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Article

Training of primary chicken monocytes results in enhanced pro-inflammatory responses

Michel B. Verwoolde[1,3]*, Robin H.G.A. van den Biggelaar[2], Jürgen van Baal[3], Christine A. Jansen[2], Aart Lammers[1]

[1] Adaptation Physiology Group, Department of Animal Sciences, Wageningen University and Research, Wageningen, the Netherlands; michel.verwoolde@wur.nl (M.V); aart.lammers@wur.nl (A.L)
[2] Department of Biomolecular Health Sciences, Division of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands; R.H.G.A.vandenBiggelaar@uu.nl (R.B); C.A.Jansen@uu.nl (C.J)
[3] Animal Nutrition Group, Department of Animal Sciences, Wageningen University and Research, Wageningen, the Netherlands; jurgen.vanbaal@wur.nl (J.B)
* Correspondence:Correspondence: michel.verwoolde@wur.nl

Abstract: Beta-glucan-stimulated mammalian macrophages show an increased responsiveness to secondary stimulation in a nonspecific manner. This phenomenon is known as trained innate immunity. Our study aimed to explore 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), 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 analysed 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 CSF1R and the activation markers CD40 and 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 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 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 the past 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 enhanced production of TNFα and IL-6 by monocytes after secondary 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 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 MHC 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 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 increased responsiveness of the cells by determining 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 generation of white leghorn chickens that were divergently selected for feather pecking behaviour. These chickens were part of a study on feather pecking behaviour (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 hatch until 8 weeks of age, 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 weeks old chickens. A heparinized syringe was used to prevent the blood from coagulation. 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 are 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 hours 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. Homogenisation 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, Missouri, USA) to separate the leukocytes by density gradient centrifugation (700 xg, 40 minutes 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% heath inactivated chicken serum and 50 U/mL penicillin and 50 µg/mL streptomycin (all from Gibco).

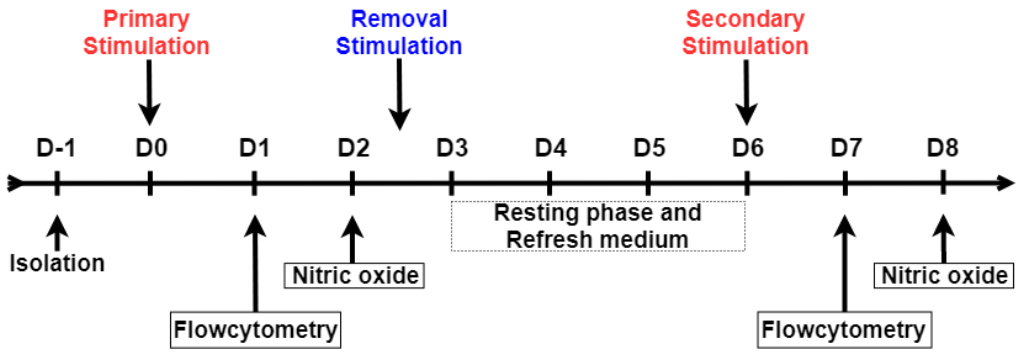

Figure 1. Schematic overview of the *in vitro* model for trained innate immunity. Adherent cells were primary stimulated with culture medium (UNSTIM), LPS, M-βG, IL-4 and M-βG + IL-4 on day 0 (D0). On D1, cells were harvested for flow cytometry. On D2, cell culture supernatant was collected to measure the release of 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.

The timeline of the *in vitro* 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, 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, Sigma L2880), recombinant chicken IL-4 (100 ng/mL, Kingfisher Biotech Inc., 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 hours after stimulation. From an identical experiment performed simultaneously, cell culture supernatant was collected 48 hours post-stimulation to measure the production of NO. After these 48 hours, 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 hours after the secondary stimulation for flow cytometry analysis. From an identical

experiment performed simultaneously, cell culture supernatant was collected after 48 hours for subsequent analysis of NO production.

2.4. Nitric oxide (NO) production assay

NO production was measured 48 hours 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 minutes at room temperature. The NO2- concentration was determined by measuring the optical density at 540nm 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 hours 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 5mM EDTA in PBS. The detached cells were transferred to a 96-well round-bottom plate (Greiner CELLSTAR®) 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% NaN3 (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), 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). 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). Data analysis was performed using FlowJo Software v. 10.5 (TreeStar Inc, San Carlo, USA).

