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

Michel B. Verwoolde [a,b,**], Robin H.G.A. van den Biggelaar [c], Ger de Vries Reilingh [a], Joop A. J. Arts [a], Jürgen van Baal [b], Aart Lammers [a,*], Christine A. Jansen [c]

[a] Adaptation Physiology Group, Department of Animal Sciences, Wageningen University and Research, Wageningen, the Netherlands
[b] Animal Nutrition Group, Department of Animal Sciences, Wageningen University and Research, Wageningen, the Netherlands
[c] Department of Biomolecular Health Sciences, Division of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

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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.

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 (Chou et al., 2017; Cox et al., 2010; Nochi et al., 2018). 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. (Bar-Shira et al., 2003; Den Hartog et al., 2016; Lammers et al., 2010). 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 (Novakovic et al., 2016; Quintin et al., 2012; van der Meer et al., 2015). However, it is known that innate immune responses vary between chickens that are genetically selected for different purposes like laying chickens and broiler chickens (Koenen et al., 2002; Leshchinsky and Klasing, 2001; Parmentier et al., 2010). 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 (Quintin et al., 2012). 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 (Cheng et al., 2014a; Domínguez-Andrés et al., 2019). This is also referred to as the immuno-epigeno-metabolic crosstalk (Hajishengallis et al., 2019). The Dectin-1/Akt/mTor/HIF-1α signalling pathway has been found to be involved in this phenomenon in response to β-glucan molecules (Goodridge et al., 2011). Hypoxia-inducible factor 1-alpha (HIF-1α) acts as a master transcription factor, not only controlling inflammatory responses, but also metabolic reprogramming (Kelly and O'Neill, 2015). Indeed, in trained innate

* Corresponding author. Adaptation Physiology Group, Department of Animal Sciences, Wageningen University and Research, P.O. Box 338, 6700 AH, Wageningen, the Netherlands
** Corresponding author. Adaptation Physiology Group, Department of Animal Sciences, Wageningen University and Research, P.O. Box 338, 6700 AH, Wageningen, the Netherlands
E-mail addresses: michel.verwoolde@wur.nl (M.B. Verwoolde), aart.lammers@wur.nl (A. Lammers).

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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

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" (Cheng et al., 2014a; Domínguez-Andrés et al., 2019). This metabolic shift results in increased lactate production (Harris et al., 2014; Kelly and O'Neill, 2015; Petit et al., 2019). Furthermore, peroxisome proliferator-activated receptors (PPARs), such as PPARγ, regulate metabolism and inflammation and are found to be involved in trained innate immunity (Christ et al., 2016; Croasdell et al., 2015). 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) (Verwoolde et al., 2020). 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 (Leshchinsky and Klasing, 2001). Moreover, broiler macrophage effector functions, such as phagocytosis, are impaired in response to LPS (Qureshi, 2003). Other studies describe variable inducible nitric oxide synthetase (iNOS) gene expression levels and NO production in chickens of several genotypes (Dil and Qureshi, 2003; Hussain and Qureshi, 1998; Qureshi, 2003). Furthermore, immunological differences between broilers and layers were found for antibody responses and ileal immune-related gene expression (Koenen et al., 2002; Parmentier et al., 2010; Simon et al., 2014). 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 (Verwoolde et al., 2020). Briefly, mononuclear cells were purified by density gradient centrifugation (700×*g*, 40 min 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 (Verwoolde et al., 2020). 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 48 h. 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 (Verwoolde et al., 2020), were restimulated with 200 μl LPS (10 μg/mL) for 24 h. We used for the NO production assay a 48 h 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 (Green et al., 1982; Parmentier et al., 2010; van der Eijk et al., 2019; Verwoolde et al., 2020). 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, AM 1906, 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 (Andersen et al., 2004).

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 h 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 min 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 min at 37 °C and followed by measurement of the absorbance at 340 nm after 30 min with the same device. The lactate concentrations were calculated using a calibrator-mix which was provided by the manufacturer.

Table 1
Primers used for RT-qPCR.

