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Non-classical monocytes contribute to innate immune training in cattle

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Abstract

Innate immune training is defined as a property of innate immune cells to react stronger to a secondary contact with pathogens. Induction of innate immune training has been reported for a variety of pathogens and selected pattern recognition receptor-ligands, such as β-glucans (βG). We examined whether *Saccharomyces cerevisiae* cell wall component βG induces training in bovine monocytes *in vitro* based on a heightened TNF secretion after stimulation by trained monocyte-derived macrophages with *Escherichia coli* LPS. Sorted CD14-expressing monocytes (classical and intermediate monocytes), as well as single populations of sorted classical, intermediate and non-classical monocytes could not be trained by βG, whereas macrophages derived from plastic-adherent mononuclear cell preparations displayed features of a trained function. The hypothesis, that non-classical monocytes need to be present in a mixed monocyte population in order to be trained by βG could be verified by a successful training of positively sorted whole monocyte populations (CD14CD16/M) containing all three monocyte subpopulations. The trainability depended on conditions favoring M1 polarization of macrophages. Altogether, innate immune training of bovine monocytes seems to depend on the presence of non-classical monocytes. This adds new information to the role of this monocyte subpopulation in the bovine immune system.

Keywords

β-glucan, bovine, immune training, macrophages, monocytes

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Introduction

The term innate immune memory describes the phenomenon where contact of innate immune cells with a pathogen or pathogen-associated molecular pattern (PAMP) leads to an altered reaction to subsequent pathogen or PAMP contact. Innate immune memory can be acquired in one of two ways, either through a lower secondary reaction, called tolerance, or through a stronger secondary reaction, called innate immune training or trained immunity.[1] Innate immune training was described as a feature of the Bacille Calmette-Guérin (BCG) vaccine, directed against human tuberculosis, as early as 2012.[2] Importantly, innate immune training also involves a heightened secondary response to different pathogens or PAMPs. In case of the BCG vaccination, human peripheral blood mononuclear cells (PBMCs) display a stronger pro-inflammatory response against *Staphylococcus aureus* and *Candida albicans*. A similar training effect was shown with *Candida albicans*, leading to a stronger reaction against *Mycobacterium tuberculosis,* mediated by *Candida albicans* cell wall β–Glucan (βG). βG induces training in human CD14+ monocytes, which is at least partially dependent on complement receptor 3 and Dectin-1.[3] In the following years, βG was used as a positive control for training experiments in human plastic-adherent monocytes and the kinetics were explored, too.[4,5] Treatment with βG leads to epigenetic changes, which form the basis for the altered reaction pattern towards pathogens later on. In addition to a different inflammatory reaction, epigenetic signatures of metabolic pathways are also changed by βG.[6]

In bovines, little is known about innate immune memory. Although old reports about innate immune tolerance exist,[7,8] innate immune training in bovines has only been evaluated with BCG. BCG induces innate immune training *in vivo* and *in vitro*, characterized by a higher IL-1β, TNF and IL-6 secretion by mononuclear cells from BCG-vaccinated animals. A different reaction was observed towards LPS and Pam3CSK4, agonists of

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Toll-like receptor (TLR)4 and TLR2/TLR1, respectively and changes in TNF secretion could be followed up for 12 weeks after vaccination.[9] In addition, innate immune training in bovine was demonstrated by a higher phagocytic activity of neutrophilic granulocytes from vaccinated animals. Of note, this was only shown for a second contact with the same pathogen.[10] While these first results in bovine look promising, their relevance for practical application is questionable. As BCG vaccination of cattle interfere with tuberculin skin tests, they cannot be established in countries using this screening method for tuberculosis.[11] This prompted us to investigate whether βG can serve as an alternative for the induction of bovine innate immune training. Just recently, Pedro et al. showed that particulate βG, as used herein, leads to a proinflammatory response by bovine monocytes and they speculated, that trained immunity could be induced by βG in bovines.[12] Furthermore, we focused on the involvement of the different monocyte subpopulations in innate immune training, an issue not addressed so far in the human, bovine or murine system.[13,14] As in humans, bovine monocytes are divided into three subsets characterized by their expression of CD14 and CD16, with classical monocytes expressing mainly CD14, intermediate monocytes expressing both surface markers and non-classical monocytes expressing mainly CD16.[13]

Methods

Isolation of bovine mononuclear cells

Heparinized venous blood from the left jugular vein was taken from 5–6 healthy, non-lactating, non-pregnant Holstein-Frisian cows, aged 8.6 ± 3.0 years, housed at the Clinic for Cattle, University of Veterinary Medicine, Foundation, Hanover, Germany. Blood was drawn into heparinized vacutainer (Becton Dickinson, Heidelberg, Germany) and PBMCs were isolated by density centrifugation over lymphocytes separation media® (Capricorn Scientific GmbH). After centrifugation (1000 x *g*, 30 Min, 4°C and 500 x *g*, 10 min, 4°C), contaminating erythrocytes were lysed by adding 20 ml distilled water for 10 s. After adding the same volume of double-concentrated PBS, cells were washed twice (250 x *g* and 120 x *g*, 10 Min, 4°C).