2.6. Statistical analysis

Statistical analysis was performed using Prism version 7.04 software (GraphPad Software, San Diego, CA). Differences in the mean among the experimental groups of the NO assay were analysed using a one-way ANOVA with the Tukey's multiple comparison tests. 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 $\frac{gMFI_{LPS}}{gMFI_{unstimulated}}$. A repeated measures one-way ANOVA with the Tukey's multiple comparison test was used for statistical analysis. $P < 0.05$ was considered a significant difference.

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 24h (Figure B1) and 7 days (Figure 2) of culture by flow cytometry to get more insight in the composition of the cell population. The cells were gated for viability (7-AAD$^-$), high forward and side light scatter (FSC vs SSC), indicative of macrophages [14]. The macrophages expressed chicken macrophage markers

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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.

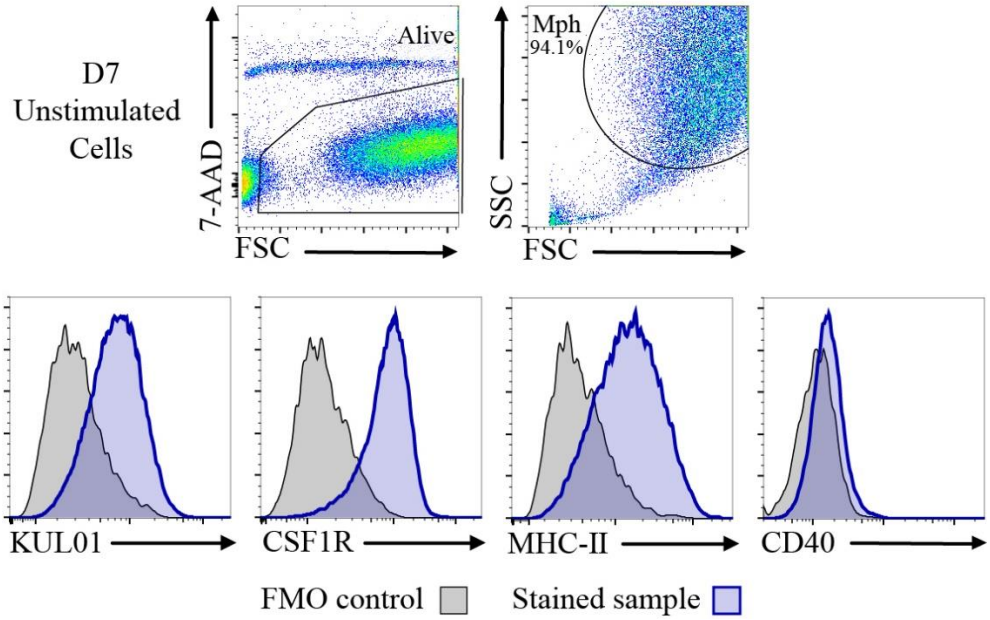

Figure 2. Adherent cells differentiated to KUL01$^+$ CSF1R$^+$ MHC-II$^+$ macrophages after 7 days of culture. Adherent unstimulated cells were characterized after 7 days of culture by flow cytometry. The cells were selected for viability (7-AAD$^-$), forward and side light scatter (FSC vs SSC), and assessed for 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. The percentage of macrophages (Mph) is shown.

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 β-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.

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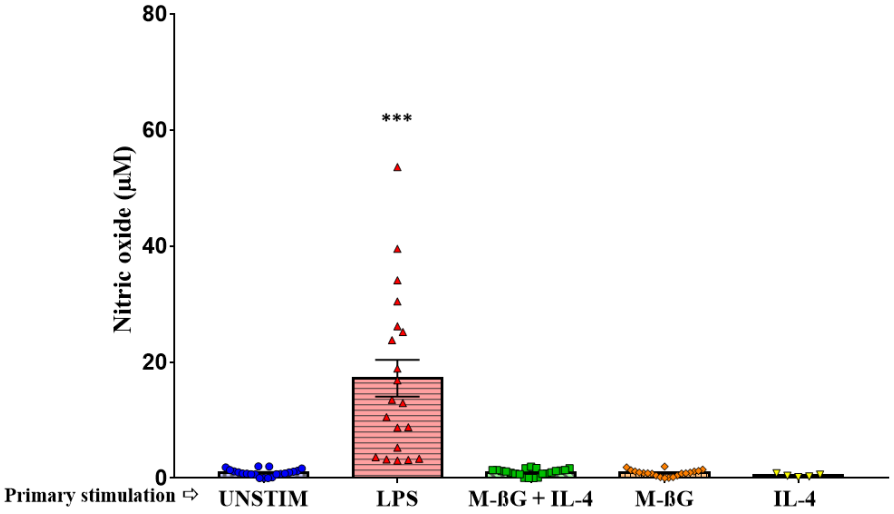