Target[a]	Sequence[b]	Accession no.
Housekeeping genes		
ACTB	F: 5′-GCCCTGGCACCTAGCACAAT-3′ R: 5′-GCGGTGGACAATGGAGGGT-3′	NM_205,518
IPO8	F: 5′-ACCTCCGAGCTAGATCCTGT-3′ R: 5′-GGCTCTTCTTCGCCAACTCT-3′	XM_015,287,054
GAPDH	F: 5′-ATCCCTGAGCTGAATGGGAAG-3′ R: 5′-AGCAGCCTTCACTACCCTCT-3′	NM_204,305
Genes associated with inflammation		
IL-1β	F: 5′-GACATCTTCGACATCAACCAG-3′ R: 5′-CCGCTCATCACACACGACAT-3′	XM_015,297,469
TNF	F: 5′-CCGCCCAGTTCAGATGAGTT-3′ R: 5′-GCAACAACCAGCTATGCACC-3′	XM_015,294,124
iNOS	F: 5′-CTACCAGGTGGATGCATGGAA-3′ R: 5′-ATGACGCCAAGAGTACAGCC-3′	NM_204,961
ARG2	F: 5′-TCTGGAAACCTCCATGGGCA-3′ R: 5′-CAGATGCTGAAAGACAGGGCT-3′	NM_001,199,704
IL-10	F: 5′-GCTGAGGGTGAAGTTTGAGGA-3′ R: 5′-TCTGTGTAGAAGCGCAGCAT-3′	NM_001,004,414
Genes associated with metabolism		
HIF-1α	F: 5′-ACGTGTAAAGGCGTGCAAAA-3′ R: 5′-CGTGAGTTGGGGTAGTCCAC-3′	XM_015,287,266
PPARγ	F: 5′-GGGCGATCTTGACAGGAA-3′ R: 5′-GCCTCCACAGAGCGAAAC-3′	XM_015,292,933

[a] 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.
[b] F: forward; R: Reverse.

2.7. Surface marker expression

Flow cytometry was performed 24 h after restimulation to phenotypically characterize the cell populations (Verwoolde et al., 2020). 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 24 h 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.

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

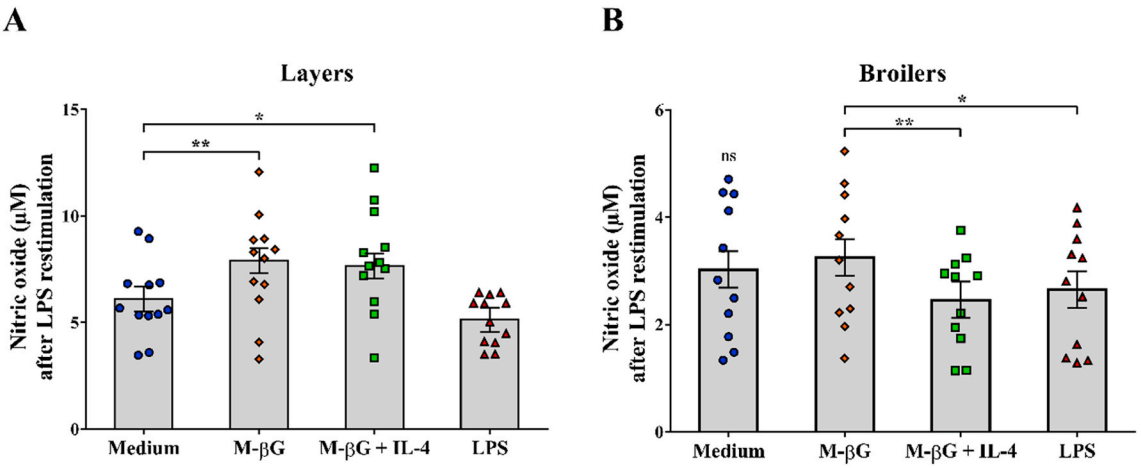

Fig. 1. Innate immune training with microparticulate β-glucan in combination with or without interleukin-4 increased NO production in macrophages from layers but not broilers. Adherent primary blood monocytes harvested from 42 days old layers (A) or broilers (B) were trained with culture medium (Medium; untrained control group), M-βG (10 μg/mL), M-βG + IL-4 (10 μg/mL + 100 ng/mL) or LPS (10 μg/mL) for 48 h on D0 and were restimulated with LPS (10 μg/mL) on D6. Presented is the NO production in the culture medium after a 48 h 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.