Generation of plastic-adherent monocytes (PaIM)

2×10^6 PBMCs per well were cultured in 24-well-plates for 20 h (37°C, 5% CO_2 in air). Non-adherent cells were removed by washing the wells with 1 ml warm PBS.

Isolation of CD14+ monocytes (classical and intermediate monocytes; CD14/M)

PBMCs were filtered through pre-separation filters (MACS Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to remove cell aggregates. Afterwards, cells were incubated with 10 µl of paramagnetic anti-CD14 beads (MACS Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) per 1×10^7 vital PBMCs for 30 min at 4°C. Unbound beads were removed by washing the cells once with 10 ml MACS buffer (PBS, 5 g/l bovine serum albumin (BSA), 1.344 g/l EDTA) (400 x *g*, 10 min, 4°C). Cells were resuspended in 3 ml MACS buffer, added to a LS separation column and mounted into a Quadro MACS station. After the elimination of flow-through cells, CD14-expressing cells were harvested by removing the column from the Quadro MACS station and rinsing it with 5 ml MACS buffer.

Isolation of classical (cM), intermediate (intM) and non-classical (ncM) monocytes

Classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical monocytes (CD14-CD16++) were isolated by a two-step MACS procedure, essentially described in Hussen et al..[15] Isolated PBMCs were labeled with FITC-conjugated anti-CD16 antibodies by adding 10 µl of antibody solution to 1×10^7 PBMCs for 30 min (4°C). Unbound antibodies were removed by washing the cells twice with 10 ml MACS buffer. Subsequently, cells were labeled with 10 µl paramagnetic anti-FITC beads (30 min, 4°C). Unbound beads were removed by washing with 10 ml MACS buffer (400 * *g*, 10 Min, 4°C). Cells were subsequently added to a LS separation column in a Quadro-MACS station. CD16-negative cells were harvested from the flow-through and CD16-positive were harvested by removing the column from the Quadro-MACS station and rinsing it with 5 ml MACS buffer. Positively selected CD16+ cells were incubated with 100 µl of release reagent (MACS Miltenyi Biotec GmbH, Bergisch Gladbach) to remove the bound paramagnetic particles and placed onto a new LS separation column. CD16+ cells with no adherent paramagnetic particles were thus collected in the flow-through. Those CD16+ cells and the CD16- cells were labeled with 10 µl of anti-CD14-beads per 1×10^7 cells (30 Min, 4°C). 50 µl stop reagent was added simultaneously to the CD16+ cells to inhibit the release reagent. Afterwards, cells were washed once to remove unbound beads (400 * *g*, 10 Min, 4°C). Cells were placed on a LS separation column in a Quadro-MACS station and flow-through (CD14-) as well as magnetically labeled cells inside the column (CD14+) collected. In detail, the flow-through of the CD16+ cells contained ncM (CD14-CD16++), the magnetically labeled CD16+ cells were intM (CD14+CD16+). Magnetically labeled CD16- cells were cM (CD14+CD16-), while the flow-through of the CD16- cells contained no monocytes (CD14-CD16-). All obtained cell suspensions were characterized by flow cytometry for their expression of CD14 and CD16 (Figure 2). Classical monocytes (CD14+CD16-; cM), intermediate monocytes (CD14+CD16+; intM) and non-classical monocytes (CD14-CD16+, ncM) were used for training experiments.

Isolation of whole monocyte populations (CD14CD16/M)

Monocytes composed of all subpopulations (cM, intM and ncM) were positively separated by a single step MACS procedure. Isolated bovine PBMC were simultaneously incubated with anti-CD14 beads and anti-CD16 beads (10 µl/1 $\times 10^7$ vital PBMC, 30 min, 4°C). Cells were washed with 10 ml MACS buffer (400 * *g*, 10 Min, 4°C) and resuspended in 3 ml MACS buffer. Sorting was done as described above. This resulted in monocyte purity of 87–94% of all measured cells and those monocytes had a viability of 99.6 to 99.9%.

Training and polarization of monocytes

For training experiments, different numbers of cells were seeded in 1 ml in 24-well plates. For PA/M, 2×10^6 PBMCs were seeded. PBMC preparations contained up to 20% monocytes. Therefore, CD14/M and CD14CD16/M were seeded in a final cell number of 4×10^5 cells. For cM, intM and ncM, 2×10^5 cells/well were seeded. Cells were cultured in modified RPMI medium (cRPMI), supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, HEPES, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol as stated in Guerra-Maupome et al.[9] On day 1 of culture, cells were washed once with 1 ml of PBS. After that, the cells were supplied with 1 ml fresh medium (control) or medium containing WGP Dispersible (InvivoGen, tlrl-wgp, wgp: whole glucan particles of *Saccharomyces cerevisiae* lacking TLR-stimulating activity; referred to as βG) at indicated concentrations. On day 2 of culture, cells were washed once with warm medium and supplied with fresh medium. On day 4 of culture, medium was removed and replaced by 1 ml fresh medium (control) or with 10 ng LPS from *E. coli* O111:B4 (Merck KGaA). To parallel set ups, recombinant bovine GM-CSF and recombinant bovine IFN-γ (Biomol, 35 µl stock solution, each 20 ng/ml final) was added daily to promote differentiation of monocytes into M1 macrophages (M1 Mph). Cultures supplemented daily with the same amount of PBS are referred to as M0 Mph (Figure 1).