Figure 3. Primary stimulation with LPS resulted in enhanced nitric oxide production. Fresh isolated adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG+IL-4 (10 µg/mL+100 ng/mL) or IL-4 (100 ng/mL). LPS induced NO production (N = 21 chickens; N IL-4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

As indicated in Figure 4, this resulted in NO production which was higher in cells primary stimulated with M-βG + IL-4 compared to cells primary unstimulated (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 for a pro-inflammatory response, after secondary stimulation with LPS in cells primary stimulated with M-βG in combination with IL-4.

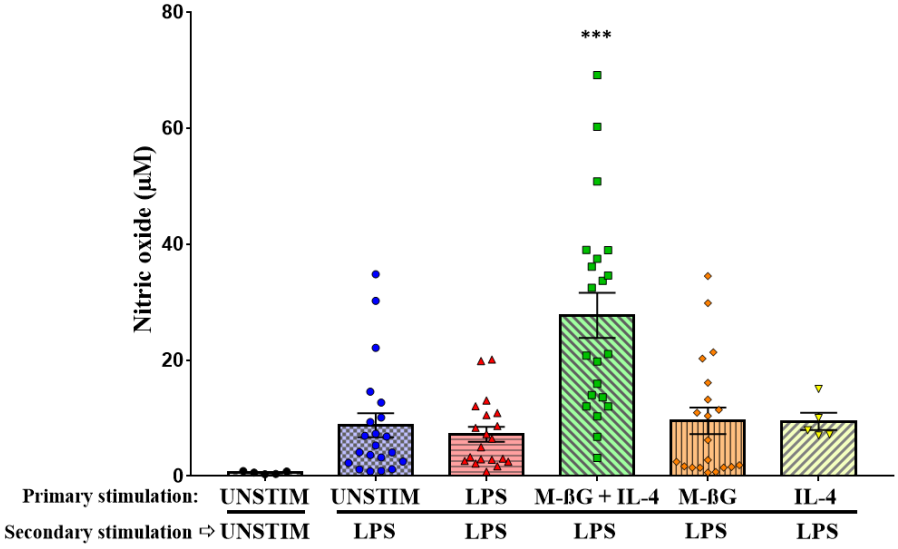

Figure 4. Primary stimulation with β-glucan microparticulates + IL-4 enhanced the NO production after secondary stimulation of LPS. Freshly isolated adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG+IL-4 (10 µg/mL+100 ng/mL) or IL-4 (100 ng/mL) on D0. The cells were secondary stimulated with LPS (10 µg/mL) except 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. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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 expression of the surface markers KUL01, CSF1R, MHC-II and CD40. Especially the surface markers CD40 and MHC-II are associated with activation of myeloid cells [17-19]. Secondary stimulation with LPS resulted in increased surface expression of CD40 compared to secondary unstimulated cells (Figure 5A and 5B). 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 and 5B). 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 5B).