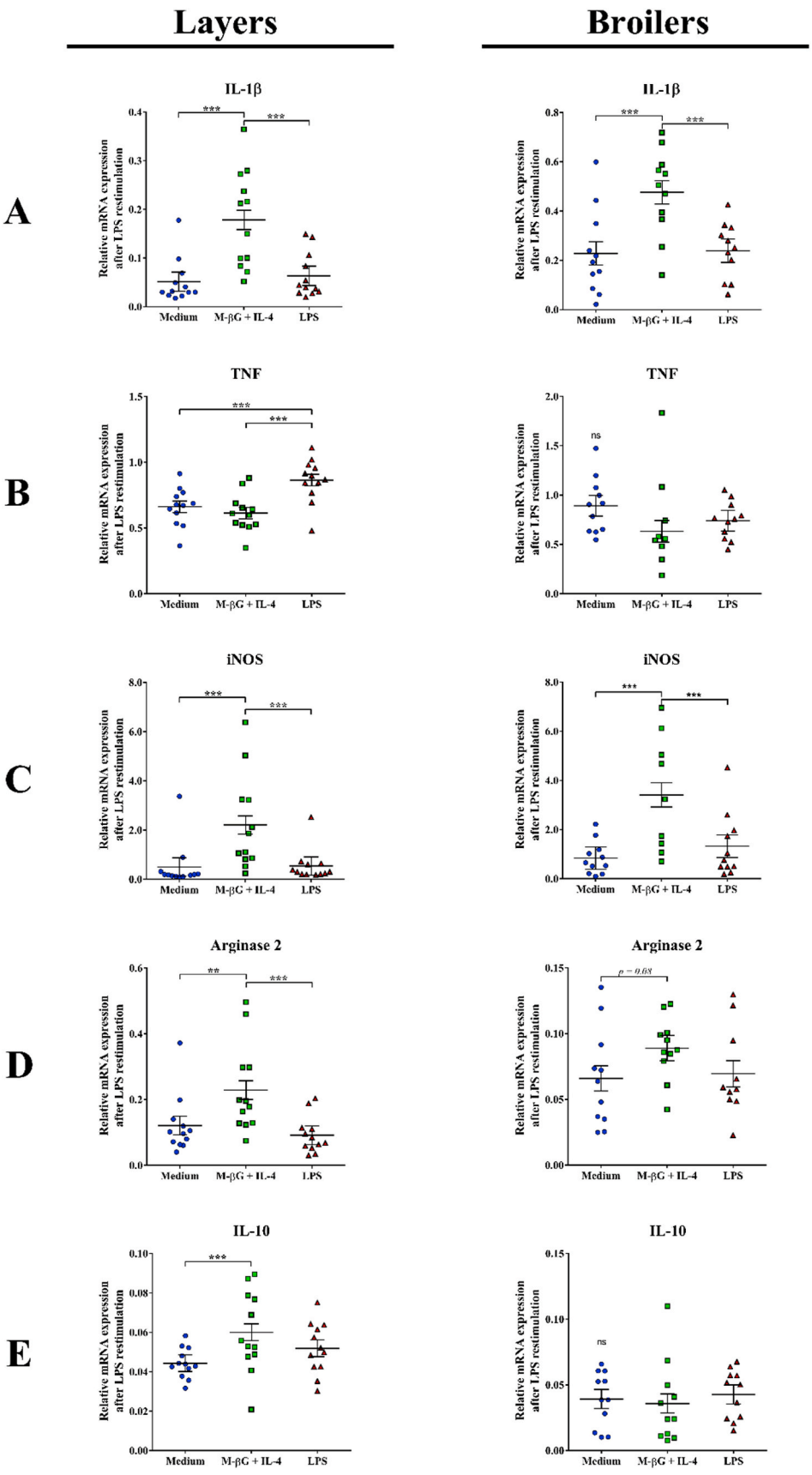

Fig. 2. Training effect on the expression levels of genes associated with inflammation in layer and broiler macrophages. Adherent blood monocytes from 42-days-old layers (A) and broilers (B) were trained with either culture medium (Medium; untrained control), LPS (10 μg/mL) or M-βG + IL-4 (10 μg/mL+100 ng/mL) for 48 h on D0 and were restimulated (i.e. challenged) with LPS (10 μg/mL) on D6. Relative gene expression levels measured 24 h 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.

