001
Treatment*Age	M-βG	2	M-βG + IL-4	0	-0.2307	0.6905	1.0000
Treatment*Age	M-βG	2	M-βG + IL-4	1	-1.0524	0.3240	0.0649
Treatment*Age	M-βG	2	M-βG + IL-4	2	0.7819	0.3293	0.4323
Treatment*Age	M-βG + IL-4	0	M-βG + IL-4	1	-0.8217	0.6864	0.9881
Treatment*Age	M-βG + IL-4	0	M-βG + IL-4	2	1.0125	0.6905	0.9463
Treatment*Age	M-βG + IL-4	1	M-βG + IL-4	2	1.8343	0.3240	<.0001

CHAPTER 5

5

Transgenerational effects of innate immune activation in broiler breeders on growth performance and immune responsiveness

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ABSTRACT

The impact of transgenerational effects on growth performance and immunity has not yet been studied extensively within the poultry husbandry sector. An important factor is the impact of the hens on the physical well-being and fitness to the environment of the offspring. This study is the first to investigate the effect of stimulating the maternal innate immune system with lipopolysaccharides (**LPS**) or β-glucan on growth performance and immune responses in the next generation. Transgenerational effects and consequences of these maternal treatments were further examined using a necrotic enteritis (**NE**) challenge model in the offspring. We show that offspring of LPS-treated broiler breeders have a higher feed efficiency from 14-21 days of age, i.e. the period just after the NE challenge. Moreover, more broiler chickens with intestinal lesions after the NE challenge were found in the offspring of the LPS-treated broiler breeders. Both the LPS and β-glucan maternal treatments resulted in transgenerational effects on blood-derived monocytes by showing a tendency of decreased IL1β mRNA levels after *ex vivo* LPS stimulation. These data are a first indication that broiler breeder hens can affect immune responsiveness and feeding efficiency of their offspring in a transgenerational manner.

Keywords: chicken, transgenerational, β-glucan, lipopolysaccharide, innate immunity

INTRODUCTION

Within the broiler husbandry sector, the containment of infectious diseases has always been a significant focus area. An important factor is the impact of the hens on the well-being and fitness of their offspring. It has been hypothesized that the hens prepares her offspring for their environment through transmission of maternal antibodies [1, 2]. When the living environments of hens and her offspring are equal, it is supposed that this next generation is better prepared. However, no research has been done yet on investigating the mode of action and potential benefits of these transgenerational effects, and within this, the role of the innate immune system.

Invertebrates and plants lack the presence of an adaptive immune system but have the ability to develop protection against pathogens in a transgenerational manner [3, 4], implying a memory function of the innate immune system. Because of this memory function in invertebrates, it has been proposed that the innate immune system of vertebrates also has a memory function, which contributes to this protection [5, 6]. Activation of the innate immune system in mammals resulting in enhanced responsiveness to subsequent triggers leading to polyspecific resistance, i.e. innate immune memory has been termed trained innate immunity [7]. Trained innate immunity in mammals involves epigenetic changes which have been found to have long term effects [8]. Therefore, it has been suggested that these epigenetic effects could also affect the innate immune system of chickens in the next generation [9], comparable to plants, invertebrates and mammals. Since broiler chickens have a short lifespan relatively to laying hens, these epigenetic effects on innate immune cells would therefore especially be more relevant for broiler chicks, knowing that the adaptive immune functions in chickens have not yet fully developed during the first weeks of their short lives [10, 11].

In previous studies we demonstrated trained innate immunity in poultry through *ex vivo* experiments with blood-derived primary monocytes [12, 13]. It is unknown, however, if trained innate immunity can influence the offspring's physiology. Therefore, the aim of the present study is to investigate if stimulation of the maternal innate immune system with microbial-associated molecular patterns (**MAMPs**) has an effect on the offspring's growth performance parameters and immune responsiveness.

We have immunized broiler breeders with two innate immune-stimulating components, the MAMPs lipopolysaccharide (**LPS**) or β-glucan to evoke possible transgenerational effects. These components act via Toll-like receptor 4 and Dectin-1 receptor signaling, respectively, which are expressed on innate immune cells including monocytes [14-17]. Although an intensive BLAST search in the *Gallus gallus* genome database (GRCg6a: build GCF_000002315.6) did not result in the identification of a dectin-1 chicken homologue, a dectin-l like β-glucan receptor is likely to be present on chicken heterophils and PBMCs (peripheral blood mononuclear cells), which have been found to respond to the dectin-1-specific agonist curdlan by an oxidative burst [17]. The transgenerational effects and consequences of these maternal treatments on growth performance and immune response parameters of their offspring were examined using a necrotic enteritis (**NE**) challenge model [18-23]. Effects of immunological stress caused by pathogens is generally

reflected in decreased growth performance parameters [24]. Differences on immune responsiveness and growth performance parameters are therefore considered as a good measurement to identify transgenerational effects of the maternal treatments.

This study should be considered as an initial step in identifying potential transgenerational effects of innate immune activation in poultry.

MATERIALS AND METHODS

Animals and Ethical Statement

This study was approved by the Animal Welfare Committee of Wageningen University and Research in accordance with Dutch laws and regulations on the execution of animal experiments (no: AVD1040020185427).

Experimental Design

Transgenerational effects of maternal stimulation of the innate immune system were studied by treatment of broiler breeder females with either LPS or β-glucan. The effects on the offspring performance were evaluated by exposing them to an NE challenge (**Figure 1)**. A schematic design of the experiment is shown in **Figure 1**. The experiment was divided into 3 phases. In phase 1, the broiler breeder phase, a randomized complete block design was applied with 3 treatments (control, LPS or β-glucan) and 162 hens per treatment, with 6 replicates per treatment. In phase 2, the egg incubation phase, a total of 90 eggs of each broiler breeder treatment group were collected, incubated, hatched and day-old chick quality was studied. In phase 3, the grow-out phase, 90 male chicks of each of the 3 broiler breeder treatment groups were raised until 36 d of age and challenged with necrotic enteritis.

Animals, Housing and Management

Broiler breeder phase (1)

A total of 486 female and 54 male Ross 308 broiler breeders at 20 weeks of age (Pluvita B.B., Apeldoorn, the Netherlands), derived from the same grandparent flock, were allocated to 18 floor pens with 27 hens and 3 male chickens per pen at the Cargill Animal Nutrition Innovation Center (Velddriel, the Netherlands). The pens were divided over 6 blocks with 3 pens per block and hens were allocated to pens based on body weight, to create blocks with similar body weights and low variation within block. Treatments were randomly allocated within weight blocks. The hens were housed in floor pens of 6.24 m2 with deep littered floor (flax) covering 1/3rd of the pen and elevated floor with plastic slats covering 2/3rd of the pen. Per pen, 240 cm of nest box was available and water was provided for 6 hours per day according the broiler breeder guidelines. Females were fed with feeding bowls and males were fed in a separate feeding trough. Diet composition and feeding regime was based on the broiler breeder guidelines [25] (see **Supplementary material**). Daily feed allowance was calculated weekly per pen, based on average pen body weights and egg production. Temperature was maintained at 20°C by floor heating.

Photostimulation started at 22 weeks of age with 10 h of light (20 lx), after which day length was gradually increased with 1 h and 10 lx per week to 12L:12D.

Egg incubation phase (2)
At 35 weeks of age, a total of 15 first class eggs per pen were randomly collected for 6 consecutive days for incubation resulting in 15 x 6 x 6 = 540 eggs per treatment. Eggs were incubated according to a standardized protocol (see **Supplementary material**).

Grow-out phase (3)
For the grow-out phase, the newly hatched chicks were feather sexed and 90 healthy male broiler chickens per broiler breeder treatment were randomly selected and allocated to 18 pens in a grow-out facility of the Cargill Animal Nutrition Innovation Center (Velddriel, the Netherlands). Per pen 15 male broiler chickens were housed that originated from the same broiler breeder pen. The 3 broiler breeder treatments were randomly allocated within 6 blocks, resulting in 6 replicates per treatment. Pens had a raised floor covered with wood shavings. Artificial lighting was set for 23 h per day from d0 to d2, 20 h per day from d2 to d6 and 18 h per day from d7 to d34. The temperature was set to gradually decrease by 0.5°C per day during the first 14 days, starting at 34°C on d0. From d14 onwards, the temperature was set to decrease gradually to a final temperature of 20°C on d36. Each pen was equipped with cup drinkers adjustable in height. During the first 3 weeks, feed was supplied using a tower feeder placed in the pen. From d21 onwards, the feed was supplied in a metal feeder trough placed in front of the pen. All treatments received the same diets and feeding regime according to the breeder guidelines (see **Supplementary material**). On d3 and d21, all broiler chickens were vaccinated against ND by means of an intramuscular injection.

Experimental Treatments

Broiler breeder phase (1): Innate stimulation with MAMPs
At 33 weeks of age, broiler breeders received 1 of the 3 treatments (Control, LPS or β-glucan). Broiler breeder hens of the LPS group were intratracheally inoculated using a blunted needle with 1 mg/kg BW LPS from *Escherichia coli* serotype O55:B5 (L2880, Sigma-Aldrich corporations, St. Louis, MO, USA) dissolved in 0.5 mL PBS (Gibco, Life Technologies Ltd., UK). Broiler breeder hens of the control and β-glucan treatment groups were inoculated intratracheally with only the solvent 0.5 mL PBS (Gibco, Life Technologies Ltd., UK). Broiler breeder hens of both the control group and the LPS group were fed a control diet, without β-glucan, formulated according to the breeder guidelines [25]. Broiler breeder hens of the β-glucan treatment group received a similar diet, in which 0.05% of corn was exchanged for the β-glucan feed additive (Macrogard, Orffa, Werkendam, the Netherlands) (**Table S2**).

Grow-out phase (3): NE challenge
All broiler chickens were challenged using an NE model based on Lensing, et al. [18]. The challenge was performed in two steps. First, a mild *Eimeria maxima* infection was used to enable colonization with *Clostridium perfringens* in the second step. All broiler chickens were orally inoculated on d7 with 1 mL of *E. maxima* (4500 sporulated oocysts per mL; Weybridge strain; Royal GD, Deventer, the Netherlands). All feed was removed 2 h prior

to inoculation. After a 7-day incubation time for coccidiosis to develop, all chicks were orally inoculated on d14 with 1 mL of a pathogenic strain of *Clostridium perfringens* (code GD 5.11.53; 10^8 CFU/mL liver broth; Royal GD, Deventer, the Netherlands) to induce the necrotic enteritis. All feed was removed 2 h prior to inoculation.

Performance Data Collection

Broiler breeder phase (1)

Broiler breeder hens were weighed individually at 33, 34, 35 and 36 weeks of age. Based on weekly body weights, ADG was calculated and feed was provided restrictedly and recorded daily. Eggs were collected twice a day and numbers of eggs were recorded daily per pen to calculate lay percentage per week (number of eggs per week divided by number of hens). One day a week, all eggs collected the previous day were weighed per pen and average egg weight was calculated. Based on egg weight and lay percentage, egg mass was calculated (egg weight multiplied by lay percentage, g). The feed conversion ratio for egg mass (**FCR**; kg of egg mass per kg of feed consumed) was calculated using calculated egg mass and ADFI. Mortality was recorded daily. Two hens per pen were randomly chosen at the start of the study and were color marked on the neck or back or both for individual identification to enable blood sampling. Blood samples were taken at 33 and 36 weeks of age. Per blood sampling 3 mL heparinized blood was collected and immediately transported to the laboratory for isolation of leucocytes. All hens were fed 2 h prior to sampling.

Egg incubation phase (2)

Prior to incubation, all eggs were weighed. Eggs were candled on d10 and d17 of incubation and empty eggs or dead embryos were recorded and removed. After hatch, total number of dead chickens, live pipped eggs and hatched eggs were recorded. Fertility (ratio of filled eggs, including embryonic mortality, of total), mortality (ratio of early and late mortality of fertile eggs) and hatchability (ratio of hatched chicks of total) were calculated. After hatch, 2 female chicks per experimental unit were randomly selected and chick quality was assessed using part of the score as described by Tona et al. [26] (see **Supplementary material**). Subsequently, the same chicks were weighed, euthanized and dissected to measure residual yolk weight and calculate yolk free body mass (**YFBM**). Yolk weight as percentage of live body weight was calculated.

Grow-out phase (3)

On d0 group BW was determined and averaged per pen. Individual body weights were determined on d7, d14, d21 and d35. Feed consumption was recorded per pen on the same days. The gain to feed ratio (**G:F**; kg of weight gain per kg of feed consumed) was calculated based on calculated ADG and ADFI. Mortality was recorded daily. At the start of the trial, five broiler chickens per pen were randomly selected and marked for blood collection on d14 (2.75 mL per broiler chicken), d21 (3 mL per broiler chicken) and d36 (5 mL per broiler chicken). Heparinized blood samples were taken from the wing vein before *C. perfringens* inoculation on d14 and before diet changes on d14 and d21. The blood samples were transported to the laboratory for isolation of leucocytes within 2 hours under controlled conditions (see below). On d15, one day after *C. perfringens* inoculation,

two broiler chickens per pen (excluding the marked broiler chickens) were randomly selected, and consequently weighed and euthanized. Lesion scoring was performed according standardized procedures [18]. First, the broiler chickens were, through a blind experimental approach, visually scored for *E. maxima* (characteristic lesions: hemorrhages, blood and orange mucus) and *C. perfringens* (characteristic lesions: white, brown and/or grey spots). The *E. maxima* was scored from 1 to 4 in the second loop of the duodenum and jejunum till the Meckel's diverticulum (score 1 = one or some hemorrhages; score 2 = several hemorrhages; score 3 = many hemorrhages, orange mucus in the lumen; score 4 = many hemorrhages, mucosal damage, free blood in the lumen). The *C. perfringens* was also scored from 1 to 4 in the gizzard towards Meckel's diverticulum (score 1 = one to five lesion present in the intestine; score 2 = more than 5 single distinguished lesions in the intestine; score 3 = lesions merge or extend to a surface of more than 1 cm2; score 4 = pseudo membranes present in the intestine causing death).

Immunological Data Collection

Isolation, culture and stimulation of primary monocytes

As described above, heparinized blood from broiler breeder hens and broiler chickens was collected at several timepoints during the experiment (see **Figure 1** for timeline). Primary monocytes from blood were isolated as described previously [13]. Briefly, mononuclear cells were purified using histopaque-1119 (density: 1.119 g/mL, Sigma-Aldrich corporations, St. Louis, MO, USA) followed by density gradient centrifugation (700 x g, 40 minutes at room temperature) and seeded at a concentration of $1*10^6$ cells per well in a 96-well flat bottom plate (CELLSTAR, Greiner Bio-One, Alphen aan den Rijn, the Netherlands), followed by incubation at 41°C in 5% CO_2 and 95% humidity overnight. The next day, non-adherent cells were washed away with pre-warmed (41°C) complete cell culture medium (i.e. RPMI 1640 supplemented with 25 mM HEPES, Glutamax™, 10% heat-inactivated chicken serum and 50 U/mL penicillin and 50 µg/mL streptomycin; all from Gibco, Life Technologies Ltd., Inchinnan, UK).

Cells were collected for RT-qPCR analysis after a 24 h stimulation with 200 µL complete culture medium (control) or 200 µL LPS from *Escherichia coli* serotype O55:B5 (f.c. 10 µg/mL, L2880, Sigma-Aldrich corporations, St. Louis, MO, USA). The cells were washed with ice-cold PBS, directly lysed with RLT lysis buffer and stored at -80°C until further RT-qPCR analysis (Qiagen, Hilden, Germany). From an identical, in parallel-performed experiment, cell culture supernatant was collected after 48 h for subsequent analysis of nitric oxide (**NO**) production. For NO production assays, cells were stimulated with LPS (1 or 10 µg/mL, or LPS (10 µg/mL) + IFNγ (0.1 µg/µL) in complete culture medium. Blood plasma collected after the density gradient centrifugation was stored at -20°C until the agglutination assay.