Antibodies and flow cytometry

PBMC and sorted monocyte populations were labeled with primary antibodies to identify monocyte subpopulations. Cells were washed once in 200 µl membrane immunofluorescence buffer (MIF buffer, PBS, 0.5% PBS, 0.01% NaN$_3$). After centrifugation (350 x *g*, 4°C, 4 Min), the supernatant was discarded and cells were resuspended for 30 Min (4°C) in 30 µl MIF buffer containing a mixture of two murine bovine cross-reactive monoclonal antibodies (anti-CD14,

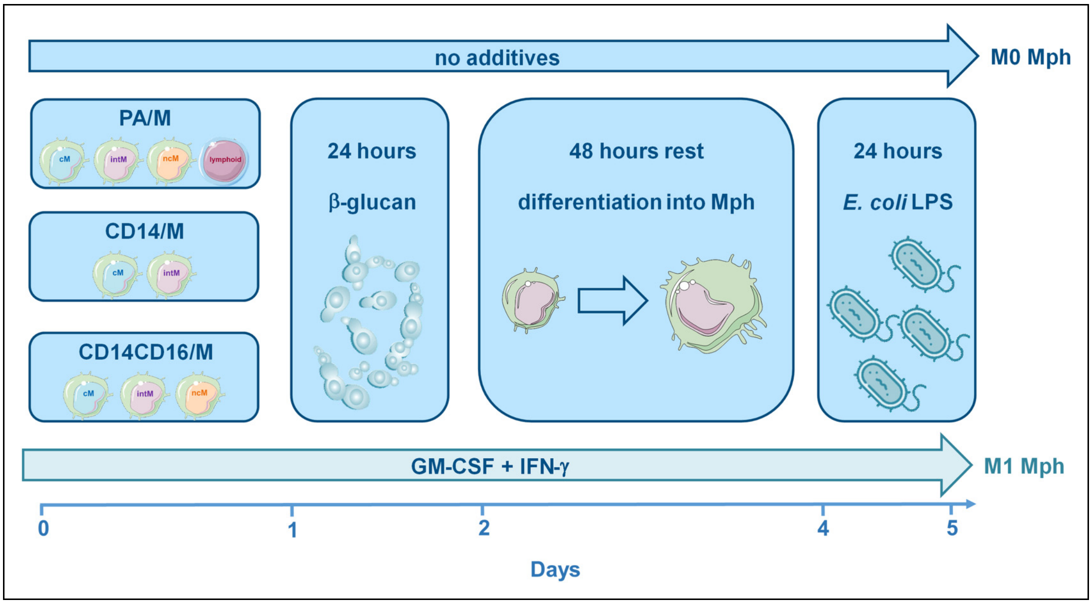

Figure 1. Protocol for βG-training. Monocytes were isolated using different techniques, resulting in different seeded cell compositions. Those cells were conditioned with βG on day 1, supernatant were removed on day 2 and fresh medium was added. On day 4, cells were stimulated with LPS. Cells were termed as M0 Mph and analyzed along with the supernatant on day 5. In another attempt, the protocol was modified by daily supplementation of GM CSF and IFN-γ. In this approach, cells were termed M1 Mph on day 5. PA/M: Plastic adherent monocytes, CD14/M: CD14 expressing monocytes, CD14CD16/M: CD14- or CD16 expressing monocytes, βG: β glucan, LPS: Lipopolysaccharide, Mph: Macrophages, E. coli: Escherichia coli, GM CSF: Granulocyte macrophage colony stimulating factor, IFN-γ: Interferon γ.