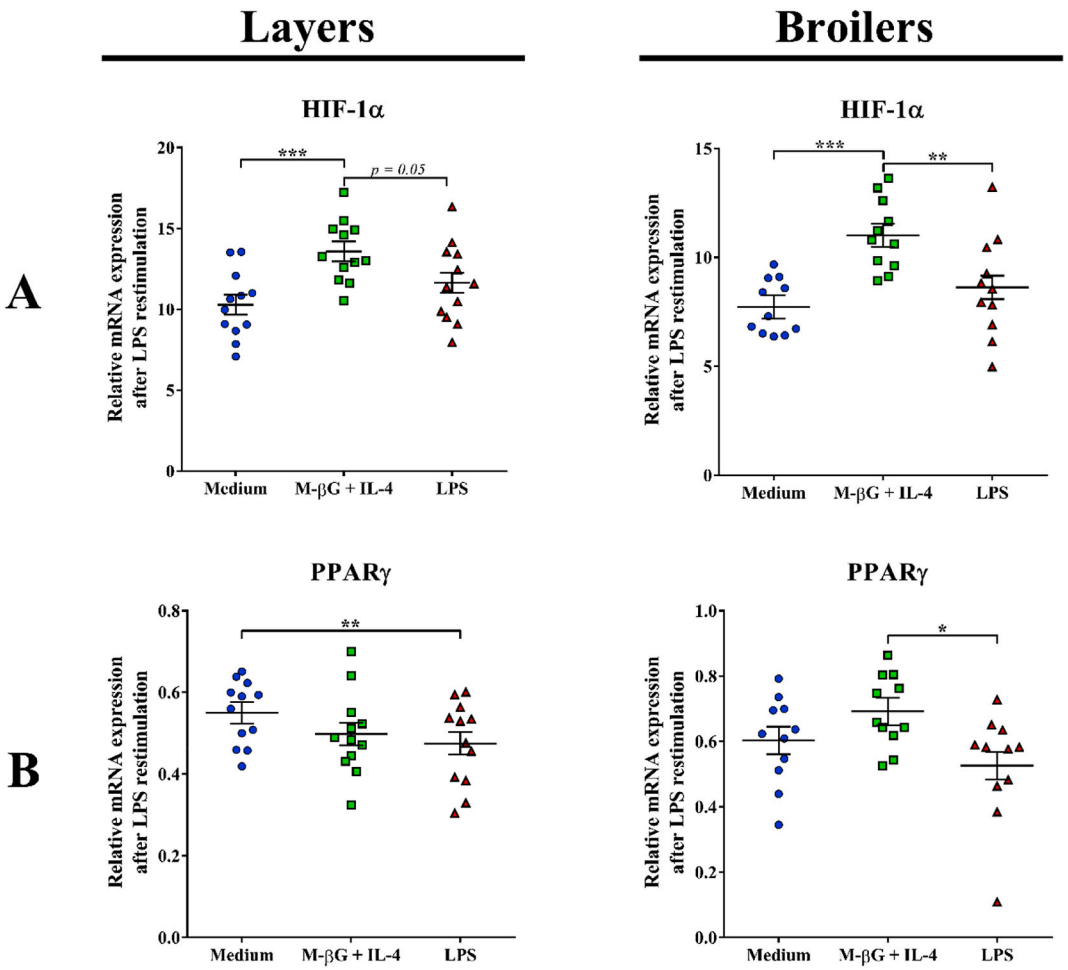

Fig. 3. Training effect on the expression levels of genes associated with cellular metabolism in layer and broiler macrophages. Adherent blood-derived monocytes from layers and broilers were stimulated with culture medium (Medium; untrained control), LPS (10 μg/mL) or M-βG + IL-4 (10 μg/mL+100 ng/mL) for 48 h 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 24 h 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. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