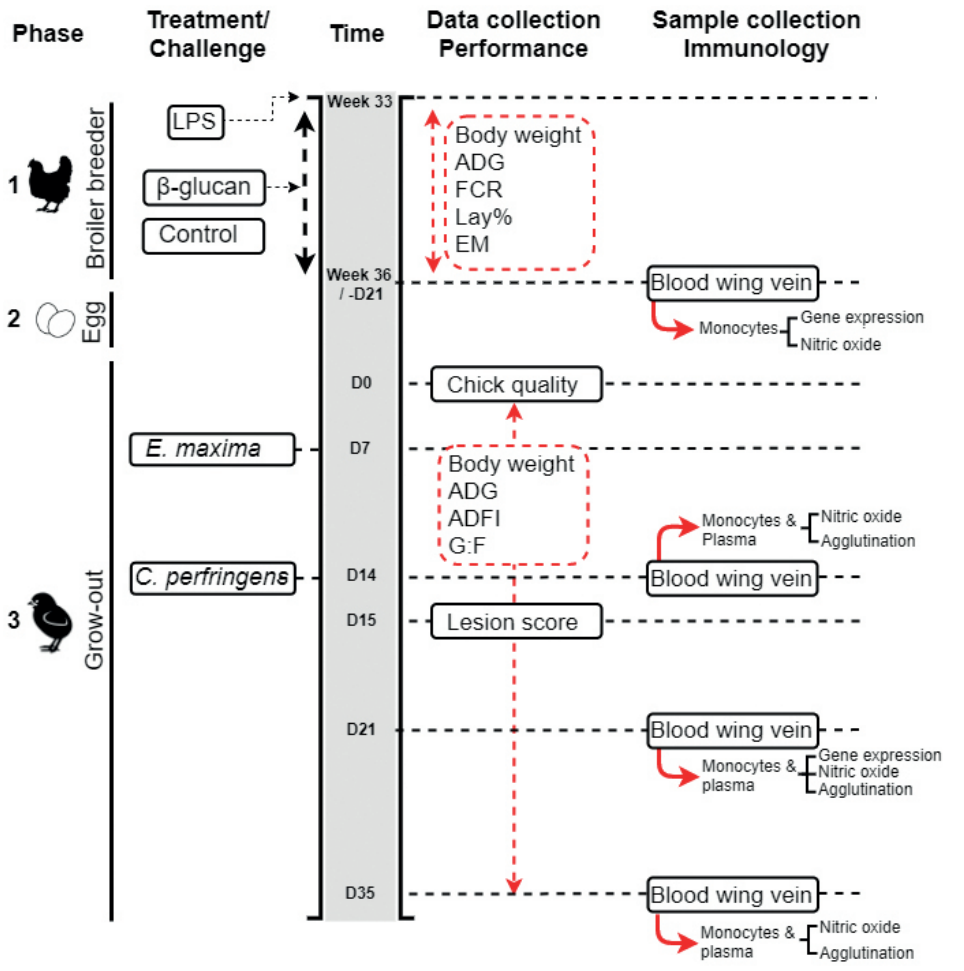

Figure 1. Timeline of the transgenerational experiment. The experiment was divided into three phases: Broiler breeder (1), Egg incubation (2) and Grow-out (3). Treatments, challenges, data collection and sample collection are shown. Time indicates the moment when the different handlings were done. Data collection for performance purposes was done during the entire experiment and indicated by the red-dashed arrows. Measurement of the Chick quality (Tona score) and intestinal lesions (Lesion score) was done ones, respectively at d1 and d15. The *E. maxima* (d7) and *C. perfringens* (d14) challenge are part of the necrotic enteritis challenge. ADG: Average Daily Gain; FCR: Feed Conversion Ratio; Lay%: Laying percentage; EM: Egg Mass ; ADFI: Average Daily Feed Intake; G:F: Gain to Feed ratio.

Nitric oxide production

Accumulated NO in the medium upon stimulation was indirectly measured by quantifying nitrite (NO_2^-) concentration in the culture medium, using the Griess reaction assay as previously described [12]. Briefly, medium was collected from stimulated macrophages and combined with Griess reagent in a 1:1 ratio. The NO_2^- concentration was determined by measuring the optical density at 540 nm with a spectrophotometer (Multiscan™,

Thermo Fisher Scientific, Waltham, MA, USA). The results were interpolated on a standard curve made by serially diluting a sodium nitrite solution ($NaNO_2$) in the range from 0 to 100 µM.

Cell viability was assessed with an Alamar Blue (**AB**) assay (see **Supplementary material**). This assay allowed the establishment of the relative variation in the number of viable cells in the wells of the 96-wells plates, because only viable cells will reduce the blue colored resazurin into the red colored resorufin. Reduction of the AB solution was quantified with a spectrophotometer. The amount of reduced resazurin, which reflects the number of viable cells, was used to normalize the corresponding NO production for the same well to assess the amount of NO per viable cell.

Total RNA isolation and qPCR analysis

Total RNA isolation and qPCR procedure are described in more detail elsewhere [12]. Briefly, total RNA was isolated and subsequently subjected to a DNase digestion treatment. RNA quantity and purity were measured with a NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies LCC, Thermo Fisher, Wilmington, Delaware, USA). RNA quality was determined using the Agilent 2100 Bioanalyzer according manufacturer's instructions (Agilent Technologies, Santa Clara, California, USA). Total RNA (50 ng) was reverse transcribed into complementary DNA (cDNA) using random hexamer primers (Roche Diagnostics, the Netherlands) and the SuperScript III Reverse Transcriptase kit (Invitrogen, 18080044). The qPCR assay was based on a 20 µL volume design using the SensiFAST™ SYBR® Lo-ROX Kit (Bioline, Meridian Bioscience Inc., Cincinnati, Ohio, USA) together with a 5 µM specific sense and antisense primer set (**Table 1**), and was performed with a QuantStudio® 5 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Corporation, Foster City, California, USA). Amplification conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s each. Melting curve analysis confirmed specific amplification of a single PCR product. The results were interpolated on a standard curve made by 10x serial dilution of a known amount of corresponding cDNA product of the target gene. Absolute mRNA quantities were normalized to the geometric mean of three internal reference genes (**Table 1**), which were identified as being the most stable genes as determined with Normfinder algorithm software [27].

Agglutination assay.

An agglutination assay was used to determine the relative amount of agglutinating antibodies against *Clostridium perfringens type A necrotic enteritis toxin B-like* strain in blood plasma. An inactivated bacterial suspension of this strain with a concentration of 10^9 bacteria/mL was commercially provided (Royal GD, Deventer, the Netherlands). The suspension was diluted to reach an optimal concentration with optical density of 0.450 (cuvette dimension of 10 x 10 mm) using a 510 nm filter (Evolution 201 UV–Visible, Thermo Scientific, Waltham, USA). Next, a volume of 25 µL of each plasma sample was two-fold serial diluted in PBS (Gibco, Life Technologies Ltd., UK) in a 96-well round bottom plate (CELLSTAR, Greiner Bio-One, Alphen aan den Rijn, the Netherlands). Every row represented one sample and the last column was used as negative control. Next, 25 µL of the optimized inactivated bacterial suspension was pipetted into the wells and suspensions were mixed for 10 seconds using a microplate shaker, followed by a 12h incubation period at 4 °C.

Antibody titers were scored after placing the plates at a 45° angle for 30 seconds in order to enhance visualization of agglutination. The antibody titer represents the number of the last column where agglutination was still present.

Table 1. Primers used for RT-qPCR. IL-1β and iNOS were used to measure an effect of maternal treatments on pro-inflammatory immunity.

Target[1]	Sequence[2]	Accession no.
Internal reference genes		
ACTB	F: 5′-GCCCTGGCACCTAGCACAAT-3′	NM_205518
	R: 5′-GCGGTGGACAATGGAGGGT-3′	
IPO8	F: 5′-ACCTCCGAGCTAGATCCTGT-3′	XM_015287054
	R: 5′-GGCTCTTCTTCGCCAACTCT-3′	
GAPDH	F: 5′-ATCCCTGAGCTGAATGGGAAG-3′	NM_204305
	R: 5′-AGCAGCCTTCACTACCCTCT-3′	
Genes associated with inflammation		
IL-1β	F: 5′-GACATCTTCGACATCAACCAG-3′	XM_015297469
	R: 5′-CCGCTCATCACACACGACAT-3′	
iNOS	F: 5′-CTACCAGGTGGATGCATGGAA-3′	NM_204961
	R: 5′-ATGACGCCAAGAGTACAGCC-3′	

[1]ACTB: Actin beta; IPO8: Importin 8; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; iNOS: Inducible nitric oxide synthetase.
[2]F: forward; R: Reverse

Statistical Analysis

Performance data:
Data were analyzed using pen as the experimental unit. Model assumptions, i.e. normality and equal variance of the error terms, were checked by inspection of the residual plots. Data were subjected to mixed model analyses, using R Studio (R Studio version 1.1 © 2009-2018 RStudio, Inc.). For the broiler breeder phase and the grow-out phase, the following statistical model was used:

$$Y_{ij} = \mu + \alpha_i + B_j + \varepsilon_{ij}$$

where Y_{ij} = dependent variable, μ = overall mean, α_i = fixed effect of treatment (i = control, LPS or β-glucan), Bj = random block effect (j = 1 - 6) and εij = residual error. For the egg incubation phase, the following statistical model was used:

$$Y_{ijk} = \mu + \alpha_i + B_j + \alpha(C)_{ijk} + \varepsilon_{ijk}$$

where Y_{ij} = dependent variable, μ = overall mean, α_i = fixed effect of treatment (i = control, LPS or β-glucan), B_j = random block effect (j= 1 - 6), $\alpha(C)_{ijk}$ = random nested effect of breeder pen within treatment effect (k =1-24) and ε_{ijk} = residual error.

Non-binomial data are expressed as least square (LS) means. LSmeans were compared after being corrected with a Tukey test for multiple comparisons and effects were considered to be significant when $P \leq 0.05$. *E. maxima*, *C. perfringens*, mortality, fertility, hatchability and Tona score data were analyzed as binomial distributed data, using the same statistical model. Tona score data was non-normally distributed and normalized using a Box-Cox transformation [28]. Effects were considered to be significant when $P \leq 0.05$.

Immunological data:
Immunological data were analyzed with SAS statistical software (SAS 9.4, SAS Institute Inc., Cary, NC, USA). The same statistical approach was used for the broiler breeder phase (1) and the grow-out phase (3) with the following statistical model:

$$Y_{ij} = \mu + \alpha_i + B_j + \varepsilon_{ij}$$

where Y_{ij} = dependent variable, μ = overall mean, α_i = fixed effect of treatment (i = control, LPS or β-glucan), B_j = *ex vivo* LPS stimulation (mRNA: j= 1 - 2; NO: j= 1 – 4; Agglutination: n/a) and ε_{ij} = residual error.

Mixed models were created with relative mRNA expression, nitric oxide and agglutination titer as dependent variables. Since pen as random effect was very small and non-significant, an ordinary linear regression model was performed. Model residuals were assessed for normality by creation of histograms and Q-Q plots. The used explanatory variable was maternal treatment. For the response variables nitric oxide and agglutination titer, the variable age was included in the model as well. Furthermore, interaction effects were tested for significance. Effect of maternal treatment and treatment in the offspring on relative mRNA expression was tested within each gene, being iNOS and IL1-β. Immunological data are expressed as means and effects were considered to be significant when $P \leq 0.05$.

RESULTS

Effects of Immunization with Innate Immune-stimulating Components on Performance and Immune Responsiveness in Broiler Breeders, Eggs and on Chick Quality
First, direct effects of the maternal treatments (Control, LPS, β-glucan) on the performance of broiler breeders themselves were investigated (**Table 2**). Intratracheal treatment with LPS resulted in decreased egg production during the first week post inoculation (≈ 8%, $P < 0.01$; wk34; **Figure 2A and Table 2**) compared to the control broiler breeder hens, but this reduced egg production was no longer significant one week later (week 35). LPS treatment resulted in an increase in ADG in the second week after inoculation (~4.1gr, $P < 0.05$; wk34 to wk35; **Figure 2B and Table 2**) compared to the control group. Effects on ADG were no longer present one week later (week 35 to 36). No differences on egg production and ADG were found for the β-glucan-treated hens. Furthermore, none of the maternal treatments showed an effect on body weight, ADFI, FCR, ME, egg weight, chick weight, YFBM, relative yolk weight of chick weight, Tona Score, fertility, mortality and hatchability (**Table 2** and **Table S3:** Egg and chick quality).

Table 2. Results of the broiler breeder phase (1). Effects of LPS and β-glucan treatment on BW, ADG, ADFI, FCR, Lay% and ME of broiler breeder hens are shown. Measurements between 25 and 33 weeks of age represents the pre-trial period data. Maternal treatment started at 33 weeks of age (control, LPS, β-glucan). Broiler breeders were weighed individually weekly post treatment. The other parameters were measured and calculated during a time interval of a week. Egg collection for the grow-out phase was done during week 35 and 36. Unless stated otherwise, results are shown as least squares means[1].

Variable	Control	LPS	β-glucan	SEM	P-Value	Average
Body Weight (kg)						
Week 33	3.797	3.778	3.799	0.055	0.542	3.791
Week 34	3.824	3.803	3.831	0.058	0.464	3.819
Week 35	3.849	3.857	3.871	0.052	0.687	3.859
Week 36	3.883	3.882	3.899	0.054	0.735	3.888
Week 25 - 33 pre-trial period						
ADG. g	11.723	11.504	11.668	0.943	0.905	11.632
ADFI. g	155.2	154.2	155.5	0.8	0.354	155
FCR	3.669	3.625	3.742	0.107	0.175	3.679
Lay%	75.99	76.92	75.84	1.81	0.626	76.25
ME	42.45	42.64	41.83	1.26	0.435	42.31
Week 33 - 34 Start LPS and β-glucan						
ADG. g	3.756	3.632	4.438	0.931	0.747	3.942
ADFI. g	161.8	160.8	161.3	0.6	0.525	161.3
FCR	3.003	3.103	3.005	0.053	0.322	3.037
Lay%	88.52[a]	83.73[b]	87.75[a]	1.09	**0.007**	86.66
ME	53.93	51.91	53.76	0.85	0.207	53.2
Week 34 - 35						
ADG. g	3.628[a]	7.762[b]	5.825[ab]	1.259	**0.037**	5.739
ADFI. g	161.8	160.8	161.3	0.6	0.525	161.3
FCR	3.050	3.149	3.070	0.070	0.588	3.090
Lay%	87.13	82.56	86.13	1.6	0.125	85.27
ME	53.08	51.18	52.79	1.13	0.457	52.35
Week 35 - 36 Egg collection for grow-out						
ADG. g	4.790	3.579	4.005	1.059	0.720	4.124
ADFI. g	161.8	160.7	161.3	0.6	0.440	161.3
FCR	2.939	2.963	2.989	0.051	0.792	2.964
Lay%	87.33	86.52	86.12	1.54	0.856	86.66
ME	55.07	54.35	54.06	0.91	0.731	54.49

[1] Each treatment consisted of 6 pens with 27 broiler breeder hens per pen; [a-c]effects were considered to be significant when $P \leq 0.05$

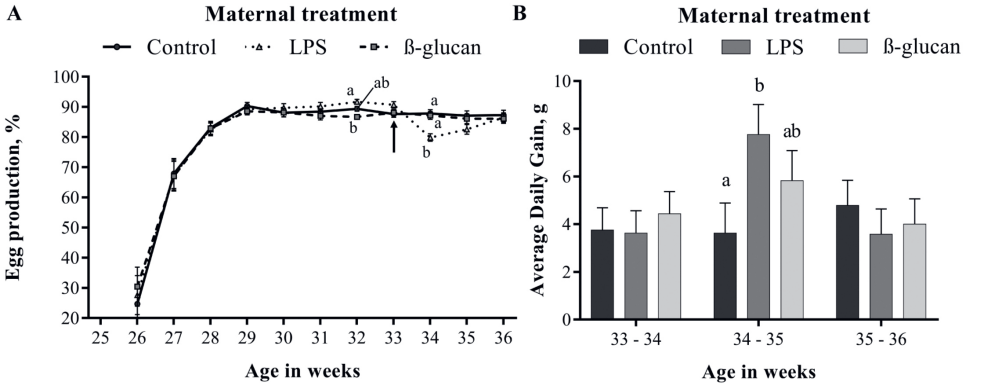

Figure 2 Maternal treatment effect on Egg production and ADG during the broiler breeder phase (1). LPS was inoculated at the beginning of week 33 (arrow) and simultaneously the treatment with β-glucan was also started at this timepoint. **A)** Egg production in week 34 for broiler breeder hens that were inoculated with LPS showed lower egg production (~8% compared to control). **B)** ADG in week 34 to 35 showed that growth was significantly higher in the LPS treatment group compared to the control. **A-B)** N=6 pens per treatment with 27 hens per pen. Data is represented as lsmeans. Effects are represented as superscripts and were considered to be significant when $P \leq 0.05$.