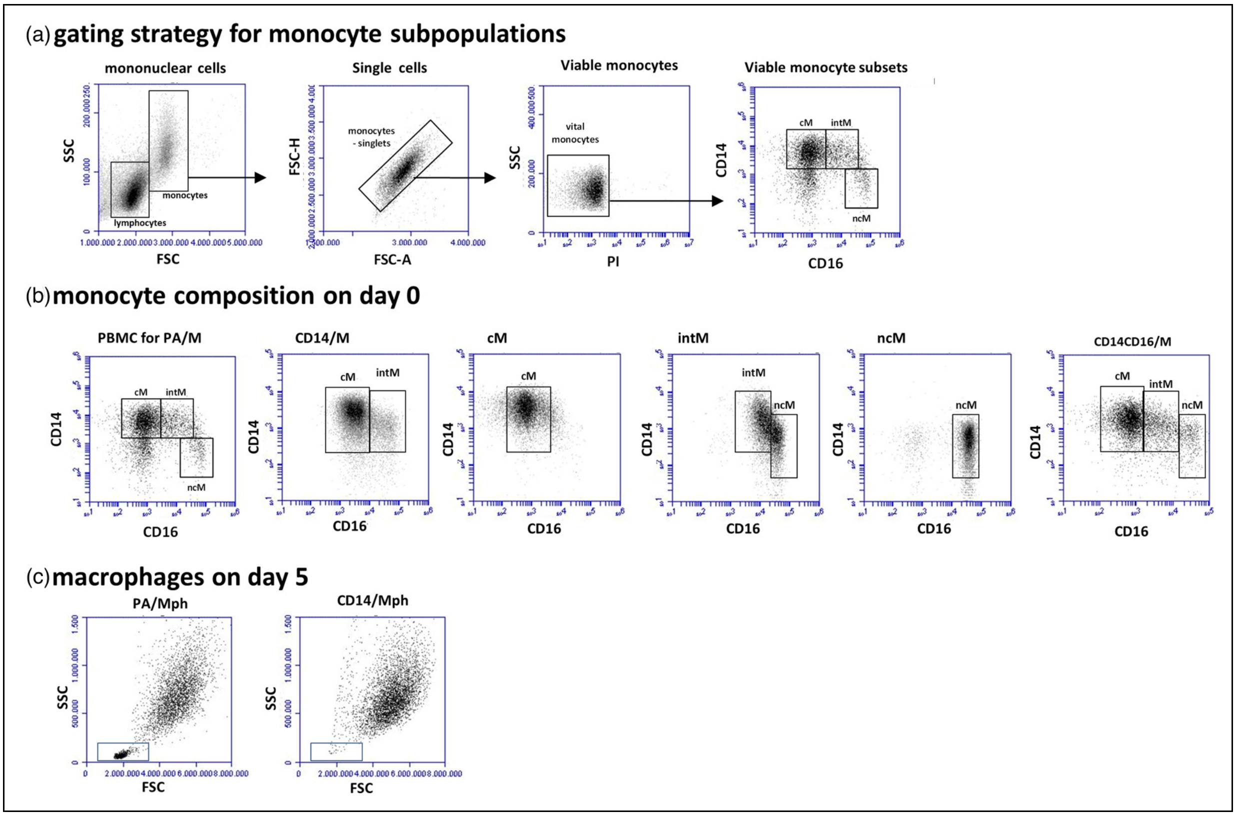

Figure 2. Flow cytometry of isolated PBMC, monocytes and macrophages. Peripheral blood mononuclear cells and monocytes were isolated and stained with antibodies against CD14 and CD16 as well as with propidium iodide. Gating of stained cells is shown in a. Monocyte subset content in the differently isolated PBMC and monocytes is shown in b. Obtained cells after culturing of PBMC and CD14/M for 5 days is shown in c. PBMC: Peripheral blood mononuclear cells, FSC: Forward scatter, SSC: Sideward scatter, FSC A: Forward scatter area, FSC H: Forward scatter area, PI: Propidium iodide, CD: Cluster of differentiation, PA/M: Plastic adherent monocytes, CD14/M: CD14 expressing monocytes, CD14CD16/M: CD14- or CD16 expressing monocytes, cM: Classical monocytes, intM: Intermediate monocytes, ncM: Non classical monocytes, PA/Mph: Macrophages derived from PA/M, CD14/Mph: Macrophages derived from CD14/M.

RRID AB_566517, IgG2a, clone TÜK4, 1:45 final; anti-CD16, RRID AB_10961759, IgG2a, clone KD1, 1:45 final, both Bio-Rad). Cells were washed twice with 200 µl MIF buffer and resuspended in 100 µl MIF buffer for analysis by flow cytometry.

Differentiated and adherent macrophages (day 5 of culture) were detached by adding 200 µl Accutase/well (20 Min, 37°C). Detachment was stopped by addition of culture medium. Cells were washed once in 200 µl MIF buffer (351 x *g*, 4 Min, 4°C). Suspended cells were incubated (30 min, at 4°C) with an ovine-specific (bovine cross-reactive) murine monoclonal antibody specific for MHC class-II (anti-MHC-II-FITC, RRID AB_323966, IgG2a, clone 37.68, final 1:45 in MIF buffer). Cells were washed twice with 200 µl MIF buffer (351 x *g*, 4 Min, 4°C) and resuspended in 100 µl of MIF buffer for analysis by flow cytometry.

Propidium iodide (2 µg/ml final) was added to labeled cells to exclude necrotic and late apoptotic cells. Cells were measured by flow cytometry (Accuri flow cytometer, BD Biosciences) and data were analyzed using the BD Accuri™ C6 software (BD Biosciences).

TNF quantification

TNF amounts were measured using TNF Duoset® ELISA (R&D Systems), according to the manufacturer's instructions. In short, 96-well Nunc Maxisorp™ plates (ThermoFisher Scientific, Waltham, MA) were coated for 18 h with a monoclonal antibody specific for bovine TNF. Plates were washed three times with a wash buffer (PBS, 0.05% Tween® 20) and blocked with reagent diluent (PBS, 5% Tween® 20) for 60 min. After washing, 100 µl of cell culture supernatants, culture medium (negative control) and a dilution series of the TNF standard were added. Plates were incubated for 120 min at room temperature with permanent lateral shaking. After washing, 100 µl of the secondary antibody diluted in reagent diluent with heat-inactivated normal goat serum were added and plates were incubated for 120 min with lateral shaking at room temperature. After washing, 100 µl Streptavidin-horseradish peroxidase (1:400 diluted in reagent diluent) was added. The plates were incubated for 20 min in the dark at room temperature and washed again. 1 ml 3,3′, 5,5′-Tetramethyl[1,1′-diphenyl]-4,4′-diamin (1 mg/ml DMSO) and 40 µl H_2O_2 (3% v/v) were mixed with 10 ml of substrate buffer (Aqua dest., 6.398 g/l citric acid, 11.866 g/l disodium hydrogen phosphate) and 100 µl were added per well. The enzymatic reaction was stopped by the addition of 50 µl 1 N H_2SO_4. The optical density was determined by a microplate reader set to 450 nm, wavelength correction was set to 540 nm. The standard curve was created by four-parameter logistic regression using GraphPad Prism.