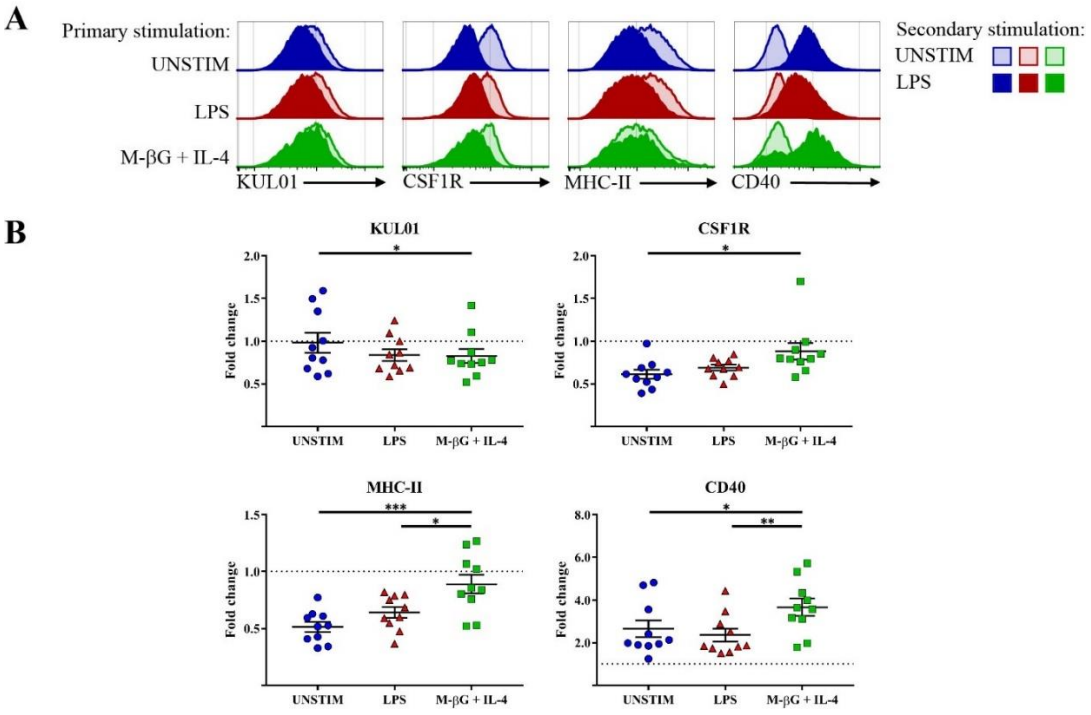

Figure 5. Primary stimulation with β-glucan microparticulates + IL-4 influenced the expression level of cell surface markers on macrophages after secondary stimulation with LPS. Surface expression of KUL01, CSF1R, MHC-II and CD40 was assessed after secondary stimulation with LPS (10 µg/mL). (A) Expression 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 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 expresses as a fold change in geometric mean fluorescent intensity (gMFI). 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.

In parallel to the training of primary monocytes, we tried to train adherent bone marrow-derived cells 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 (Figure C1 and C2). The responses to the primary stimulations were similar to the 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 C1). 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 C2).

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 analysed surface expression of markers associated with monocyte activation, indicative of a pro-inflammatory response [17,18]. 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 [20]. Furthermore, trained macrophages from mice and humans show enhanced production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα [3,21]. 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 [22]. Both the results on expression of the cell surface markers and NO production show that primary chicken monocytes can be trained similar to their mammalian counterparts.

NO production of trained monocytes may lead to increased killing capacity upon phagocytosis [23]. 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* [24]. In that study this was referred as innate immune training.

It is known that increased phagocytosis results in enhanced antigen presentation [25]. 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 [17,18,26,27]. The study with bovine monocyte-derived macrophages also indicates a relationship between trained innate immunity and increased antibody levels of the adaptive immune response [24].

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. β-glucan is a known agonist of the pattern-recognition receptor dectin-1 found on mammalian phagocytes (Brown GD. et al., 2003). 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 4h [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 [28]. Whether dectin-1 or related receptors play a role in chicken innate immune training by β-glucan and IL-4 has to be elucidated and awaits further studies.

In humans, primary stimulation with LPS resulted in a tolerogenic state of the macrophages [21]. In the current study no tolerance was observed since no significant decrease was observed for LPS primary stimulated cells in 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 [29,30]. 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 [31,32].

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 5B). This suggests that training affects the regulation of macrophage survival, differentiation and proliferation [15]. In line to our observation, reduced KUL01 expression was also found on bone marrow-derived monocytes after LPS stimulation [33,34].

We showed trained innate immunity by using a relative 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 [35]. These changes are independent from 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 mechanism 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 [13,36-38]. 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. Possible interactions of enhanced innate immunity with metabolic and/or behavioural physiology should be considered [39,40].

Author Contributions: Conceptualization, M.V., C.J. and A.L.; methodology, M.V., J.B., C.J. and A.L.; validation, M.V. and R.B.; formal analysis, M.V.; investigation, M.V., R.B. and A.L.; resources, M.V., C.J. and A.L.; data curation, M.V. and A.L.; writing—original draft preparation, M.V.; writing—review and editing, R.B., J.B., C.J. and A.L.; visualization, M.V. and R.B.; supervision, J.B., C.J. and A.L.; project administration, M.V. and A.L.; funding acquisition, A.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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

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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.