Table 3. Results of the grow-out phase (3) whereby the transgenerational effects of the maternal treatments (control, LPS, β-glucan) are investigated. Effects of LPS and β-glucan treatment on BW, ADG, ADFI and G:F of the offspring before and after the NE challenge (d15) are shown. On d0 group BW was determined and averaged per pen. Individual BW were determined on d7, d14, d21 and d35. The other parameters were measured and calculated during a time interval of 7 days. Unless stated otherwise, results are shown as least squares means[1].

Variable	Control	LPS	β-glucan	SEM	P-Value	Average
Body Weight (g)						
Day 0	45.1	45.2	45.4	0.5	0.878	45.3
Day 7	169.4	170.2	167.0	3.4	0.713	168.9
Day 14	420.2	413.0	418.3	12.4	0.816	417.2
Day 21	860.2	847.1	855.0	23.9	0.889	854.1
Day 35	2334.8	2300.6	2340.9	57.2	0.832	2325.4
Day 0 to 7						
ADG. g	17.75	17.86	17.22	0.451	0.533	17.61
ADFI. g	17.4	17.4	17.1	0.4	0.694	17.3
G:F	1.017	1.027	1.008	0.015	0.601	1.017
Day 7 to 14						
ADG. g	35.83	34.68	35.91	1.60	0.612	35.47
ADFI. g	49.1	49.2	49.4	1.7	0.981	49.3
G:F	0.730	0.705	0.724	0.016	0.229	0.720
Day 14 to 21						
ADG. g	62.41	61.98	61.78	2.01	0.971	62.06
ADFI. g	86.2	82.0	86.6	2.8	0.403	84.9
G:F	0.725[a]	0.757[b]	0.713[a]	0.011	**0.017**	0.731
Day 21 to 35						
ADG. g	104.55	101.39	105.69	2.62	0.492	103.88
ADFI. g	154	150.5	156.7	4.0	0.462	153.7
G:F	0.679	0.674	0.675	0.009	0.888	0.68

Variable	Control	LPS	β-glucan	SEM	P-Value	Average
Day 0 to 35						
ADG. g	60.56	58.93	60.81	1.34	0.558	60.10
ADFI. g	85.2	82.8	86.3	2.0	0.454	84.8
G:F	0.711	0.712	0.705	0.005	0.367	0.71

[1] Each treatment consisted of 6 pens with 15 male chickens per pen; [a-b]effects were considered to be significant when $P \leq 0.05$

Next, effects of the *in vivo* maternal treatments (Control, LPS, β-glucan) on *ex vivo* responsiveness of monocytes in the blood was investigated. Blood-derived monocytes were stimulated *ex vivo* and the mRNA expression of the inflammation-associated genes IL-1β and iNOS, and the production of NO were measured (**Figure 3**). *Ex vivo* stimulation with LPS resulted in increased IL-1β mRNA compared to e*x vivo* stimulation with only culture medium ($P < 0.01$), while the amount of iNOS mRNA remained unaffected (**Figure 3A** and **Table S4**). Furthermore, after the *ex vivo* LPS stimulation, no differences between the *in vivo* maternal treatments with either LPS or β-glucan has been found on IL-1β and iNOS expression levels compared to the control treatment (**Figure 3A** and **Table S4**).

Next, we measured the amount of NO accumulated in the culture medium upon agonist stimulation (**Figure 3B**). Irrespective of the source (control, LPS or β-glucan treatment) of the monocytes, NO production increased after *ex vivo* stimulation with LPS (1μg/mL, 10μg/mL) or a combination of LPS (10μg/mL) + IFNy (0.1 μg/mL) compared to the culture medium (control) group, whereby LPS + IFNy showed the largest NO production ($P < 0.01$; **Figure 3B** and **Table S5)**. Interestingly, monocytes isolated from β-glucan-treated broilers displayed a significant ($P < 0.05$) higher NO production in response to *ex vivo* stimulation with LPS + IFNy, compared to monocytes from control or LPS-treated hens **(Figure 3B and Table S5)**.

Transgenerational Effect of Maternal Immunization on Growth Performance and Immune Responsiveness

The offspring of the LPS-treated broiler breeders showed an increased gain feed ratio (G:F) during d14 to d21 compared to their associates of control and β-glucan-treated broiler breeders ($P < 0.05$; **Table 3** and **Figure 4A**). NE intestinal lesions were measured on d15, which was one day after the *C. perfringens* challenge and 8 days after the *E. maxima* challenge (**Figure 4B)**. More broiler chickens with *E. maxima* lesion scores higher than 0 were found in the LPS treated broiler breeder offspring than in the control and β-glucan offspring ($P < 0.05$ with intestinal lesion score > 0; **Figure 4B** and **Table S6**). Furthermore, numeric differences were found between the severeness of the intestinal lesions, as visualised by the scores (0,1,2,3) (**Figure 4B** and **Table S6**). No effect of treatment was observed of *C. perfringens* infection on the intestinal lesion scores.

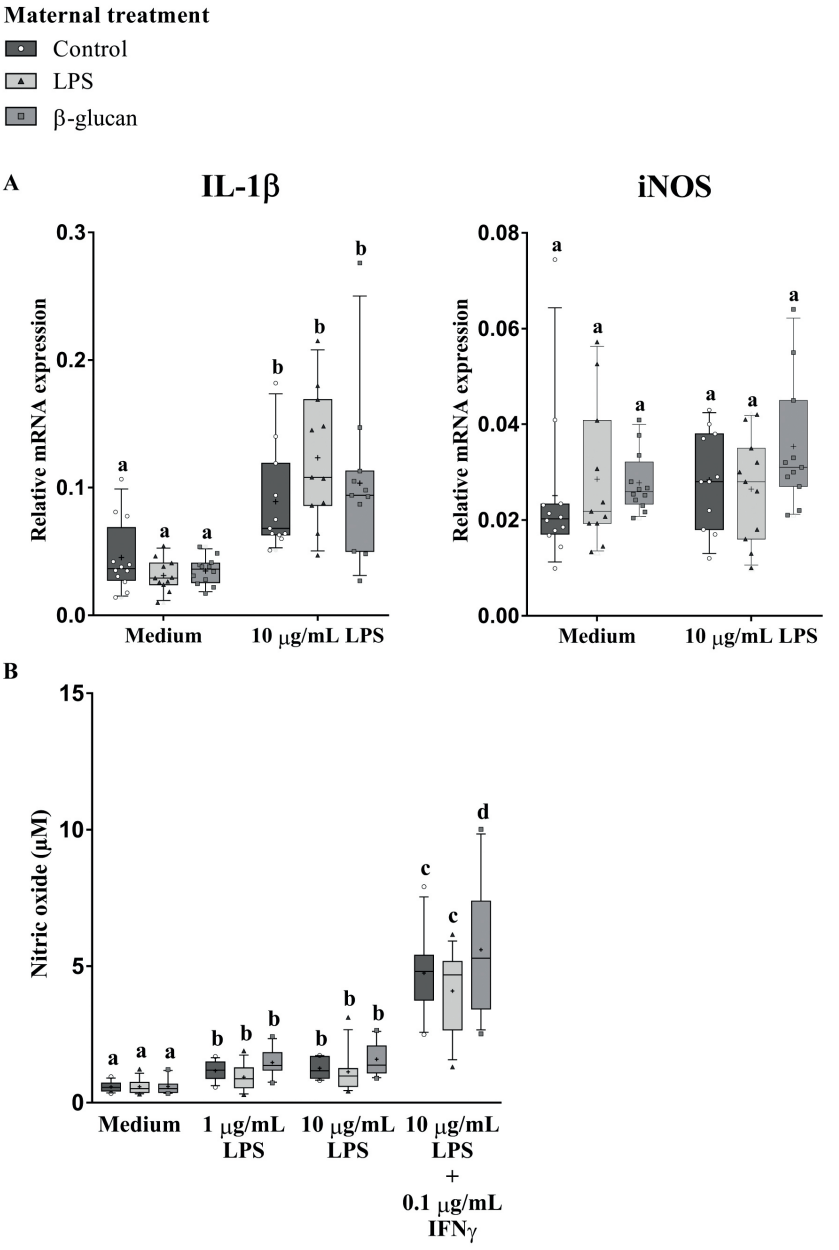

Figure 3. Effect of maternal treatments on inflammation associated parameters in blood-derived primary monocytes of broiler breeders. The broiler breeders received the maternal treatments at 33 weeks of age and monocytes were collected at the age of 36 weeks. **A)** Relative mRNA expression levels of IL-1β and iNOS were measured after a 24h *ex vivo* stimulation with medium or LPS (10 μg/mL), followed by collection of total RNA. **B)** NO production levels in culture medium were measured after a 48h *ex vivo* stimulation with Medium or LPS (1 μg/mL, 10 μg/mL) or a combination of LPS (10μg/mL) + IFNy (0.1 μg/mL). **A-B)** N=12 animals per treatment. For all figures the box extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median and '+' at the mean. Whiskers represents 10-90 percentile. Effects are represented as superscripts and were considered to be significant when $P \leq 0.05$.

Ex vivo LPS stimulation of blood-derived monocytes from 21 day-old offspring resulted in increased IL-1β and iNOS mRNA levels compared to the culture medium-treated group ($P < 0.01$; **Figure 5A** and **Table S7**). Furthermore, both *in vivo* maternal treatment LPS and β-glucan tended ($P = 0.08$) to decrease IL-1β mRNA in monocytes exposed to *ex vivo* LPS stimulation (**Figure 5A** and **Table S7**). The LPS-induced iNOS mRNA expression, on the contrary, was not affected by the maternal treatments.

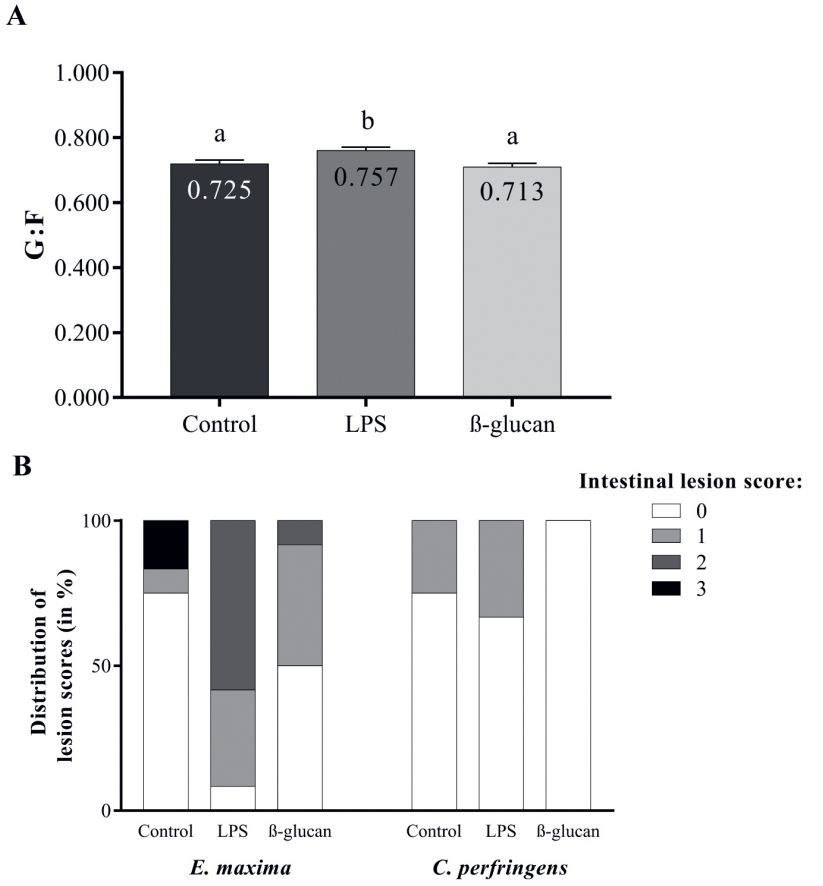

Figure 4. Effects of maternal treatment in the next generation on gain-feed ratio (G:F), *E. maxima* lesion scores and *C. perfringens* lesion scores, after the Necrotic enteritis (NE) challenge. The NE challenge consists of a *E. maxima* challenge at 7 days of age and a *C. perfringens* challenge at 14 days of age. **A)** Representation of the G:F from d14 to d21. Offspring related to the LPS-treated mother hens become more efficient in using feed after the NE challenge compared to the control group. N=6 pens per treatment with 15 hens per pen. Data is represented as lsmeans. Effects are represented as superscripts and were considered to be significant when $P \leq 0.05$. **B)** Representation of the Necrotic enteritis lesion scores on d15, one day after the *C. perfringens* challenge and 8 days after the *E. maxima* challenge. Data is represented as percentage of the total number of animals per treatment group. The distinction between the severeness of the intestinal lesions was also included in the figure (not included in statistical analysis). 0 indicates no lesions and 3 indicates the most severe intestinal lesions. More chicks showed *E. maxima* lesions that are related to the LPS treated broiler breeders compared to the control ($P < 0.05$). However, no significant effect of the *C. perfringens* infection was found with respect to the intestinal lesions. N=6 pens per treatment with 2 hens per pen.