Statistical analysis

Statistical analysis was performed using SAS Enterprise Guide, Version 7.12 (SAS Institute Inc.). In case of normal distribution, paired Students t-test and ANOVA was applied. In case of non-normal data distribution, Wilcoxon signed-rank test and Friedman test were used to test for significance of differences. The relation between memory effect and cell subpopulations was checked for significance using the Spearman signed rank test. A Memory Effect (M_e) was calculated as stated in equation (1).

$$M_e = \frac{(pg/ml)TNF_{(cond,\,stim)}}{(pg/ml)TNF_{(stim)}} \tag{1}$$

Calculation of memory effect

TNF concentrations in supernatants of βG-conditioned (cond) and LPS-stimulated (stim) monocyte-derived macrophages were divided by TNF concentrations in supernatants of non-conditioned and LPS-stimulated monocyte-derived macrophages.

Results

Monocytes were separated in different ways to be used for training experiments. Isolated PBMCs contained 11.5–22.0% monocytes, composed of 48.3–71.3% cM, 16.8–44.1% intM, and 7.6–13.6% ncM as well as lymphocytes (Figure 2b, PBMC for PA/M). Those PBMCs were cultured overnight and washed the following morning to select adherent cells. This procedure led to a fraction of contaminating lymphocytes in the macrophages at day 5 between 3.9% and 12.8% (Figure 2c). Monocytes isolated as CD14+PBMCs had a purity of 52.4–91.1% (% monocytes of all cells, data not shown) and contained no lymphoid cells on day 5 (Figure 2c). Those monocytes are referred to as CD14/M and were composed of cM and intM, while ncM were absent (Figure 2b CD14/M). Isolation of single monocyte subsets resulted in populations of cM (median 74.1% cM of vital cells; Figure 2b cM), whereas intM and ncM (Figure 2b intM/ncM) had a lower purity (56.9% intM of vital cells; 51.4% ncM of vital cells). The established method to isolate mixed populations composed of cM, intM, and ncM resulted in purities of 87–94%. Those cells are referred to as CD14CD16/M and were composed of 50.3–76.8% cM (coefficient of variation 14.93%), 11.6–35.3% intM (coefficient of variation 45.89%), and 6.3–17.6% ncM (coefficient of variation 31.88%). CD16+CD335+natural killer cells were absent in this monocyte preparation (Figure 2b CD14CD16/M, Figure S2).

βG conditioning differentially affects macrophage viability

Numbers of vital macrophages differentiated from PA/M, CD14/M, or CD14CD16/M under M1-polarizing conditions were higher than those of Mph differentiating under M0 conditions (Figure 3). The number of viable M0 or M1 Mph were not altered significantly by βG-conditioning (Figure 3). If Mph were stimulated with LPS, βG-conditioning resulted in a significantly higher number of harvested M0 and M1 Mph derived from CD14CD16/M (Figure 3e and f).

Influence of βG-conditioning on M1-marker expression depends on the monocyte preparation

M1 Mph significantly expressed higher levels of MHC class II molecules. Based on mean fluorescence intensity levels, the fold changes (M1 versus M0) were 1.77 (PA/Mph), 2.68 (CD14/Mph), and 2.23 (CD14CD16/Mph) (Figure 4). LPS stimulation significantly lowered the MHC class II expression of M0 PA/Mph but not M0 CD14/MPh and M0 CD14CD16/MPh (Figure 4a, c and e). In addition, only M0 PA/Mph displayed a significantly reduced MHC II expression after conditioning with βG (Figure 4a). LPS stimulation of all macrophage populations differentiating under M1-polarizing conditions resulted in a significantly reduced MHC-class II expression density (Figure 4b, d and f). βG-conditioning reduced MHC class II expression only in M1 PA/Mph and M1 CD14/Mph (Figure 4b and d). In M1 CD14/Mph the βG conditioning significantly pronounced the LPS stimulation-induced drop of MHC class II expression.

Monocyte preparations differ in their response to βG

PA/M cultured with or without M1-polarizing cytokines secreted TNF in response to βG conditioning (Figure 5a and b), whereas βG-induced TNF secretion could not be observed by CD14CD16/M (Figure 5c and d). After a resting period of two days, neither control nor βG-conditioned M0 or M1 CD14/M secreted TNF in detectable amounts (Figure 6a and b). After the resting period, no TNF was detectable in medium controls or βG-conditioned M0 or M1 CD14CD16/Mph (Figure 6h and i).