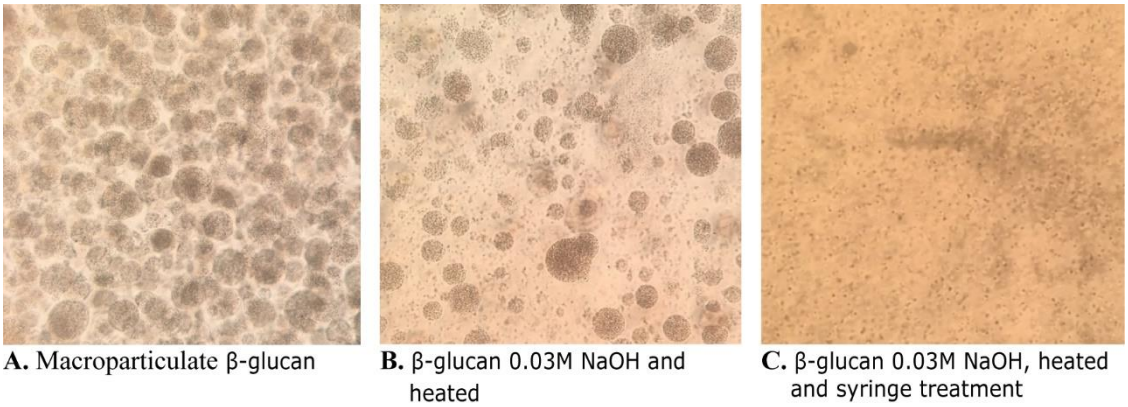

Figure A1. Microscopic pictures of β-glucan containing material from the *Saccharomyces cerevisiae* cell wall (Macrogard, Orffa). Macroparticulate β-glucan (**A**), β-glucan 0.03M NaOH and heated (**B**) and β-glucan 0.03M NaOH, heated and syringe treatment (**C**).

Aggregates of β-glucan macroparticulates in an aquatic suspension (Figure A1A). Beta-glucan macroparticulates were resuspended in 0.03M NaOH and heated at 70 °C for 2.5 hours. Aggregates starting to fall apart resulting in microparticulates (Figure A1B). A microparticulate suspension was created by using a sterile syringe (BD Microlance 27G ¾ - nr 20) by drawing it up and down 2 times (Figure A1C). Microscope magnification = 200 x, using an inverted microscope (Zeiss Primovert Inverted Microscope, Carl Zeiss Microscopy GmbH, Germany).

Appendix B

Flowcytometry data after 24h of culturing

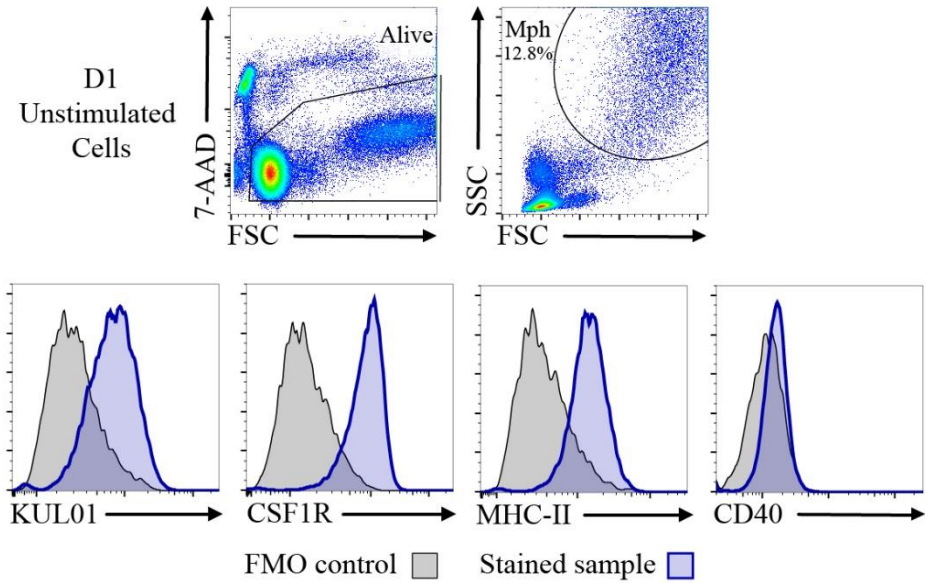

Figure B1. Adherent cells were characterized after 24 h of culture by flow cytometry. The cells were selected for viability (7-AAD$^-$), forward and side light scatter (FSC vs SSC), and assessed for expression of KUL01, CSF1R, MHC-II and CD40. The histograms show expression of the macrophage markers in blue and fluorescent-minus-one (FMO) staining controls in grey. The percentage of macrophages (Mph) is shown.