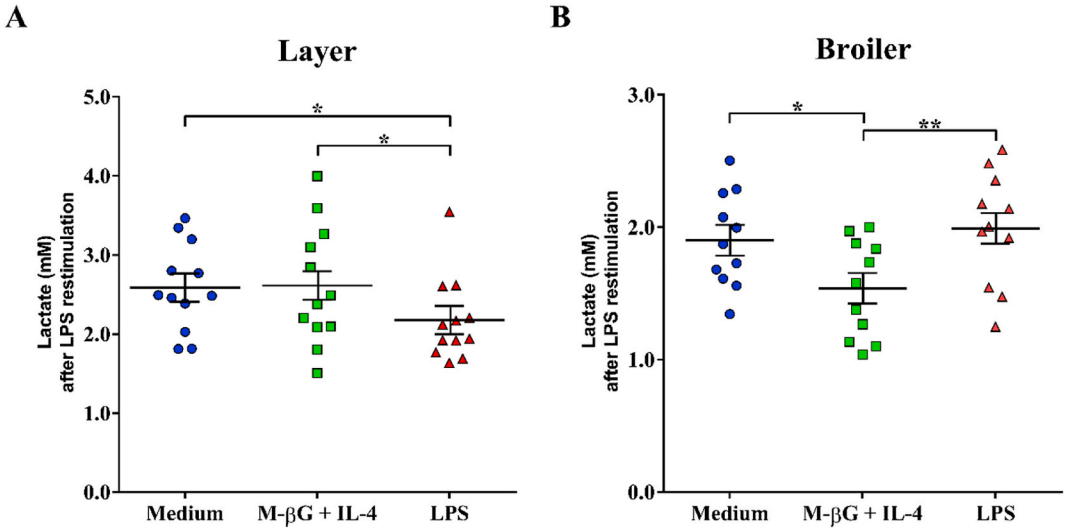

Fig. 4. Extracellular lactate production of trained macrophages from layers and broilers. Monocytes collected from 42 days old layers (A) and broilers (B) were *in vitro* trained with either culture medium (Medium; untrained control), M-βG (10 μg/mL), M-βG + IL-4 (10 μg/mL+100 ng/mL) or LPS (10 μg/mL) for 48 h on D0. The cells were restimulated with LPS (10 μg/mL) for 24 h 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. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

control group.

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 (Balic et al., 2014; Mast et al., 1998) (Fig. 5B).

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 24 h (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).