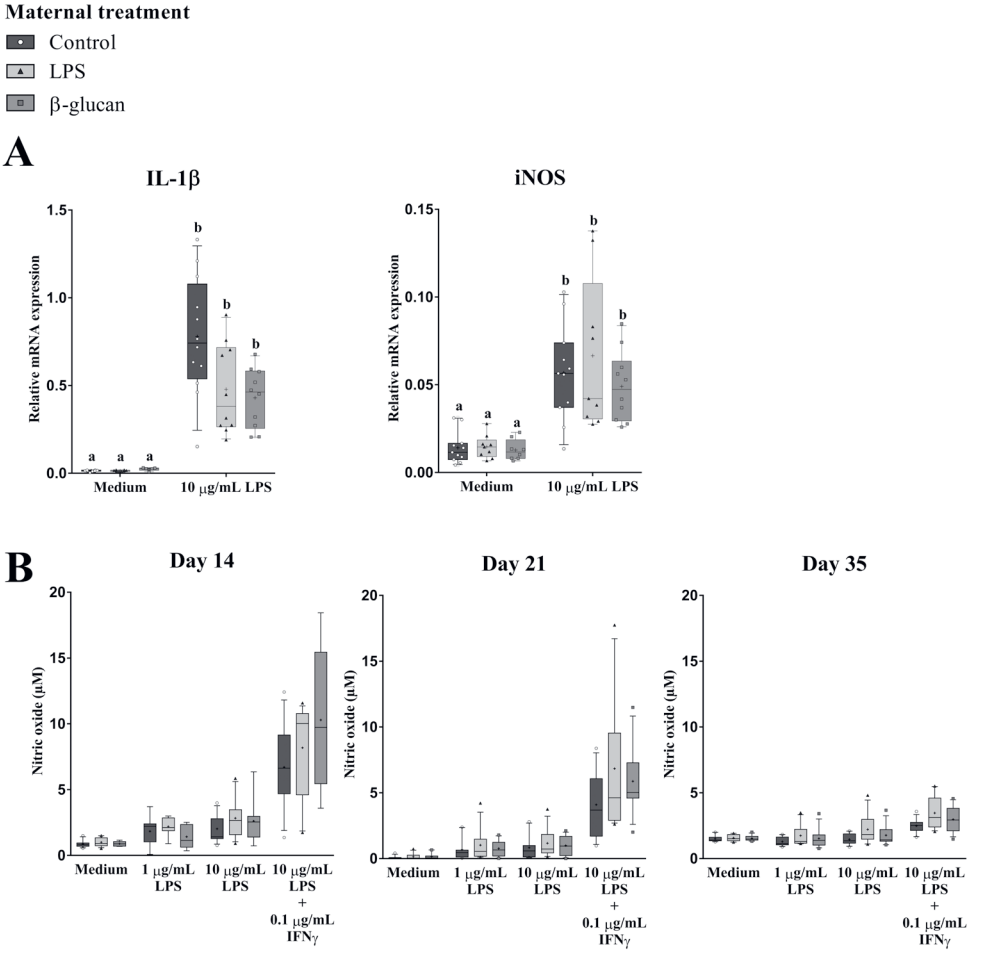

Figure 5 Effects of maternal treatment on the responsiveness of blood-derived primary monocytes isolated in the next generation (grow-out phase 3). Gene expression levels and NO production were measured after *ex vivo* stimulation with Medium or LPS. Offspring received *in vivo* at d14 the *C. perfringens* infection. **A)** Relative mRNA expression levels of IL-1β and iNOS were measured in the monocytes that were collected at the age of 21 days (7 days after the *in vivo* challenge). Monocytes received *ex vivo* stimulation with medium or LPS (10 μg/mL) for 24hrs. **B)** Nitric oxide production of primary monocytes collected at d14, d21 and d35. Monocytes were *ex vivo* stimulated with Medium or LPS (1μg/mL, 10μg/mL) or a combination of LPS (10μg/mL) + IFNy (0.1 μg/mL) for 48hrs. **A-B)** N=12 animals per treatment. The box extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median and '+' at the mean. Whiskers represents 10-90 percentile. Effects are represented as superscripts and were considered to be significant when $P \leq 0.05$.

Notably, NO production in response to LPS decreased in cells derived from 35 days-old broiler chickens compared to those from 21 or 14 days-old. ($P < 0.01$). Monocytes from chickens at 14 days of age showed a higher ($P < 0.01$) LPS-induced NO production compared to those from chickens of 21 or 35 days of age (**Figure 5B** and **Table 4**). Furthermore, NO production was higher in *ex vivo* LPS or LPS+IFNy-stimulated cells compared to the culture medium group ($P < 0.01$, **Figure 5B** and **Table 4**). Highest NO

production was observed in cells stimulated with LPS+IFNy. NO levels were higher in the *in vivo* maternal LPS treatment group compared to the control group (P < 0.05; **Figure 5B** and **Table 4**). No difference between the β-glucan maternal treatment group was found compared to the control (**Figure 5B** and **Table 4**)

Table 4. Statistical output grow-out phase (3): Nitric oxide production. Accumulated NO in the medium upon Ex vivo LPS stimulation in the grow-out phase (3) was determined and used as a tool to investigate transgenerational effects of the maternal treatments (control, LPS, β-glucan).

Variable	Category	n	LSMeans	SEM	P-value
Maternal treatment	β-glucan	141	2.45[a]	0.17	**0.0141**
	LPS	136	2.73[b]	0.17	
	Control	131	2.02[a]	0.17	
Age	14	124	3.36[a]	0.18	**0.0077**
	21	144	1.88[b]	0.16	
	35	140	1.96[b]	0.17	
Ex vivo LPS stimulation	0	104	0.86[a]	0.19	**<0.0001**
	1	96	1.45[ab]	0.20	
	10	104	1.72[b]	0.19	
	10+0.1 IFNy	104	5.57[c]	0.19	
R-square model		0,52			

a,b,c: different letters P < 0.05

interaction age**Ex vivo* LPS stimulation P < 0.01

Transgenerational Effect on Agglutinating Antibody Titers.

We measured agglutinating antibody titers as a serologic marker to investigate if maternal treatments would influence the humoral response in the offspring after a challenge with *C. perfringens*. Antibody titers against *C. perfringens* increased in all chickens of all ages. The antibody titers increased with age (P < 0.01), but were not affected by the *in vivo* maternal treatments (**Figure 6**).

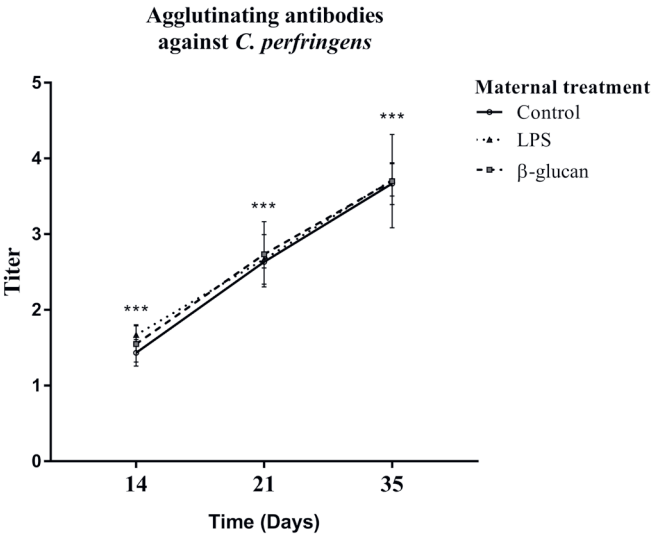

Figure 6. Agglutinating antibody titers against *C. perfringens* in the offspring's blood plasma. Offspring received an oral inoculation of *C. perfringens* at d14. Blood plasma was collected at d14 (before infection), d21 and d35 of age and subjected to an agglutination assay. Each bar represents the mean of agglutinating antibody titers ± SEM with N=30 animals per treatment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

DISCUSSION

In this study, we aimed to investigate if maternal stimulation of the innate immune system with MAMPs in broiler breeders could have an effect on growth performance parameters and immune responsiveness of their offspring. Both MAMP treatments of the broiler breeder phase are extensively used in research on trained innate immunity in mammals [29, 30]. These researchers found epigenetic reprogramming of trained monocytes, indicating a role for innate training in disease protection at the longer term. β-glucan has been found to induce trained innate immunity in mammals and chickens, which results in an increased immune response after a second stimulation with an unrelated antigen [12, 31]. Within the broiler breeder phase, dietary treatment with β-glucan did result in increased NO production levels of monocytes, suggesting innate immune activation. However, no further effects of β-glucan treatment on growth performance and immune responses were observed. LPS stimulation has also been found to initiate functional reprogramming of innate immune cells, including monocytes in mammals [29]. In contrast to the β-glucan treatment, the LPS treatment reduced egg production and increased growth directly after administration. Intratracheal challenges with LPS has pathological consequences for chickens, whereas oral administration of beta-glucans has not [32-34]. These pathological consequences are due to the pro-inflammatory effects of LPS [35]. Maternal LPS treatment may therefore result in stronger transgenerational effects compared to the β-glucan treatment. Indeed, the transgenerational effects in the present study are specifically found in the offspring of LPS-treated broiler breeder hens.

The greater G:F ratio in the offspring of LPS-treated broiler breeder hens during the first week following the *C. perfringens* challenge, could be ascribed to a transgenerational effect on feed conversion efficiency. It is possible that the retainment of nutrients recovers more quickly in broiler chickens from the maternal LPS treatment group despite the higher occurrence of intestinal lesions. Since the G:F was measured during 7 day-intervals and intestinal lesion score only at d15, it is tempting to speculate that the intestinal tract of the offspring of the LPS-treated broiler breeders had an improved and faster recovery and therefore a greater G:F ratio, while the recovery of the control group was slower. It would be interesting to investigate whether the recovery rate of the capacity of nutrient absorption in the offspring is enhanced by maternal LPS treatment of broilers in a future study. The observation of the intestinal lesions caused by *E. maxima* could be an indication of transgenerational innate training effects due to maternal immunization with LPS. For future research it is recommended to measure lesion scores at shorter intervals after challenge to visualize the dynamics of the appearance of intestinal lesions in time and consequently see intestinal recovery in time. Intestinal recovery from a bacterial challenge may be an evaluation parameter to explore transgenerational effects.

The tendency for a treatment effect of maternal immunization with MAMP on IL-1β mRNA expression by *ex vivo* LPS stimulated monocytes of offspring needs to be confirmed. Furthermore, it is unclear whether reduced IL-1β transcription levels also result in reduced IL-1β protein levels and as a consequence reduced inflammation. Whether this reduced IL-1β mRNA expression is caused by epigenetic modifications in monocytes or by attenuated infection due to increased resistance is currently not know.

We applied β-glucan through the feed, since it is a commonly used immune modulating feed additive [36-38]. We used LPS intratracheally, because this infection model was already successfully used in the past to stimulate the immune system of chickens [39, 40]. Considering the results of LPS and β-glucan in the offspring of the current study, it is conceivable that a more pathological treatment, such as LPS, has the potential to initiate a more detectable transgenerational effect compared to a more mild treatment such as dietary β-glucan. Differences in animal age, β-glucan dosage, exposure time, β-glucan purity and β-glucan origin might be key factors in inducing transgenerational effects [34, 41-44].

We found low mortality rates and relatively low intestinal lesion scores upon the NE challenge in the offspring, indicating that we applied a sub-clinical infection. Sub-clinical conditions can be preferable to study immune-related effects. In contrast to overstimulation of the immune system induced by a severe challenge, a mild infection may increase the chance of finding more subtle differences in immune responses. Furthermore, sub-clinical conditions represent the most frequent situation under practical circumstances. The infection model used in this study, is therefore highly suitable for testing the effect of feed induced improvements of animal health.

Within the current study, it was also hypothesized that differences in monocyte activity would influence the agglutinating capability against *C. perfringens*. The cytokine IL-1β is an activator of the humoral response and would therefore also influence the production

of antibodies against *C. perfringens* [45, 46]. However, despite the fact that we did observe decreased mRNA levels of IL1β after the *C. perfringens* infection in the LPS treatment group, no differences in the level of specific antibodies was found.

The importance of transgenerational effects has not been recognized for a long time, but it now becomes more clear that these effects have long lasting effects on the physiology of an organism [1, 9, 47]. In this study, we aimed to investigate if activation of the maternal innate immune system with MAMPs affects performance and innate immune responsiveness of the offspring. These data are a first indication that broiler breeder hens can affect immune responsiveness and feeding efficiency of their offspring in a transgenerational manner. Studies with pied flycatchers and domesticated zebra finches describe transgenerational effects of maternal immunization with LPS on antibody levels in the offspring [48-50]. However, no mechanism behind this transgenerational effect has been described. It is possible that modification of the innate immune system by molecules such as LPS are responsible for the described effects. For future studies, we would recommend additional MAMPs that are known to be able to train the innate immune system [51] in combination with additional read-out parameters. More read-out parameters which have already described to be associated in visualizing trained innate immunity and transgenerational epigenetics in mammals, including histone modification analyses or transcriptomics, may be worth considering [30, 52]. In addition, it should be realized that vaccinations of breeder hens could also have transgenerational effects on offspring immune phenotype [2]. Administration of standard vaccines to broiler breeders could therefore be an useful strategy to study transgenerational effects. More knowledge about transgenerational effects of maternal immunization or infection will contribute to a better understanding of the variation in immune phenotypes, disease resistance and metabolic disorders.

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SUPPLEMENTARY MATERIAL

Materials & Methods

Tables & Figures

Table S1. Ingredient and nutritional composition of the starter, grower and finisher diets during the Grow-out phase.

	Starter	Grower	Finisher
Soybean meal	305.1	250.6	246.6
Maize	285.9	356.4	352.7
Wheat	200.0	200.0	200.0
Soybeans, heat treated	80.0	80.0	80.0
Rapeseed, solvent extracted	50.0	50.0	50.0
Animal fat,	20.1	16.1	20.5
Soybean oil	20.1	16.1	20.5
Limestone	14.76	11.78	11.81
Monocalciumphosphate	6.69	2.51	2.58
Sodiumbicarbonate	2.6	2.66	1.99
Salt	1.9	1.93	1.88
DL-Methionine	2.14	1.53	1.29
L-Lysine HCL	0.51	0.37	-
L-Threonine	0.11	0.00	-
Hostazym X 15000	0.1	0.1	0.1
Phyzyme XP 10000 TPT - 500 FTU	0.05	0.05	0.05
Premix Broiler Starter[1]	10	-	-
Premix Broiler Grower[2]	-	10	10
CP	243	221	219
Crude fat	77	71	79
Ash	0	0	0
DM	888	884	885
ME[3], kcal/kg	2850	2900	2950
Dig. Lys[3]	12.0	10.6	10.2
Ca	8.8	6.8	6.8
P	5.7	4.6	4.6
CP	247	227	225
Crude fat	80	73	94
DM	885	881	882
Ca	8.2	6.5	6.7
P	5.2	4.3	4.6

[1]Supplied per kg diet: thiamine, 1.0 mg; riboflavin, 4.5 mg; niacinamide, 40 mg; D-pantothenic acid, 9 mg; pyridoxine-HCL, 2.7 mg; choline chloride, 500 mg; cyanocobalamin, 20 µg; vitamin E (DL-α-tocopherol), 33 IU; menadione, 2.3 mg; vitamin A (retinyl-acetate), 12,000 IU; cholecalciferol, 5,000 IU; biotin, 100 µg; folic acid, 0.5 mg; FeSO4·H2 O, 150 mg; MnO2, 100 mg; CuSO4·5H2 O, 40 mg; ZnSo4·H2 O, 145 mg; Na2 SeO3, 0.56 mg; KI, 2.0 mg; antioxidant (oxytrap PXN), 125 mg.