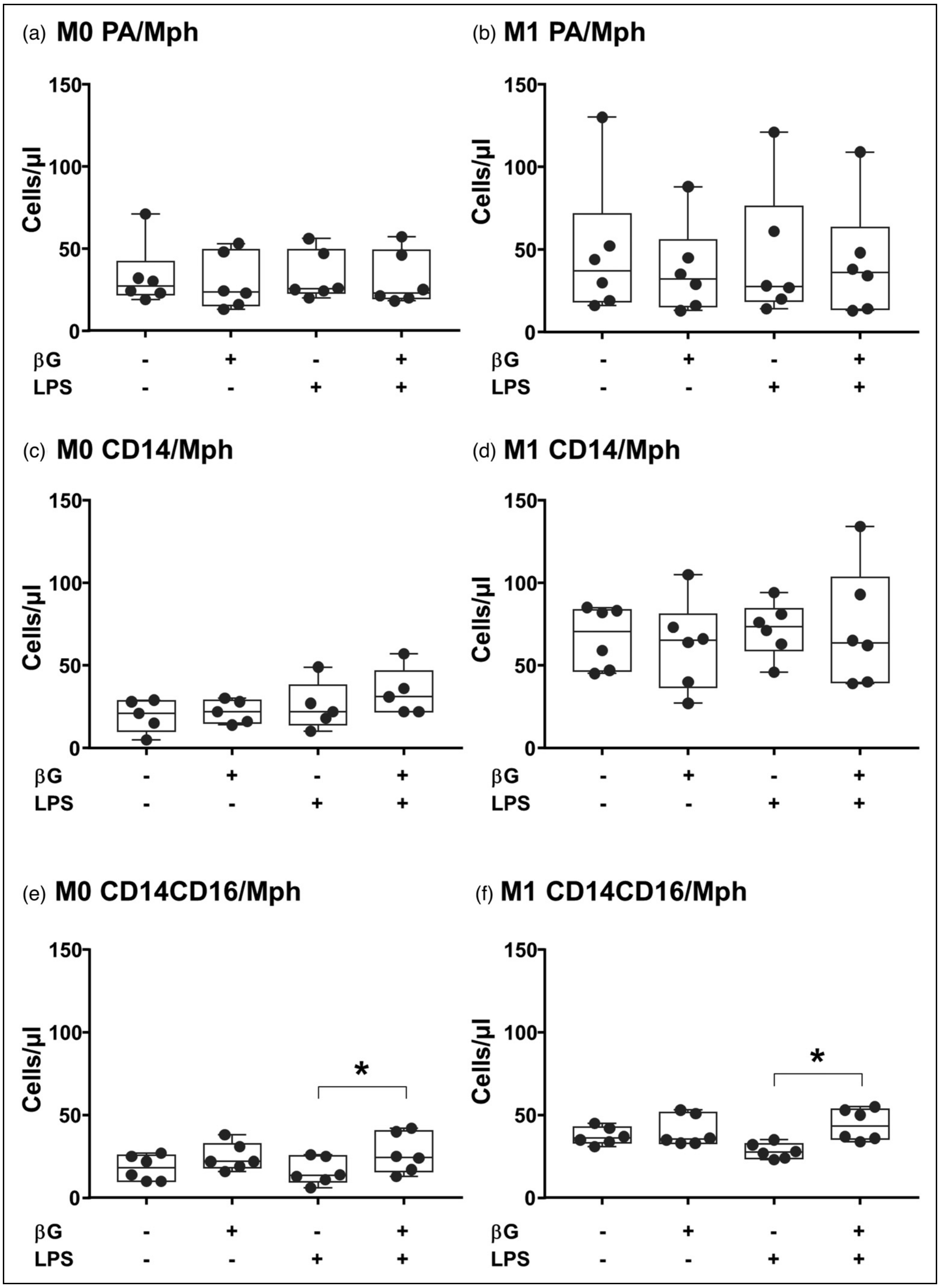

Figure 3. Viable macrophages. Monocytes were selected as PA/M (a, b), CD14/M (c, d) or CD14CD16/M (e, f) and submitted to the protocol seen in Figure 1, shortly cells were conditioned with 10 µg of βG and stimulated with 10 ng of LPS, with a 48 h-break in between. Daily supplementation with GM-CSF and IFN-γ resulted in M1 Mph, displayed in b, d and f. Viable macrophages were measured by flow cytometry, morphologically gating on macrophages and excluding propidium-iodide-positive cells. Shown are the number of macrophages in one metered µl. Statistical differences are indicated (*$P < 0.05$). PA/Mph: Plastic adherent macrophages derived from PA/M, CD14/Mph: Macrophages derived from CD14/M, CD14CD16/Mph: Macrophages derived from CD14CD16/M, βG: β-glucan, LPS: Lipopolysaccharide, Mph: Macrophages, GM-CSF: Granulocyte macrophage colony stimulating factor, IFN-γ: Interferon γ.