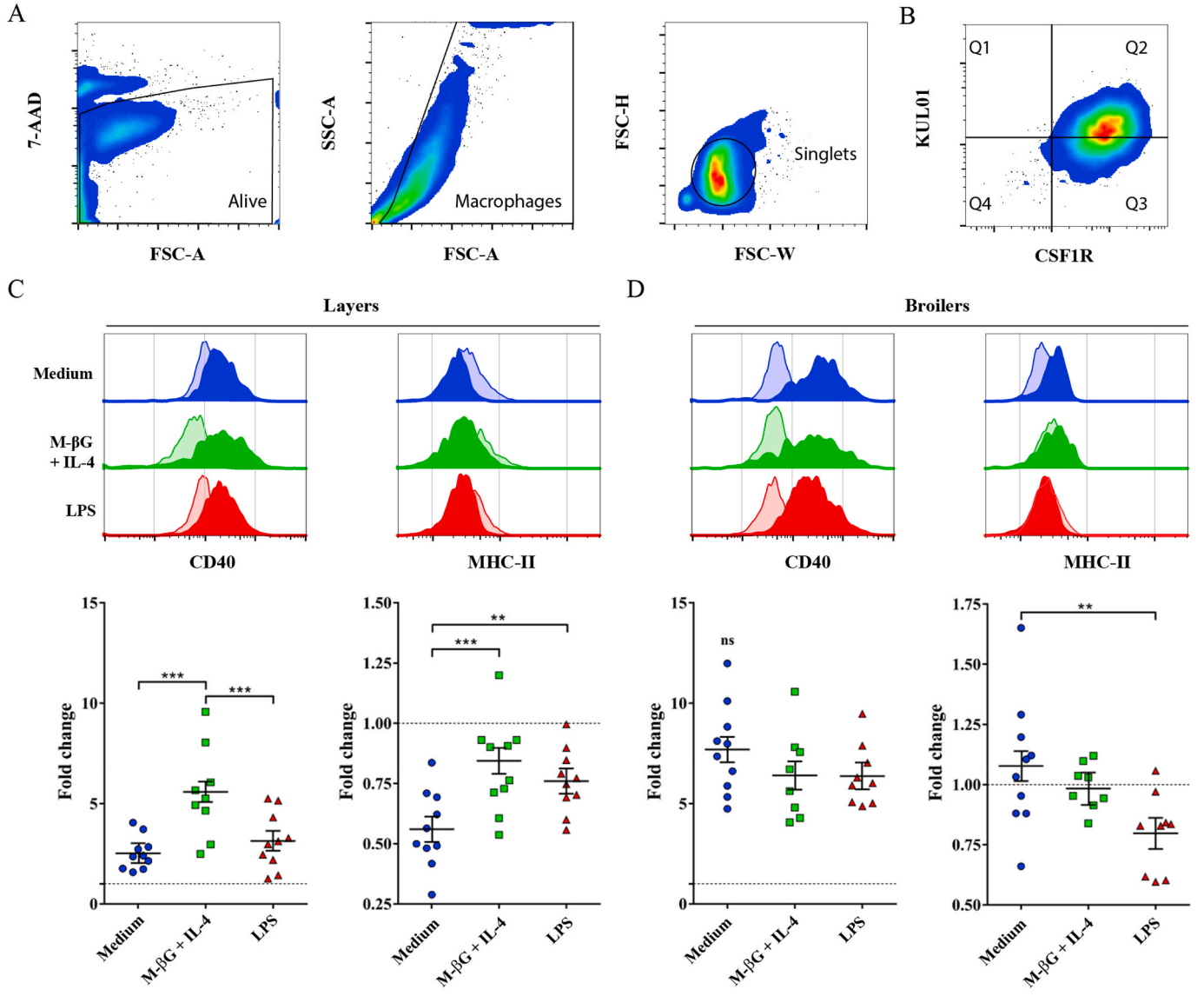

Fig. 5. Surface expression of CD40 and MHC-II on macrophages from layers and broilers. Macrophages were stained and analysed by imaging flow cytometry. The cells were gated for viability using 7-AAD (left panel), by their scatter profile (middle panel), and for single cells (right panel) (A). The gated 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 24 h 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