[2]Supplied per kg diet: vitamin A (retinyl-acetate), 10,000 IU; vitamin D3 (cholecalciferol), 2,000 IU; vitamin E (DL-α-tocopherol), 20 mg; vitamin K3 (menadione), 2.3 mg; vitamin B1 (thiamine), 0.8 mg; vitamin B2 (riboflavin), 4.5 mg; vitamin B6 (pyridoxine-HCL), 1.9 mg; vitamin B12 (cyanocobalamine), 20 µg; niacine, 30 mg; D-pantothenic acid, 8 mg; choline chloride, 400 mg; folic acid, 0.5 mg; biotin, 50 µg; FeSO4.H2O, 150 mg; CuSO4.5H2O, 40 mg; MnO, 100 mg; ZnSo4.H2O, 145 mg; KI, 1.9 mg; Na2SeO3, 0.50 mg; antioxidant (oxytrap PXN), 125 mg
[3] calculated according to CVB (2018).

Table S2. Ingredient (g/kg) and nutritional composition of experimental diets during the broiler breeder phase.

	Control and LPS	**β-glucan**
Maize	405.0	404.5
Wheat	200.0	200.0
Soybean meal	129.7	129.7
Wheat middlings	100.0	100.0
Sunflowerseed meal	50.0	50.0
Soybean oil	22.3	22.3
Limestone	76.3	76.3
Sodiumbicarbonate	2.57	2.57
Salt	2.27	2.27
Monocalciumphosphate	0.40	0.40
DL-Methionine	1.19	1.19
L-Threonine	0.17	0.17
Hostazym X 15000	0.10	0.10
Phyzyme XP 10000 TPT - 500 FTU	0.05	0.05
Premix[1]	10.0	10.0
β-glucan (MacroGard)	-	0.50
CP	157.0	157.0
Crude fat	44.0	44.0
Ash	105.0	105.0
DM	889.0	889.0
ME[2], kcal/kg	2850.0	2850.0
Dig. Lys[2]	5.7	5.7
Ca	30.0	30.0
P	4.2	4.2
CP	157.4	157.4
Crude fat	44.4	44.4
DM	889.4	889.4
Ca	30.0	30.0
P	4.2	4.2

[1]Supplied per kg diet: Vitamin A (retinyl-acetate), 12,500 IU; vitamin D3 (cholecalciferol), 2,500 IU; vitamin E (DL-α-tocopherol), 80 mg; vitamin K3 (menadione), 2.5 mg; vitamin B1 (thiamine), 2.0 mg; vitamin B2 (riboflavin), 9.0 mg; vitamin B6 (pyridoxine-HCL), 4.5 mg; vitamin B12 (cyanocobalamine), 30 µg; niacine, 40 mg; D-pantothenic acid, 12 mg; choline chloride, 600 mg; folic acid, 1.5 mg; biotin, 150 µg; FeSO4.H2O, 135 mg; CuSO4.5H2O, 40 mg; MnO, 130 mg; ZnSo4.H2O, 206 mg; KJ, 2.6 mg; Na2SeO3, 0.9 mg.
[2] calculated according to CVB (2018).

Materials & Methods

Animals, Housing and Management

Broiler breeder phase (1)
Diets were formulated based on digestibility and nutrient data provided by CVB (CVB. 2018b) in Bestmix (version 3.30.93). Diet composition was based on broiler breeder guide recommendations (version 2017, Aviagen-EPI, Roermond, The Netherlands) for the starter phase up to 5% lay and for the peak phase (5% lay to end of the trial). Feed was produced in mash form by Research Diet Services (Wijk bij Duurstede, the Netherlands). Target body weights were the same for all broiler breeders and followed breed recommendations (version 2017, Aviagen-EPI, Roermond, The Netherlands).

Egg incubation phase (2)
The eggs were stored at 20°C when stored for 3 days or 15°C when stored up to 6 days, room humidity was maintained between 70 and 80%. After 6 days, eggs were incubated at 37.5°C and 55% humidity using two incubators (NatureForm Inc., Jacksonville, FL). The 15 eggs per pen were distributed over 18 trays, with in each tray 6 blocks (collection day) with 15 eggs each. Broiler breeder pens were randomly allocated within blocks. After the setter period (d0 to d17 of incubation), eggs were transferred to baskets, based on the blocking and tray structure in the setter. From d19 to d21 of incubation, temperature was decreased to 36.7°C and 70.0% humidity.

Grow-out phase (3)
All treatments received the same diets, which were formulated based on digestibility and nutrient data provided by CVB (CVB. 2018b) in Bestmix (version 3.30.93). Diet composition was based on CVB recommendations for broilers: maximum growth (CVB. 2018a) and fed in three phases: starter (d0 to d7), grower (d7 to d21) and finisher (d21 to 35) (**Table S1**). All diets were produced in pelleted form (2.5 mm starter and grower, 3.0 mm finisher) by Research Diet Services (the Netherlands).

Performance Data Collection

Egg incubation phase (2)
Chick quality was assessed using part of the score as described by Tona et al. (2003). Activity was scored 0 (good) or 6 (weak), down and appearance was scored 10 (clean and dry), 8 (wet) or 0 (dirty and wet) and the navel was scored 12 (closed and clean), 6 (not completely closed but not discolored) or 0 (not closed and discolored). The total score was calculated by adding the individual scores per chick. Subsequently, the same chicks were weighed, killed by cervical dislocation and dissected to measure residual yolk weight and calculate yolk free body mass (YFBM). Yolk weight as percentage of live body weight was calculated.

Immunological Data Collection

Alamar blue assay

Alamar blue (AB) is a non-toxic cell-permeable solution and was added to the cells directly after the collection of the culture media for the NO assay. Stock solution was made by dissolving 1 g of Resazurin Sodium Salt (BioReagent, R7017, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) in 100 mL sterile PBS (Gibco) followed by sterile filtering through a 0.22 µm pore size syringe filter (Acrodisc, Pall Laboratory Corporation, New York, NY, USA) and stored at -20°C protected from light until further use. A 250x dilution in pre-warmed culture medium was used for the analysis with a total end volume of 100 µl per well. Cells with AB solution were incubated at 41°C in 5% CO_2 and 95% humidity for 4 hours protected from light. Reduction was quantified by measuring the optical density at 570 nm and 600 nm with a spectrophotometer at 0 h and 4 h (Multiscan™, Thermo Fisher Scientific, Waltham, MA, USA). Percentage of reduced AB solution for both time point was calculated using the following formula, based on AB assay manual (Biosource version PI-DAL1025/1100Rev 1.0, Invitrogen, Carlsbad, California, USA):

$$percentage\ reduced = \frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 \ - \ (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{RED})\lambda_1 A'\lambda_2 \ - \ (\varepsilon_{RED})\lambda_2 A'\lambda_1}$$

λ_1 = 570 nm λ_2 = 600 nm

($\varepsilon_{OX)}$ λ_2 = molar extinction coefficient of AlamarBlue oxidized form 600 nm

($\varepsilon_{OX)}$ λ_1 = molar extinction coefficient of AlamarBlue oxidized form 570 nm

($\varepsilon_{RED)}$ λ_2 = molar extinction coefficient of AlamarBlue reduced form 600 nm

($\varepsilon_{RED)}$ λ_2 = molar extinction coefficient of AlamarBlue reduced form 570 nm

A λ_2 = absorbance of test wells 600 nm

A λ_1 = absorbance of test wells 570 nm

A λ_2' = absorbance of negative control well 600 nm

A λ_1' = absorbance of negative control well 570 nm

Results

Table S3. Effects of maternal treatment on egg and chick quality.

Variable	Control	LPS	β-glucan	SEM	P-Value	Average
Egg Weight Start, g	62.45	62.10	62.66	0.38	0.53	62.41
Chick Weight d0, g	45.22	45.34	45.41	0.40	0.93	45.32
YFBM, %	39.54	39.52	39.71	0.34	0.87	39.59
Relative Yolk Weight, %	12.35	12.50	11.66	0.36	0.18	12.17
Tona Score [1]	17.49	17.28	17.40	15.17	0.55	17.39
Fertility [2]	0.981	0.972	0.983	0.007	0.401	0.979
Mortality [2]	0.043	0.030	0.045	0.009	0.415	0.040
Hatchability [2]	0.932	0.921	0.924	0.012	0.763	0.926

[1] Box-Cox transformation was applied to indicated variables owing to their non-normal distribution. Reported LSMeans and Average have been back-transformed for ease of interpretation

[2]Probability of a lower score

Table S4. Broiler breeder phase (1): Relative mRNA expression (Age=week 36)

		Gene iNOS				Gene il1β			
Variable	**Category**	**n**	**LSMeans**	**SEM**	**P-value**	**n**	**LSMeans**	**SEM**	**P-value**
Treatment	Control	23	0.027	0.003	0.4055	23	0.07	0.01	0.6999
	LPS	22	0.028	0.003		22	0.08	0.01	
	β glucan	23	0.031	0.003		23	0.07	0.01	
Ex vivo LPS stimulation	0	35	0.027	0.002	0.3275	35	0.04	0.01	<0.0001
	10	33	0.030	0.002		33	0.11	0.01	
R-square model		0.04				0.43			

Table S5. Broiler breeder phase (1): Nitric oxide production (Age=week 36)

Variable	Category	n	LSMeans	SEM	P-value
Treatment	Control	48	1.94[b]	0.15	0.0117
	LPS	48	1.68[b]	0.15	
	β glucan	48	2.31[a]	0.15	
Ex vivo LPS stimulation	0	36	0.59[a]	0.12	<0.0001
	1	36	1.19[b]	0.12	
	10	36	1.33[b]	0.12	
	10+0.1 IFNy	36	4.82[c]	0.12	
R-square model		0.74			

a,b,c: different letters $P < 0.05$

Table S6. Grow-out phase (3): NE challenge lesion scores in percentage (Age=day 15)

E. maxima				C. perfringens			
	treatment				treatment		
Lesion score	**Control**	**LPS**	**β-glucan**	**Lesion score**	**Control**	**LPS**	**β-glucan**
0	75.00	8.33	50.00	0	75.00	66.67	100.00
1+2+3	25.00[a]	91.67[b]	50.00[a]	1+2+3	25.00[a]	33.33[a]	0.00[a]
Total	**100.0**	**100.0**	**100.0**	**Total**	**100.0**	**100.0**	**100.0**
0	75.00	8.33	50.00	0	75.00	66.67	100.00
1	8.33	33.33	41.67	1	25.00	33.33	0.00
2	0.00	58.33	8.33	2	0.00	0.00	0.00
3	16.67	0.00	0.00	3	0.00	0.00	0.00
Total	**100.0**	**100.0**	**100.0**	**Total**	**100.0**	**100.0**	**100.0**

Table S7. Grow-out phase (3): Relative mRNA expression (Age=day 21)

		Gene iNOS				Gene il1B			
Variable	**Category**	**n**	**LSMeans**	**SEM**	**P-value**	**n**	**LSMeans**	**SEM**	**P-value**
Treatment	Control	23	0.044	0.009		20	0.41	0.06	0.0842
	LPS	19	0.038	0.010		18	0.24	0.06	
	β-glucan	19	0.040	0.010		16	0.26	0.07	
Ex vivo LPS stimulation	0	28	0.014	0.008	<0.0001	21	0.01	0.06	<0.0001
	10	33	0.068	0.008		33	0.60	0.05	
R-square model		0.30				0.59			

References

CVB. 2018a. Tabellenboek veevoeding pluimvee.
CVB. 2018b. Veevoedertabel.

CHAPTER 6

6

General Discussion

Introduction

The main research goal of this thesis was to study trained innate immunity and transgenerational effects in chickens. In my thesis I tried to answer the following research questions:

1. Does trained innate immunity exist in chickens?

2. Can transgenerational effects be induced by the innate immune system in chickens and is trained immunity involved in this process?

The results described in this thesis showed evidence for trained innate immunity and transgenerational effects in chickens. Maternal immune activation with LPS resulted in higher levels of HuSA specific antibodies in the offspring. Furthermore, maternal immune activation with LPS inhibited the stimulating effect of dietary β-glucan on the anti-HuSA antibody response. These results showed that stimulation of the maternal innate immune system with LPS resulted in influenced antibody response in the offspring. Based on these results, I hypothesized the presence of transgenerational effects where a non-specific innate stimulation with LPS has induced a memory-like function against HuSA specific antibody responses in the offspring. i.e., one innate antigenic stimulus can lead to an amplified response against other unrelated stimuli. This aspecific response is known as one of the main consequences of trained innate immunity (**Chapter 2**). The observed transgenerational effects with LPS in this thesis also suggest the existence of a non-genetic memory-like mechanism,

Trained innate immunity is characterized by an immuno-epigeno-metabolic crosstalk of cellular pathways in monocytes, macrophages and NK cells, which involve the following core mechanisms: 1) "immune adaptations", 2) "metabolic reprogramming" and 3) "epigenetic DNA modifications" (**Figure 3, general introduction**). Indeed, within this thesis, I was able to demonstrate trained innate immunity in primary chicken monocytes based on readouts specific for immune adaptations (e.g. IL1-β, MHCII, CD40, iNOS, NO) and metabolic reprogramming (HIF-1α, PPARγ, lactate) (**Chapter 3 and 4**). Furthermore, I showed that maternal stimulation with LPS and β-glucan resulted in decreased gene expression levels of IL-1β in blood-derived monocytes in the offspring, suggesting a reduced pro-inflammatory phenotype. This effect indicated again transgenerational effects of the innate immune system (**Chapter 5**). Next to that, maternal stimulation with LPS resulted in an improved feed efficiency in the offspring in the first week after a *necrotic enteritis* pathogenic challenge (**Chapter 5**). Altogether, I found evidence for trained innate immunity in chickens as well as modulating effects on innate immunity in a transgenerational manner (**Chapter 2, 3, 4 and 5**).

In this general discussion I will discuss the findings within this thesis, formulate new knowledge gaps and propose directions for future research. Also, the impact of my research on the poultry husbandry sector will be discussed including possible contributions to disease resistance, optimal performance and increased welfare by changing animal husbandry measures like feeding and vaccination strategies.

Trained innate immunity in chicken

Trained innate immunity in chickens was investigated using an *in vitro* cell model, which I developed and utilized in all studies on trained innate immunity in this thesis (**Chapter 3**). In this model I demonstrated trained innate immunity based on parameters associated with immune function (IL1-β, MHCII, CD40, iNOS, NO) and metabolism (HIF-1α, PPARγ, lactate). The measured immune parameters can primarily be assigned specific to trained innate immunity core mechanism 1) "immune adaptations" and to core mechanism 2) "metabolic reprogramming" (**Figure 3, general introduction**). Crosstalk between these core mechanisms exists via cellular pathways. Indeed, I showed stimulating effects on parameters that are correlated with the immune adaptive mechanisms and metabolic reprogramming. Trained macrophages, showed an increase in mRNA levels of the pro-inflammatory parameters, including IL1-β, but also affected the metabolic associated parameters, including Hypoxia-inducible factor 1-alpha (HIF-1α). However, a functional correlation between these parameters needs to be established. Since I did not perform measurements on epigenetic DNA modifications in my studies, direct evidence for a role of core mechanism 3) "epigenetic DNA modifications" on trained innate immunity in chicken is not present yet. Nevertheless, based on analogy of trained innate immunity in mammalian species I expect a role for epigenetic programming in chickens as well. In mammals, adaptations on immunity and cellular metabolism are essential to induce trained innate immunity, including epigenetic DNA modifications (e.g. Histone modification and DNA methylation), whereby both IL-1β and HIF-1α play a pivotal role [1-3]. Indeed, within this thesis, both IL-1β and HIF-1α gene expression levels were elevated under training conditions with β-glucan+IL-4 after a secondary LPS stimulation (**Chapter 3 and 4**). HIF-1α acts as a master transcription factor not only controlling inflammatory responses, but also metabolic reprogramming [1, 2, 4-6]. I found increased HIF-1α gene expression levels in trained macrophages. This suggests that the Dectin-1–Akt–mTOR–HIF-1α signaling pathway is involved in trained innate immunity in chickens (**Figure 6, general introduction**). An intensive BLAST search in the most recent *Gallus gallus* genome database (GRCg6a:build GCF_000002315.6) by myself did not result in the identification of a dectin-1 homologue in chickens. However, stimulation of chicken heterophils and peripheral blood mononuclear cells (PBMCs) with β-1,3-glucan curdlan, a potent agonist of human and murine dectin-1 receptors, led to an oxidative burst [7]. These results indicate the existence of a functional dectin-1 homologue in chicken immune cells. In mammals, the Dectin-1–Akt–mTOR–HIF-1α pathway plays a role in the interplay between epigenetics and metabolism. Therefore, a similar interplay may also be present in chicken, because gene expression levels of HIF-1α was also affected upon innate training by β-glucan.