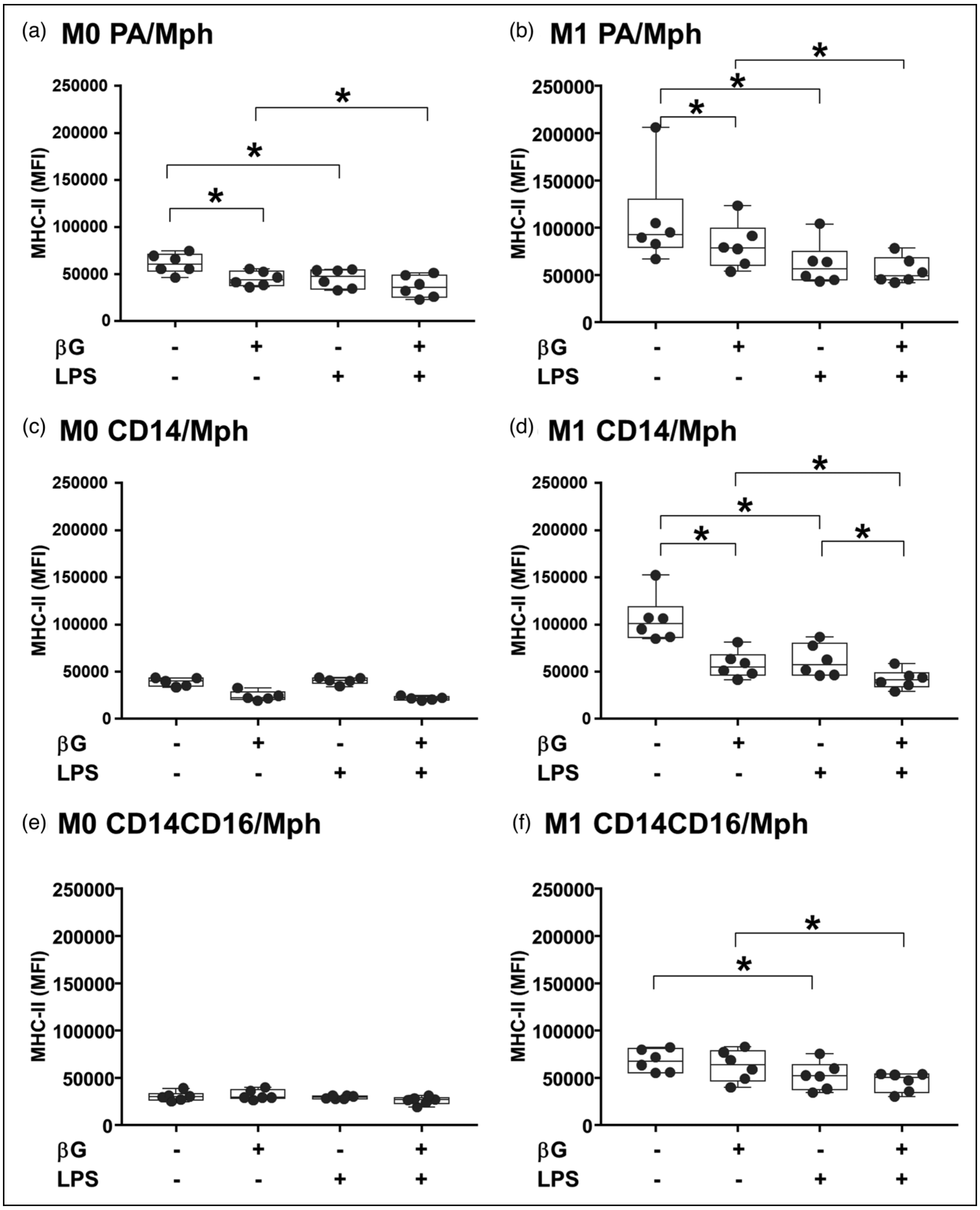

Figure 4. Phenotype of bovine macrophages. Monocytes were selected as PA/M (a, b), CD14/M (c, d) or CD14CD16/M (e, f) from six different animals and submitted to the protocol seen in Figure 1, shortly cells were conditioned with 10 μg of βG and then stimulated with 10 ng of LPS, with a 48 h-break in between. Daily supplementation with GM-CSF and IFN-γ occurred in b, d and f. Expression of MHC II was measured by immunofluorescence and flow cytometric detection after exclusion of dead cells. Statistical significant differences are indicated (*$P < 0.05$). MHC-II: Major histocompatibility complex II, PA/Mph: Plastic adherent macrophages derived from PA/M, CD14/Mph: Macrophages derived from CD14/M, CD14CD16/Mph: Macrophages derived from CD14CD16/M, βG: β glucan, LPS: Lipopolysaccharide, GM CSF: Granulocyte macrophage colony stimulating factor, IFN-γ: Interferon γ.

Purified bovine monocyte subsets and CD14 + monocytes cannot be trained by ßG

Whereas M1 CD14/Mph secreted TNF after LPS stimulation (Figure 6B), neither M1 cMph, M1 intMph, nor M1 ncMph secreted TNF after LPS stimulation (Figure 6c, d and e). βG-conditioned Mph derived from CD14+ monocytes (CD14/MPh, Figure 6a) and Mph derived from purified cM, intM, and ncM (cMph, intMph, ncMph, Figure 6c, d and e) displayed no enhanced TNF secretion after LPS stimulation.