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Appendix C

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 days 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 xg, 10 minutes 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

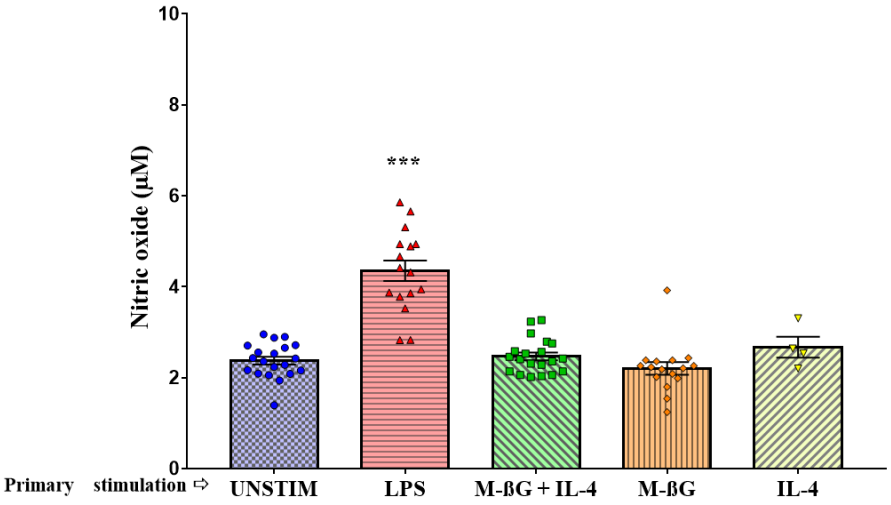

Figure C1. Bone marrow-derived adherent cells primary stimulation with LPS resulted in enhanced NO production. Fresh isolated chicken bone marrow-derived adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG+IL-4 (10 µg/mL+100 ng/mL) or IL-4 (100 ng/mL). LPS induced NO production (N = 12-20 chickens; N for IL-4 = 4 chickens). Each bar represents mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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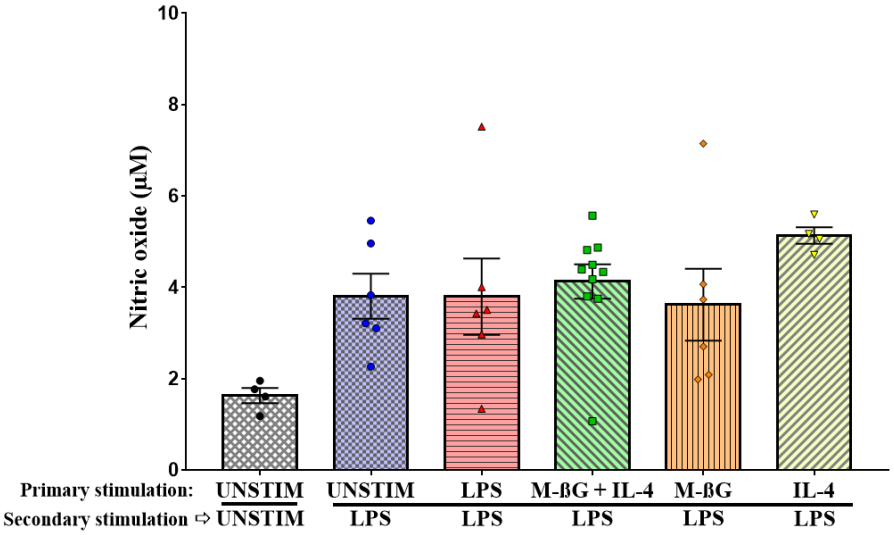

Figure C2. Bone marrow-derived adherent cells primary stimulated with M-βG + IL-4 failed to enhance NO production after secondary stimulation of LPS. Freshly isolated chicken bone marrow-derived adherent cells were primary stimulated with culture medium (UNSTIM), LPS (10 µg/mL), M-βG (10 µg/mL), M-βG+IL-4 (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. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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