In mammals trained macrophages shift to aerobic glycolysis [1] (**Figure 6, general introduction**). mTORC1 drives HIF-1α protein accumulation through enhanced transcription of HIF-1α mRNA and decreased rate of proteolytic degradation of HIF-1α protein.. Activation of HIF-1α under circumstances of aerobic glycolysis stimulate the conversion of pyruvate into lactate and several turn-over metabolites of the TCA cycle such as fumarate, citrate and consequently acetyl-CoA [8] (**Figure 1**). Fumarate and acetyl-CoA are associated with histone methylation and histone acetylation of genes associated with

trained innate immunity. Acetyl-CoA is the major substrate for histone acetyltransferases and fumarate an indirect inhibitor (via KDM5 family) of histone demethylases [9, 10]. In mammals, binding of β-glucan to dectin-1 triggers a calcium influx in the cell resulting in an increased accessibility of DNA for transcription factors and as a consequence enhanced transcription of genes after a secondary stimulus, with MAMP such as LPS [8]. Netea et al. found increased expression of inflammatory associated genes, leading to production of NO, reactive oxygen species (ROS) and expression of pro-inflammatory cytokines (e.g. IL-1β) [8]. Within this thesis I also used β-glucan as a primary stimulus and LPS as a secondary stimulus and I also observed enhanced NO production and increased gene expression levels of IL-β, suggesting that trained innate immunity is very similar in chickens compared to mammals.

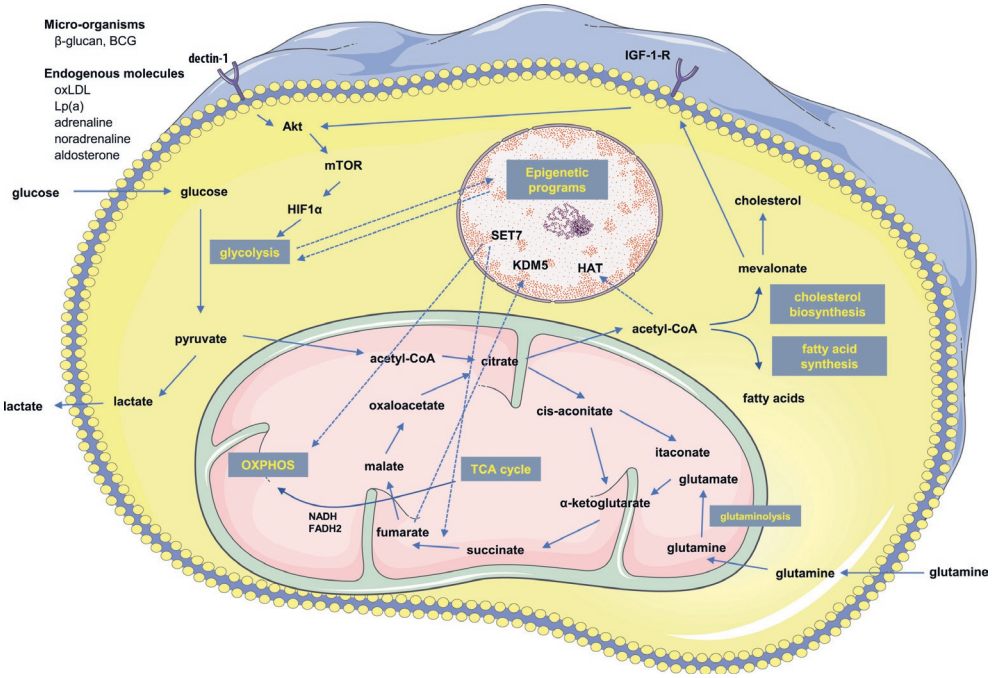

Figure 1 The role of Dectin-1–Akt–mTOR–HIF-1α pathway on the interplay between epigenetics and metabolism in immune cells. mTORC1 drives HIF-1α protein accumulation through enhanced transcription of HIF-1α mRNA and decreased rate of proteolytic degradation of HIF-1α protein. Under aerobic glycolysis conditions, HIF-1α stimulates the conversion of pyruvate into lactate and the increase in several metabolites of the TCA cycle, including fumarate, citrate and consequently acetyl-CoA. Fumarate and acetyl-CoA are associated with histone methylation and histone acetylation of genes associated with trained innate immunity. IGF1 receptor plays, besides dectin-1 receptor, also a role in the activation of the Akt-mTOR- HIF-1α pathway. Reprinted by permission from Elsevier and Creative Commons:Elsevier, Molecular Aspects of Medicine, [9], CC Copyright©, 2022.

Macrophages are apt to infiltrate hypoxic tissues whereby their metabolic program switches to glycolysis, probably under the tight control of HIF-1α [11]. Since hypoxic circumstances are correlated with tissue injury and concequently inflammation, it is

not suprising that trained macrophages, with a higher expression level of HIF-1α, have improved phagocyte and killing capacities and beeing more effective at the site of inflammation [12]. I hypothesize that trained macrophages are not only more effective in killing of invaded pathogens, but that trained macrophages are also faster in recruitment to the site of injury because of the improved activation of HIF-1α. I hypothesize that faster recruitment of trained macrophages will result in a faster inflammatory response. However, currently no evidence is availabe to support this hypothesis. It would be interesting to investigate wheter the number of activated macrophages in tissue under training conditions is also enhanced, whereby not only the activated state of the macrophage but also the number of recruited macrophages is increased.

I reported similarities in pro-inflammatory and metabolic associated parameters between chickens and mammals of trained macrophages. However, I also found differences between chickens and mammals. For instance, I was not able to demonstrate increased lactate production in β-glucan-trained chicken macrophages, as reported for their mammalian and teleost counterparts [1, 13, 14]. Trained macrophages shift to an aerobic glycolysis state and this increased lactate production was associated with an upregulation of glycolysis, controlled by acetyl-CoA [1]. It is unclear why this difference between mammals and chicken in lactate production exists. A possible explanation can be that trained macrophages in mammals have an increased aerobic glycolysis through the consumption of extracellular glycose. Besides glycolysis also the lipogenesis is an important route within macrophages that is affected by trained innate immunity [8, 15-17] (**Figure 2**). During aerobic glycolysis, acetyl-CoA is consumed in the TCA cycle for lactate production but act also as an important precursor for the biosynthesis of cholesterol, fatty acids and triacylglycerol. Indeed, as acetyl-CoA comes from pyruvate conversion whereby acetyl-CoA acts as an important catalysator of lipogenesis, trained macrophages display an enhanced ability to accumulate cholesterol and oxidize LDL internally [18]. It is possible to speculated that chicken macrophages do favor acetyl-CoA controlled accumulation during lipogenesis above the consumption acetyl-CoA for lactate production. This may explain the observed difference in lactate production between trained macrophages from chickens, mammals and fish. The preference for lipogenesis may be genetically regulated. However, it could also be dependent on availability of extracellular glucose or lipids. Although glucose is a component of the RPMI 1640 cell culture media (11.11 mM D-Glucose, Life Technologies Ltd., UK) it is possible that glucose was limited at a certain moment during cell culture which affected the interplay between glycolysis and lipogenesis. Lipids are provided via fetal calf serum in the culture media and therefore available in the cell culture during the *in vitro* experiments.

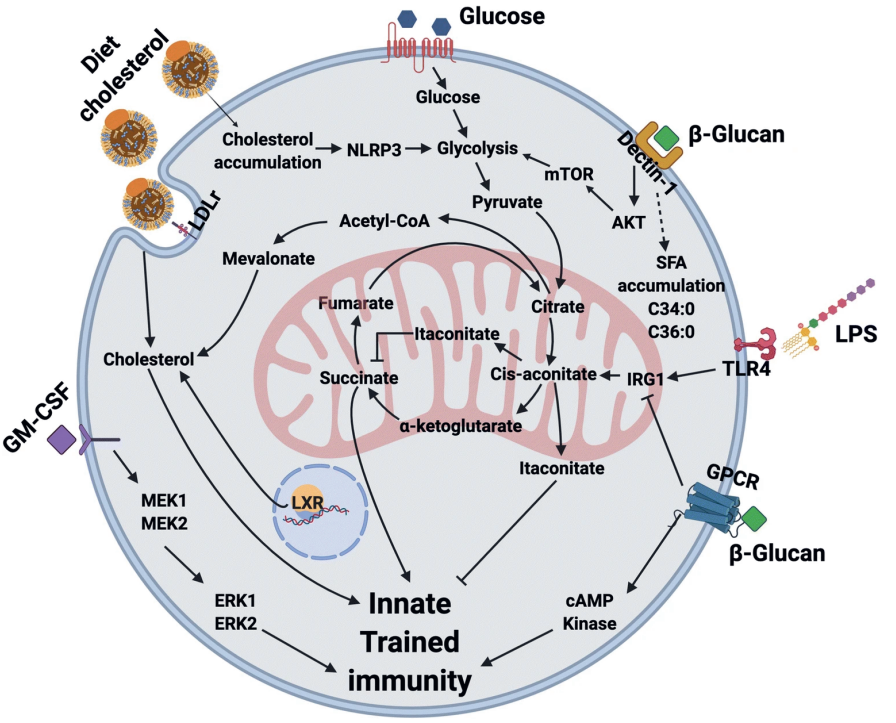

Figure 2 The interplay between glycolysis and lipogenesis is drastically affected by trained innate immunity in macrophages. Besides lactate production, acetyl-CoA is also responsible for the biosynthesis of cholesterol. Acetyl-CoA is a product of pyruvate conversion whereby acetyl-CoA act as an important catalysator of lipogenesis and correlated cholesterol metabolism. Reprinted by permission from Springer Nature and Copyright Clearance Center:Springer Nature, Molecular Neurobiology, [19], CCC Copyright©, 2022.

Also IL-4 may have played an important role in the observed absence of lactate accumulation during trained immunity in chickens. I found evidence of trained innate immunity in chicken macrophages by using β-glucan together with IL-4. Cytokine IL-4 is able to polarize monocytes towards a M2 phenotype (**Figure 2, general introduction**). In the literature it is described that the IL-4-STAT-6-PPARγ signaling axis (**Chapter 3**) correlates with the maturation M2 macrophages (alternatively activated macrophages) [20]. Interestingly, PPARγ is also associated with fatty acid accumulation and thereafter the production of Acetyl-CoA [21]. It is therefore possible that IL-4 triggered lipogenesis and induced together with β-glucan trained innate immunity in chicken cells. Another study reported a functional link between M2 macrophages ("immune adaptation"), lactate production ("metabolic reprogramming") and epigenetic modifications ("epigenetic DNA modifications") [22]. These researchers demonstrated that IL-4 polarized M2 macrophages increased lactate utilization by converting lactate into pyruvate to generate enough Acetyl-CoA for histone acetylation–dependent gene expression when glucose is scarce. This may explain why I did not observe enhanced lactate accumulation in my

experiments and that, consequently, lipogenesis was enhanced as an alternative cellular route. Therefore, IL-4 may be a key player in the crosstalk between immune, epigenetic and metabolic pathways as a result of training in chicken monocytes.

I found increased gene expression levels of HIF-1α in trained macrophages. Noe et al., proposed a lactate-dependent histone acetylation in M2 macrophages under the control of HIF-1α [22]. It is described that enhanced histone acetylation increases accessibility of DNA and related transcription of pro-inflammatory genes by a secondary stimulus, such as LPS [8]. Indeed, within this thesis I have found that application of β-glucan together with IL-4 induces trained innate immunity in chicken macrophages based on an increased pro-inflammatory response. It is therefore interesting to hypothesize that IL-4 polarized anti-inflammatory macrophages (M2), shifts to a pro-inflammatory state after training with β-glucan, resulting in enhanced pro-inflammatory responses after a LPS stimulation. Namely, the switch of M2 macrophages from an anti- to a pro-inflammatory phenotype after a secondary LPS stimulation was also described in an earlier study [23], where it was suggested that this shift plays a role in the pathogenesis of atherosclerosis. Indeed, others also showed that the occurrence of atherosclerosis is related with training of macrophages [8, 17, 24, 25]. In people suffering from atherosclerosis, trained macrophages show enhanced lipogenesis, resulting in the formation of foam cells together with the known pro-inflammatory profile. The latter suggests that, in human, trained innate immunity may not always be beneficial for the host [8, 16]. In fact, atherosclerosis is also very relevant for poultry [26, 27]. Therefore, the insights of trained innate immunity could shed more light on the research of atherosclerosis in poultry. Furthermore, more knowledge on lipid deposition, energy usage and inflammatory effects is relevant because these aspects will have effects on performance and production of meat and eggs. In fact, within the poultry industry, abdominal fat deposition is an undesirable factor [28]. More knowledge on the mechanism around lipogenesis is of high value to improve meat quality of broiler chickens. One of the parameters to investigate is mevalonate. In human, the mevalonate pathway is important in the induction of trained innate immunity, lipogenesis and cholesterol synthesis and the increased expression of IGF1 receptor [9, 29](**Figure 1**). Triggering of the IGF1 receptor activates, in addition to the dectin-1 receptor, also the Akt-mTOR- HIF-1α pathway.

Altogether, I conclude that trained innate immunity in monocytes and macrophages shows a lot of plasticity and variability on different cellular signaling routes. Variation between species (e.g. chicken, mammals, fish) or between breeds (e.g. layer, broiler) may contribute to more insight in the cellular and molecular pathways involved in trained innate immunity. Understanding *how* and *why* differences exists between trained macrophages can add to understanding the mechanism behind trained innate immunity in chicken. To shed more light on this matter, I set out to study trained innate immunity in layer and broiler monocytes to compare the robustness and/or variation of the trained innate immunity concept in different poultry breeds (**Chapter 4**).

Trained innate immunity in phenotypically different chickens: Layers vs broilers

Within this general discussion I stated that a polarization of macrophages towards anti-inflammatory phenotype (M2) with IL-4, likely shifts to a pro-inflammatory state after training with β-glucan. Interestingly, broilers have been reported to display a reduced production of pro-inflammatory cytokines and increased production of immunosuppressive cytokines compared to laying hens, resulting in a reduced inflammatory response [30]. Furthermore, effector functions of broiler macrophages like phagocytosis and killing of bacteria and tumor cells, are variable in different broiler lines [31]. Also variations in gene expression levels of iNOS and NO production have been reported between different genetic chicken lines [31-33]. Next to that, broilers and layers differed in antibody responses and expression of immune-related genes in the ileum [34-36]. Since broilers are genetically selected for development of meat production, and layers are selected for egg production, energy usage of broilers and layers is different [37]. Based on these immunological and metabolic differences between broilers and layers I expected to find differences between layers and broilers in the immuno-epigeno-metabolic crosstalk and, consequently, trained innate immunity. The *in vitro* studies in this thesis showed that IL-4 may play a role in initiating trained innate immunity. It has been described that IL-4 regulated polarization of macrophages results in an anti-inflammatory phenotype (M2) [38] (**Figure 2, general introduction**). Therefore, I hypothesize that broiler macrophages are more susceptible to train, because these macrophages are already more anti-inflammatory orientated as described above. I observed differences in surface expression of MHC-II between macrophages from layers and broilers (**Chapter 4, figure 5**). Surface expression of MHCII was reduced after the primary LPS stimulation in layer chickens, but increased after a second LPS stimulation. In broilers, however, the MHCII expression levels remained the same after the primary LPS stimulation, but decreased after a second LPS stimulation. This indicates possible variation in the induction and mechanism of trained innate immunity and/or tolerance in different chicken breeds.