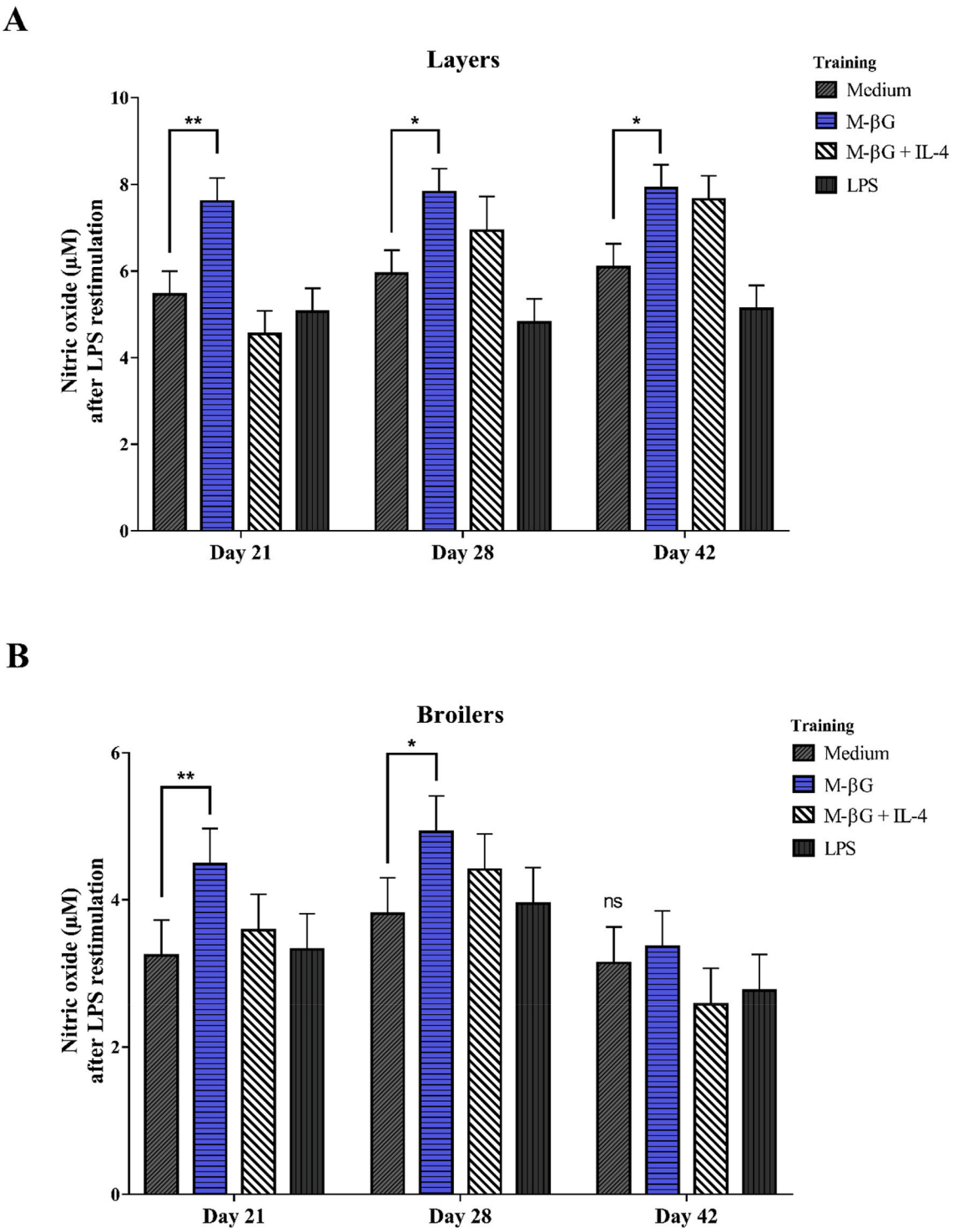

Fig. 6. Training effects on monocytes from chickens with different ages on nitric oxide production. Primary monocytes collected from layers (A) and broilers (B) aging 21, 28 and 42 post-hatch were *in vitro* trained with either culture medium (Medium; untrained control), M-βG (10 μg/mL), M-βG + IL-4 (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 48 h 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).

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).

As for broilers, we also found an increase in NO production after training with M-βG in macrophages derived from 21 to 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 (Verwoolde et al., 2020). 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 (Bekkering et al., 2018; Cheng et al., 2014a; Domínguez-Andrés et al., 2019; Kelly and O'Neill, 2015; Moorlag et al., 2018).

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) (Bekkering et al., 2016; Byrne et al., 2020; Juste et al., 2016; Petit et al., 2019; Quintin et al., 2012). 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 (Leshchinsky and Klasing, 2001). This could be attributed to differential expression of TLR4 between genetical different chicken breeds (Dil and Qureshi, 2002).

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 (Bekkering et al., 2016), mice (Novakovic et al., 2016; Quintin et al., 2012) and carp (Petit et al., 2019). 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 (Cooney et al., 2011; Fenn et al., 2012; Mahbub et al., 2012). 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 (Verwoolde et al., 2020), needs to be verified in other studies.

Nitric oxide, produced by the enzyme iNOS, is a prominent mediator of inflammatory reactions (Pautz et al., 2010). 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 (Kleinert et al., 2003; Pautz et al., 2010).

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 (Nishi et al., 2008). 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 (Cheng et al., 2014a; Domínguez-Andrés et al., 2019). 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 (Cheng et al., 2014b; Kelly and O'Neill, 2015; Mitroulis et al., 2018). They not only orchestrate inflammation reactions, but also metabolic processes (Kelly and O'Neill, 2015; Moorlag et al., 2018). Macrophages are apt to infiltrate hypoxic tissues whereby they switch their metabolic program to glycolysis, probably under the tight control of HIF-1α (Cramer et al., 2003). This tunes with the evidence that LPS increases the level of tricarboxylic acid (TCA) cycle intermediates succinate, malate, fumarate, α-ketoglutarate and citrate (Arts et al., 2016; Menon et al., 2015; Tannahill et al., 2013). 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 (Selak et al., 2005). 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) (Corcoran and O'Neill, 2016; Palsson-McDermott et al., 2015). PDK

inhibits pyruvate dehydrogenase, thereby preventing the formation of acetyl-CoA from pyruvate (Kim et al., 2006; Tannahill et al., 2013). 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 (Arts et al., 2016; Domínguez-Andrés et al., 2019). 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 (Cheng et al., 2014b). Several metabolites of the TCA cycle serve as cofactor for chromatin modification-catalyzing enzymes (Etchegaray and Mostoslavsky, 2016). α-ketoglutarate, for example, is a co-factor that can favor histone demethylation (Su et al., 2016), and fumarate and succinate, contrariwise, are described as inhibitors of demethylation processes (Arts et al., 2016; Lu et al., 2012; Xiao et al., 2012). 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 (Mills and O'Neill, 2014). 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 (Arts et al., 2016; Cheng et al., 2014b; Petit et al., 2019). 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 (Novakovic et al., 2016; Quintin et al., 2012). 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 (Couper et al., 2008). 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 (Marathe et al., 2006; Yang and Ming, 2014).

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 (Novakovic et al., 2016; Quintin et al., 2012). 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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Declaration of competing interest

None.

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Appendix A. Supplementary data

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