Tolerance and trained innate immunity

In the trained innate immunity chicken model developed and used in this thesis, primary stimulation with β-glucans was used to induce activation (trained innate immunity) of the monocyte and primary stimulation with LPS was used to induce tolerance. Indeed, I found *in vitro* evidence of trained innate immunity upon stimulation with β-glucan, however, I did not find any evidence for tolerance upon stimulation with LPS. For instance, IL-1β levels, an important pro-inflammatory parameter, were not reduced after a second stimulation with LPS compared to the control. The fact that we did not find evidence of LPS induced tolerance in the current study is in contrast with previous *in vivo* studies in birds and in mammalian species [39, 40] and with previous *in vitro* studies in mammals [41, 42].

A possible explanation for the differences on tolerance between my chicken study and studies with mammals could be explained by the involvement of epigenetic mechanisms.

Trained innate immunity and tolerance go side by side with respect to macrophage plasticity and variation. It was, for instance, described that there is a dynamic interaction between LPS and β-glucan by inducing and damping tolerance [42]. LPS plays also an important role within trained innate immunity, tolerance and related epigenetic effects by DNA epigenetic changes of the H3K27ac and H3K4me markers [8, 43]. A possible explanation for the differences in tolerance induction in my works compared to studies on mammalian cells can be the regulation of noncoding RNAs [44]. Namely, tolerance is controlled by a variety of TLR ligands, cytokines, and soluble mediators via the regulation of non-coding RNAs (ncRNAs). Interesting, long ncRNAs are found to establish and maintain epigenetic modifications and therefore also described as an important mechanistical link in trained immunity [45]. Research on ncRNAs goes beyond the scope of this thesis, but is highly recommended for further investigation of trained innate immunity in chickens.

Another explanation for the absence of tolerance within this thesis can be the role of T-cells. Most of the research described in literature on tolerance was performed *in vivo*. In these *in vivo* studies not only macrophages but also T cells are involved in the induction of tolerance. It was previously proposed that, in chickens, activated Th2 cells and possible also Tregs may initiate the tolerogenic state of the macrophages [46]. This is very different form the *in vitro* stimulation with purified monocytes performed in this thesis. On the other hand, tolerance has been found in *in vitro* experiments in absence of T cells with human/mouse isolated macrophages [41, 42]. This difference between the studies with human and mouse macrophages and my work may be related to possible differences in the receptor binding affinity of TLR4 for LPS, or more downstream cellular pathways. It is described that the activation of TLR4 and the dectin-1 receptor by LPS or β-glucan respectively share crosstalk [47]. Furthermore, within this thesis I investigated gene expression levels of HIF-1α and consequently the possible dectin-1–Akt–mTOR–HIF-1α pathway. In literature it is described that parallel with the dectin-1–Akt–mTOR–HIF-1α pathway a second Dectin-1–Syk–CARD9–TRAF6–NFκB pathway was found in mammals [48] and carp [49]. In here, the LPS and β-glucan share TRAF6-NFκB in their downstream cellular pathways, as part of the MyD88 dependent pathway, and possible have inhibiting or stimulating effects among each other. Furthermore, a third Dectin-1–Raf-1–NFκB pathway might also have similar crosstalk effects [50]. Inhere, the Raf-1 pathway integrates with the Syk pathway at NFκB level.

The described pathways shows a crosstalk of TLR4, Dectin-1 and other related signaling cellular components. This crosstalk may result in either training, tolerance or reversed tolerance even in the next generation [8, 42, 51]. Factors that may differ between tolerance and training can be age, time of stimulation and dosage of the used components (e.g. β-glucan and LPS) [52, 53]. Although immune stimulation can induce training, stimulation with a higher dose of the same stimulating compound can also induce tolerance [51]. Bauer et al. proposed an antagonistic functioning effect on immune response with mTOR and AMP-activated protein kinase (AMPK), whereby mTOR via HIF-1α will induce training, while AMPK through deacetylase SIRT1 activation will induce tolerance (**Figure 3**). A pathogenic or stimulating component overdose will possibly result in damage and death of the cells. More research on the dose-dependency of trained innate immunity and/or tolerance would be of great value in unraveling its mechanism.

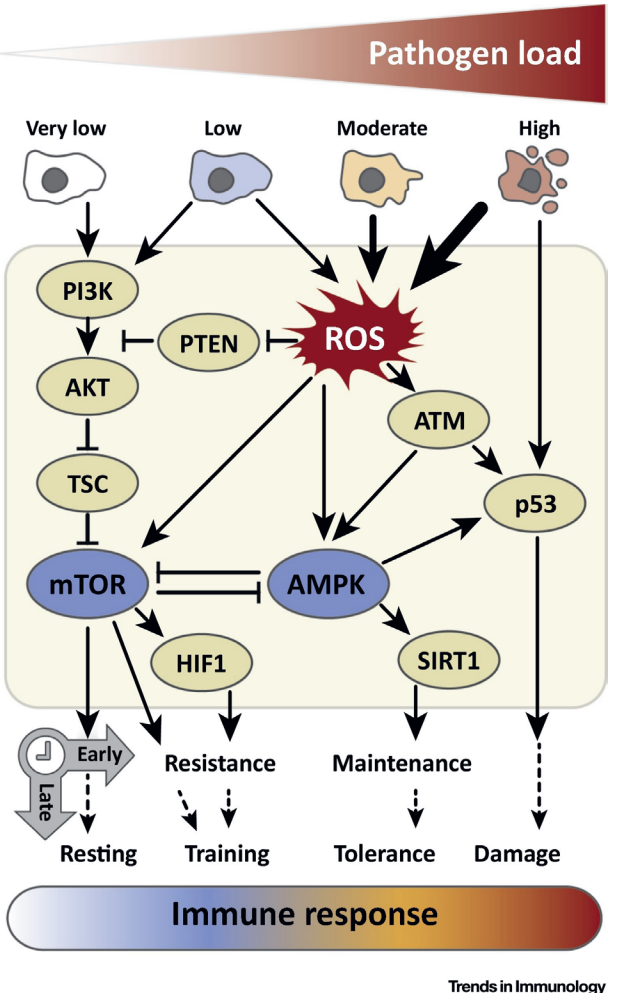

Figure 3 Proposed cellular pathways in macrophages involved in pathogen load response. Pathogen load or antigen dose can induce training (low dose), tolerance (moderate dose) or damage (high dose). Here, mTOR via HIF-1α will induce training, while AMPK via SIRT1, a deacetylase, will induce tolerance. Reprinted by permission from Elsevier and Copyright Clearance Center:Elsevier, Trends in Immunology, [51], CCC Copyright©, 2022.

Transgenerational effects and trained immunity

Transgenerational effects

The importance of transgenerational effects has not been recognized for a long time, but it now becomes more clear that transgenerational effects have long lasting influences on the whole physiology of an organism [54-56]. Based on the results described in **chapter 2 and 5** it can be concluded that maternal immune activation with LPS has influenced the immune system of the offspring. In **chapter 2**, maternal immune activation with LPS increased HuSA-specific IgY responses (**experiment 1**). Furthermore, maternal immune activation with LPS inhibited the stimulating effect of dietary β-glucan on the specific IgY anti-HuSA response (**experiment 2**). Unfortunately, the results of experiment 1 and 2 were not completely consistent, which suggests that other unknown factors also play a role in these transgenerational effects (**Chapter 2**). Nevertheless, both experiments showed transgenerational effects on immune responses by maternal LPS activation. In **chapter 5**, I described that maternal immune activation with LPS resulted in an increased G:F ratio in the offspring in the first week after the *C. perfringens* challenge. Simultaneously, in the same treated group (LPS), a higher number of broiler chickens showed intestinal lesions caused by *E. maxima* compared to the control group (**Chapter 5**). Furthermore, IL-1β mRNA levels in *ex vivo* stimulated monocytes from offspring of LPS and β-glucan-treated broiler breeder hens tended to be lower compared to the control group. This reduced capacity to produce Il-1β may indicate a transgenerational tolerogenic effect.

Considering the results of these transgenerational studies, it seems that LPS treatment results in more pronounced transgenerational effects compared to a treatment with β-glucan. Two important questions concerning *how* transgenerational effects can be induced remain: 1) Is it possible to explain *how* LPS or β-glucan maternal immunization influenced the immune system in the offspring?, and 2) Is it possible to explain *how* LPS maternal immunization influenced antibody responses against HuSA in the offspring? In chicken, it has been described that non-genetic memory like effects are transferable to the next generation via, for example, components in the egg [57] or via epigenetic inheritance in the fertilized zygote [58]. The observed transgenerational effects with LPS in this thesis also suggest the existence of a non-genetic memory-like mechanism, i.e., one innate antigenic stimulus can lead to an amplified response against other unrelated stimuli. Based on my findings, however, I cannot completely rule out, that LPS or LPS specific maternal antibodies were transferred via the egg. However, it is very likely that LPS has been enzymatic degraded and removed out of the chicken's body before fertilization and egg collection took place. In mice it has been found that LPS has a half-life of 3–4min in the blood and that enhanced innate proinflammatory responses were back to normal within 4 h [59, 60]. Also a study with chickens show that the enhancing effect of LPS administration in the trachea on TLR4 expression were back to original state within 72 h [61]. I collected the fertilized eggs 3–5 weeks after maternal immune activation, which makes it unlikely that LPS was still present in the yolk. With respect to the specific antibodies, transgenerational studies with pied flycatchers and domesticated zebra finches describe that maternal LPS immunization increased specific LPS antibody levels in offspring [62-64]. However, the level of LPS specific maternal antibodies in the offspring is likely negligible, because no differences in LPS antibody titers were observed

in the offspring at day 0 (data not shown). Therefore, the transgenerational effects of LPS within hatchlings that received β-glucan enriched feed, cannot be explained by LPS specific maternal antibodies. The report on transgenerational studies with the pied flycatchers [62] suggest that the maternal antibodies are not the only explanatory factor for the observed transgenerational effects but no other transgenerational mechanisms is described. Therefore, I postulate that the second route, i.e. via epigenetic inheritance, is the explanatory mechanism of the transgenerational LPS effect within respect the current thesis.

Transgenerational epigenetic inheritance

Transgenerational epigenetic effects are previously defined by Berghof et al. (2013) as: "Effects based on information 1) carried by a cell/individual, but 2) that is not encoded by changes in the nucleotide sequence of the DNA and 3) that is transferred to successive generation(s) 4) without the necessity of the original environmental stimulus."[54]. Another definition was by Wu and Morris (2001): "Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [65]. Interestingly, it was already suggested that transgenerational effects of LPS can be transferred by epigenetic DNA modifications to next generations [66, 67]. Evidence for the existence of epigenetic DNA modifications to next generations has indeed been observed in chickens [68] where zygotic gene activation in chicken was reported to occur in two waves. Interestingly, only maternal alleles and not paternal alleles are activated during the first wave and seem to be related to early cell division in embryogenesis. This indicates that solely maternal alleles are activated in the chicken zygote upon fertilization. Within the second wave, after the maternal-to-zygotic transition, both maternal and paternal genomes were involved in the expressed genes. The results of Hwang et al. [68] suggest that the maternally derived first wave is a moment for early development and epigenetic traits in chickens. Therefore, I hypothesize that components such as LPS that activate the maternal innate immune system influence the neonatal immune system in a transgenerational manner including epigenetic inheritance.

Transgenerational epigenetic inheritance of trained innate immunity

If epigenetic inheritance is possible within chickens, as described above, then trained innate immunity via epigenetic inheritance is also possible. However, in my research I did not study DNA modifications in the offspring or on the oocyte within the female chicken or the sperm of the male chicken. With respect to transgenerational epigenetic inheritance, epigenetic modifications are possible the only inheritable via the oocyte or sperm route to reach the next generation.

Within this thesis I did not investigate if LPS and β-glucan reached and affected the oocyte of the maternal hens to initiate trained innate immunity. The stimulating component β-glucan were administered as a feed additive and LPS intratracheally. The question is whether these components via the dietary or intratracheal route are able to cause epigenetic DNA modifications in oocytes of the maternal hens. In chicken, dietary β-glucan will indeed reach the blood via the intestinal tract [69]. Particulate β-glucan is primarily absorbed in the intestine by dendritic cells via Microfold cells (M-cells) [70, 71]. Fragmented β-glucan are subsequently excreted to the blood. Furthermore, macrophages

show the ability to take up antigens via gap junctions in the intestinal barrier [72]. Recent study in humans and mice, have shown that injected β-glucan is able to reach the bone marrow and subsequently initiates trained innate immunity [73]. With respect to LPS, a study with chickens shows that intratracheally applied beads were found back in large numbers in lungs, bursa, air sacs, humerus and radius within hours after administration [74-76], indicating that intratracheally administered antigens are easily absorbed. Taken together, it is likely that administration of β-glucan or LPS via the diet or airways are able to reach the gametocytes and/or hematopoietic stem cells in the bone marrow.

In **chapter 3 and 4** of this thesis I showed that blood-derived monocytes can be trained with β-glucan. Since mature myeloid cells are not transferred to the next generation, this route is probably not the mechanism that initiate transgenerational epigenetic effects in a chicken. Another route that is described to initiate trained innate immunity in macrophages is targeting the hematopoietic stem cells in the bone marrow [3, 8]. The route via the hematopoietic stem cells is a promising route to maintain and preserve the effects of trained innate immunity, but probably not for epigenetic inheritance. However, it is reasonable to expect that if epigenetic effects can be initiated in hematopoietic stem cells, it would also be possible to initiate epigenetic modulating effect on gametocytes, like the oocytes within female chickens and spermatogonium germ cells of male chickens. In chickens, the effect epigenetic inheritance via bone marrow on hematopoietic stem cells, oocytes and spermatogonium germ cells is not well investigated. Within this thesis I performed some experiments in which I investigated trained innate immunity in bone marrow-derived cells of layer chickens (**Chapter 3**). In these experiments I did not find evidence for trained innate immunity in chicken bone marrow-derived cells, which is different from previous studies with murine bone marrow cells [77]. In my studies, I measured NO production as read-out parameter and did not include analysis of the surface markers MHCII and CD40 or the gene expression levels of iNOS and pro-inflammatory cytokine IL-1β. Analysis of these parameters, including NO, was used to demonstrate trained innate immunity in blood-derived macrophages (**Chapter 3 and 4**). Based on my findings I cannot concluded that chicken bone marrow derived cells are not trainable. With other read-out parameters it may be possible to demonstrate *ex vivo* trained innate immunity in chicken bone marrow-derived cells.

A question that is still unanswered is: Is it possible to explain *how* LPS maternal immunization influenced antibody responses against HuSA in the offspring? (**Chapter 2**). I hypothesize that LPS may induce inheritable epigenetic changes that influenced the hematopoietic stem cells maturation of innate immune system in the offspring. Monocytes and macrophages, differentiated from hematopoietic stem cells, play an important role within antigen presentation, T cell and B cell interaction and finally the antigen specific antibodies (**Figure 1, general introduction**). Therefore, I hypothesize that inheritable epigenetic changes caused by maternal LPS immunization, influenced the antigen specific antibody production against HuSA but also the stimulating effect of β-glucan in the offspring (**Chapter 2**). Comparable with chapter 2, the results of chapter 5 could probably also be explained by epigenetic modifications that are introduced at the level of stem cell and subsequently also hematopoietic stem cells effecting the physiology, including growth performance and feed efficiency parameters, of the offspring.

In **chapter 5**, offspring of the LPS treated mother hens showed improved feed efficiency after a pathogenic challenge, whereas the same chicks showed more intestinal lesions. Normally, effects of immunological stress caused by pathogens is generally reflected in decreased growth performance parameters [78]. As proposed in chapter 5, it is tempting to speculate that the intestinal tract of the offspring of the LPS-treated broiler breeders had an improved and faster recovery and therefore a greater G:F ratio, while the recovery of the control group was slower. It would have been interesting to measure, beside the expression of pro-inflammatory cytokine IL-1β, also the expression of the anti-inflammatory cytokine Transforming growth factor beta (TGF-β). An interplay has been described in which downregulation of TGF-β is associated with an upregulation of IL-1β after a NE challenge [79]. This resulted in intestinal inflammatory immune responses and subclinical NE effects in the intestinal tract of chickens. Vice versa, intestinal recovery after a NE challenge is associated with upregulation of TGF-β production and downregulation of IL-1β by inducing Th2 and Th17 cytokine secretion [79-82]. Therefore, it would be interesting to investigate whether the recovery rate of the capacity of nutrient absorption in the offspring is faster by maternal LPS treatment of broilers in a future study. This faster recovery could be the result of a trained innate immune system caused by transgenerational epigenetic inheritance and, although minor, a first indication of enhanced disease resistance against the used pathogenic challenge. Modulating effects of the innate immune system were probably also found in **chapter 2** and suggest a similar mechanism.

The finding within this thesis that activation of the maternal innate immune system with LPS inhibited the enhancing effect of dietary β-glucan on HuSA specific antibody response in the offspring, suggest the important role of innate immune cells. The reason for the contradictory results between the two experiments (**Chapter 2**) is, however, still unclear. Whereas maternal immune activation with LPS resulted in an increased HuSA specific IgY titer in the first experiment, this effect was not found in the second experiment. The differences between these experiments shows that immune modulation is variable and indicate the plasticity of the mechanisms behind these modulations. Differences in animal age, β-glucan/LPS dosage, exposure time, β-glucan/LPS purity and β-glucan/LPS origin might be explanatory factors within these variabilities [83-87]. In **chapter 4**, for instance, it was found, based on NO production, that age and breed had enhancing or decreasing effects on the NO production and training effect. It was therefore concluded that age and breed are variables that can influence immune modulating capacity. Variability and plasticity of immune modulation, including trained innate immunity, are therefore important features to take into account when implementing trained innate immunity as a vaccine and/or feed strategy. What the optimal circumstances are awaits further research wherein this thesis provided valuable information and insights on this.

Knowledge gaps, future research and applications.

The research presented in this thesis, to my knowledge, studies for the first time trained innate immunity and non-genetic transgenerational effects of the innate immune system in chickens. The interest in the research fields of epigenetics, trained immunity

and transgenerational effects is, exponentially increasing. Especially in human, research focusses on *how* trained immunity can be used and *how* it could provide a novel view on health and disease [8, 88-90]. For future research within the avian immunology field, the poultry animal husbandry sector and veterinary vaccine development it is required to identify and understand the current knowledge gaps and potentials of trained innate immunity and transgenerational non-genetic inheritance.

Trained innate immunity *in vitro* model

During the development of the chicken trained innate immunity *in vitro* model, I focused first on inflammatory read-out parameters, such as IL-1β, MHCII, CD40, iNOS and NO. In time, the model was expanded with more read-out parameters on metabolism and key factors within the immuno-epigeno-metabolic crosstalk, like HIF1α and lactate. Parallel with the dectin-1–Akt–mTOR–HIF-1α pathway a second Dectin-1–Syk–CARD9–TRAF6–NFκB pathway was found in mammals [48] and carp [49]. Furthermore, the Dectin-1–Raf-1–NFκB pathway might also have similar training effects [41]. Different cellular pathways related to trained innate immunity are worthwhile to investigate. However, the 'toolbox' available for immunological parameters in chickens is limited, compared to human and mice. Within the current thesis I was mainly restricted to gene expression levels of anti- and pro-inflammatory cytokines. Although gene expression provides valuable information about involved signalling routes, it not necessarily means that transcribed genes are also translated in functional proteins, such as cytokines. The development of robust and publicly available ELISA methods to measure a variety of important cytokines would be very helpful for this type of research. Furthermore, I recommend more research on relevant key factors within metabolic reprogramming (e.g. mevalonate, acetyl-CoA, pyruvate, cholesterol) to identify the cellular pathways within chicken monocytes and macrophages.

Although, within this thesis, great similarities were found in trained innate immunity between chickens and mammals, differences were also found. With respect to lactate, I recommend to measure lactate production also within an *in vivo* experimental setting. Furthermore, in humans, inhibiting mevalonate with methyltioadenosine (MTA) showed that mevalonate plays an important role in the induction of trained innate immunity [29]. Research on inhibiting potential cellular key players would therefore be important to investigate cellular pathways in chicken macrophages. Next to that, as already proposed in this discussion, it is interesting to hypothesize that IL-4 polarized anti-inflammatory macrophages (M2), shifts to a pro-inflammatory state after training with β-glucan, resulting in enhanced pro-inflammatory responses after a LPS stimulation. This hypothesis is supported by the finding of Tits et al. (2011) [23] and awaits more investigation in chickens.

In the studies described in this thesis I focused on β-glucan and LPS. However, more components are known to have the potential to induce trained immunity in macrophages. For human monocytes four categories of components that potentially are involved in trained innate immunity have been described [16]. These are metabolites & signalling

molecules (e.g. Fumarate, mevalonate, IGF1, GM-CSF, IFNγ), sterile inducers (e.g. oxLDL, lipoproteins, uric acid), infectious inducers (e.g. Bacillus Calmette-Guerin, Candida albicans, Saccharomyces cerevisiae, β-1.3-(D)-glucan) and PAMPs (e.g. LPS, Pam3CSK4, Poly(I:C), Flagellin, MDP, TriDAP). All these components are able to induce epigenetic reprogramming. I did not include analysis of DNA methylation, histone acetylation or formation of regulatory micro-RNA molecules in my studies [68, 91]. Including these parameters in future studies in training innate immunity in chicken is highly recommended since epigenetic inheritance is probably the most important mechanism responsible for transgenerational trained innate immunity.

Transgenerational effects of the innate immune system

Within this thesis, I have not studied how exactly epigenetic modification will reach the oocyte or the spermatogonium germ cells. More research on these epigenetic changes will be of great value to understand the mechanism behind transgenerational trained innate immunity in chickens and non-genetic inheritance. I stated within this discussion that the epigenetic profile of the maternal or paternal line has significant impact on the induction and/or effectiveness of transgenerational trained innate immunity. Identification and categorization of epigenetic profiles in chickens that are associated with life history would help to predict immune modulating effects on an individual level. Epigenetic profiles may exist of a collection of different genes involved in the crosstalk of the three core elements of trained innate immunity. Differences in animal age, β-glucan/LPS dosage, exposure time, β-glucan/LPS purity and β-glucan/LPS origin might all cause changes that affect gene transcription. Also environmental factors such as temperature, hygienic circumstances and the effects of intestinal microbiota do affect the physiology of an individual and are probably also important explanatory factors for variation in transgenerational effects and immune responses [92, 93]. One may speculate that differences in epigenetic profiles between the two experiments in chapter 2 may explain the differential effects on HuSA-specific IgY responses of the offspring. This may be explained by the biological variation and/or life history of the hens. which impact gene transcription resulting in different epigenetic profiles. It would, for instance, interesting to design a transgenerational experiment with germ free chickens to eliminate any caused variation by previously infections.

Based on the results in this thesis I conclude that LPS is able to induce transgenerational effects. Since LPS is located in the cell wall of gram-negative bacteria, maternal infection with a gram-negative bacterial pathogen, which occurs frequently in practical circumstances, may influence the immune system in the offspring. It is even tempting to speculate that maternal treatment with LPS may have advantageous effects on health of chickens in the next generation and therefore on the disease resistance. After all, within this thesis I found in offspring higher levels of HuSA specific antibodies after a maternal LPS treatment (**Chapter 2, experiment 1**) and improved feed efficiency under low sanitary conditions of a NE challenge (**Chapter 5**). Effects of immunological stress caused by pathogens is generally reflected in decreased growth performance parameters [78]. Maintaining normal growth performance parameters is an indication of a better and

more efficient immunological response under pathogenic circumstance, which I describe as disease resistance. In **chapter 3 and 4** the immune modulating potentials of β-glucan became clear, but why dietary β-glucan had no transgenerational effects (*in vivo)* in **chapter 5** on feed efficiency and intestinal lesions awaits more research.

Together, all these data show that trained innate immunity and transgenerational inheritance is a complex and variable mechanism and very much dependent on multiple factors. Pathogen load or antigen dose can induce training (low dose), tolerance (moderate dose) or damage (high dose) [51]. More research on the optimal antigenic dose resulting in trained innate immunity and/or tolerance would therefore be of great value. Next to that, differences in training between macrophages from 21-, 28- and 42-days old chickens shows that the level of training differed for monocytes obtained at different ages, suggest that immunomodulatory approaches to train immune cells could be age dependent (**Chapter 4, figure 6**). Differences between breeds, age, life history or other environmental factors are therefore important factors to take into account [89].

Feed additive and vaccine application

Trained innate immunity is characterized by its poly-specific protection and has potential to improve disease resistance of poultry in a nonspecific manner. Improved disease resistance is especially important at a young age when the adaptive immune system has not yet fully developed [36, 94-96]. Dietary additives or vaccinations based on β-glucan could potentially be applied to train innate immune cells and improve resistance to a variety of pathogens rather than the current vaccination schemes which are pathogen specific [89]. As proposed in this discussion, immune modulating components could target the hematopoietic stem cells in the bone marrow [3, 8]. Using feed additives or special designed vaccines to target hematopoietic cells, oocytes or spermatogonium germ cells and induce trained immunity would be a very effective way to induce long term immune memory and protection [8]. However, within chickens, targeting the hematopoietic stem cells is not well investigated and results within this thesis on bone marrow-derived cells (**Chapter 3**) are not conclusive and awaits more research. The latter could be essential for future implementation of long-term effects of trained innate immunity as a feed or vaccine approach within the poultry husbandry sector. It is important to investigate variability and plasticity of the immune system at early and later life physiology as immune modulating effects also have consequences on metabolism (performance) [92] and behavior (endocrine function) and vice versa [97, 98]. What the optimal circumstances are awaits further research wherein this thesis provided valuable information and insights on this.

Concluding remark

The results described in this thesis showed evidence for trained innate immunity and transgenerational effects in chickens. Overall, I conclude that maternal immune experiences, such as infections or vaccinations, influence the immune system in the offspring. This also influence the effects of dietary interventions with feed additives on the immune system in the offspring. Within this, trained innate immunity has great potentials to be used in adapting current vaccination and feeding strategies to further improve disease resistance, animal performance and animal welfare in the poultry husbandry industry. More knowledge about transgenerational effects of maternal immune activation or infection will contribute to a better understanding of the variation in immune phenotypes, disease resistance and metabolic disorders. This knowledge may have consequences for the animal husbandry sector, how to optimize their vaccination strategies and how to apply immunomodulatory dietary additives more effectively.

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LIST OF PUBLICATIONS

Peer reviewed scientific publications

Verwoolde MB, Arts J, Jansen CA, Parmentier HK, Lammers A. Transgenerational Effects of Maternal Immune Activation on Specific Antibody Responses in Layer Chickens. Front Vet Sci. 2022 Feb 17;9:832130. doi: 10.3389/fvets.2022.832130. PMID: 35252424; PMCID: PMC8891521.

Verwoolde MB, van Baal J, Jansen CA, Graat EAM, Lamot DM, Lammers A, van Eck L. Transgenerational effects of innate immune activation in broiler breeders on growth performance and immune responsiveness. Poult Sci. 2021 Nov;100(11):101413. doi: 10.1016/j.psj.2021.101413. Epub 2021 Jul 30. PMID: 34601443; PMCID: PMC8531860.

Verwoolde MB, van den Biggelaar RHGA, van Baal J, Jansen CA, Lammers A. Training of Primary Chicken Monocytes Results in Enhanced Pro-Inflammatory Responses. Vet Sci. 2020 Aug 19;7(3):115. doi: 10.3390/vetsci7030115. PMID: 32825152; PMCID: PMC7560005.

Verwoolde MB, van den Biggelaar RHGA, de Vries Reilingh G, Arts JAJ, van Baal J, Lammers A, Jansen CA. Innate immune training and metabolic reprogramming in primary monocytes of broiler and laying hens. Dev Comp Immunol. 2021 Jan;114:103811. doi: 10.1016/j.dci.2020.103811. Epub 2020 Aug 1. PMID: 32750399.

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Conference proceedings and abstracts

Verwoolde, M. B., van den Biggelaar, R. H. G. A., van Baal, J., Jansen, C. A., & Lammers, A. (2019). The influence of genetic background on trained innate immunity in chicken macrophages. Abstract from 4th International Conference on Innate Immune Memory, Nijmegen, Netherlands.

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WIAS Education & training certificate

Description	year
The Basic Package (3.0 ECTS[1])	
WIAS Introduction Day	2016
Research Integrity & Ethics and Animal Science	2017
Course on essential skills (Frank Little) *(recommended) (26, 28-29 April)*	2016
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WIAS Course Design of Experiments	2016
WIAS Course Statistics for the Life Sciences	2016
Animal Health and Immunology Discussion Group (AHIDG)	2016-2019
Course Epigenesis & Epigenetics, Physiological consequences of perinatal nutritional programming	2017
Flow Cytometry Workshop	2018
SUSINCHAIN Workshop - Setting Quality Criteria for Insect-Based Food Products	2020
Literature Survey: Transgenerational nutritional programming, epigenetic inheritance and imprinting of the immune system in chicken	2020
Professional Competences (7.6 ECTS[1])	
Project and Time Management (PTM)	2016
The Essentials of Scientific Writing and Presenting (ESWP)	2016
Scientific Writing (SWR)	2018
Posters & Pitching 1	2018
Course Supervising BSc & MSc thesis students	2019
Scientific Publishing	2019
WIAS Course The Final Touch	2019
Course Brain friendly working and writing	2019
Course Brain Training	2019
Presentation Skills (3.0 ECTS[1])	
In vitro model to study trained innate immunity in chicken primary monocytes; XV Avian Immunology Research Group meeting, Oxford, UK, poster presentation	2018
Trained innate immunity in chicken macrophages, Boosting macrophages with β-glucans; WIAS science day 2019, Lunteren, 18 March 2019, oral presentation	2019
The influence of genetic background on trained innate immunity in chicken macrophages; 4th International Conference on Innate Immune Memory, Nijmegen, poster presentation	2019
Teaching competences (6.0 ECTS[1])	
Supervising practical	2016-2019
Supervising students	2016-2019

[1]One ECTS credit equals a study load of approximately 28 hours

Completion of the training activities is in fulfilment of the requirements for the education certificate of the Graduate School of the Wageningen Institute of Animal Sciences (WIAS).

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