PA/M and CD14CD16/M can be trained by βG

βG-conditioned PA/Mph and M1 CD14CD16/Mph secreted significantly higher TNF amounts after LPS

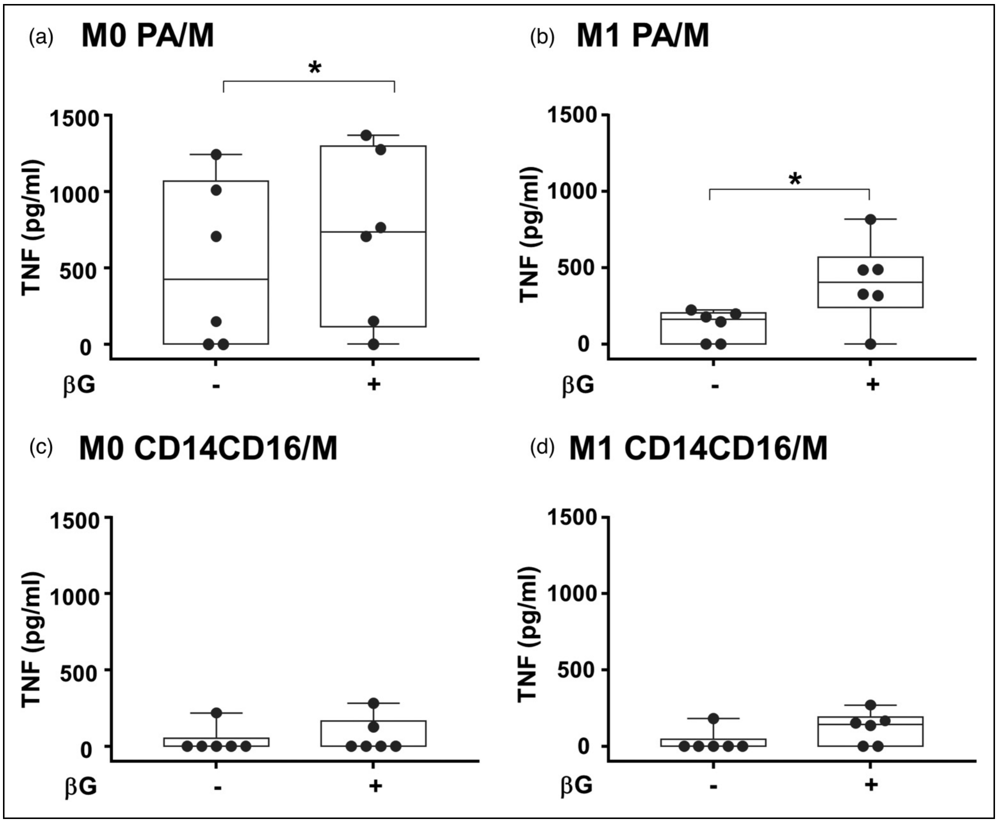

Figure 5. TNF secretion after βG treatment. Monocytes were selected as PA/M (a, b) or CD14CD16/M (c, d) and treated with 10 µg βG on day 1 for 24 h. Supernatant was collected and TNF contents measured by ELISA. Daily supplementation with GM-CSF and IFN-γ occurred in b and d. Statistical significant differences are indicated ($*P < 0.05$). PA/M: Monocytes isolated by plastic adherence, CD14CD16/M: Monocytes expressing CD14 and / or CD16, βG: β-glucan, GM-CSF: Granulocyte macrophage colony stimulating factor, IFN-γ: Interferon γ, ELISA: Enzyme-linked immunosorbent assay, TNF: Tumor necrosis factor.

stimulation. Although the training effect of βG could be observed in M0 PA/Mph (Figure 6f), the LPS response was more pronounced with M1 Mph (Figure 6g and i; Table 1).

To evaluate the impact of the cellular composition of PA/M at the time of βG conditioning for the memory effect (M_e), we correlated M_e with fractions of $CD2^+$ lymphoid cells ($CD2^+$, $CD2^+CD4^+$, $CD2^+CD8^+$), cM, intM, and ncM among the PBMC before seeding. The fraction of ncM showed a high positive and significant correlation with the training effect ($R = 0.89$, $p = 0.02$) (Supplemental Table 1).

Discussion

The analysis of trained innate immunity with focus on primary macrophages requires the separation of their precursors from blood[5,16–18] or bone marrow[19] followed by defined culture conditions. The protocols described for human monocytes usually involve the purification of monocytes by plastic-adherence or by magnetic-activated negative selection of monocytes.[17,18] Our initial protocol was based on studies with human monocytes[5] and murine macrophages.[19] In accordance to[5] we incubated bovine cells with βG for 24 h and stimulated macrophages with 10 ng LPS for 24 h. This approach did not induce a consistent TNF release in M0 MPh, however, trained immunity could be detected in bovine macrophages despite this suboptimal LPS concentration. Between conditioning/training with βG and LPS stimulation we chose a resting period of 48 h, as a study in mice showed a training effect with 24 h and 72 h resting periods.[19] For training, we used βG from *Saccharomyces cerevisiae* preparation used for training in murine macrophages,[19] which was also shown to induce cytokine secretion by bovine monocytes.[12] The concentrations of βG were chosen according to concentrations used in training experiments with human monocytes.[5,17,18] Whereas a trained phenotype of human macrophages could be achieved with 1 µg βG derived from *C. albicans*, bovine monocytes had to be trained with at least 5 µg βG from *Saccharomyces cerevisiae* to achieve a more robust TNF release after LPS stimulation (Supplemental Figure 1). This could be due to a species-specific different sensitivity of monocytes towards βG or may reflect different affinities

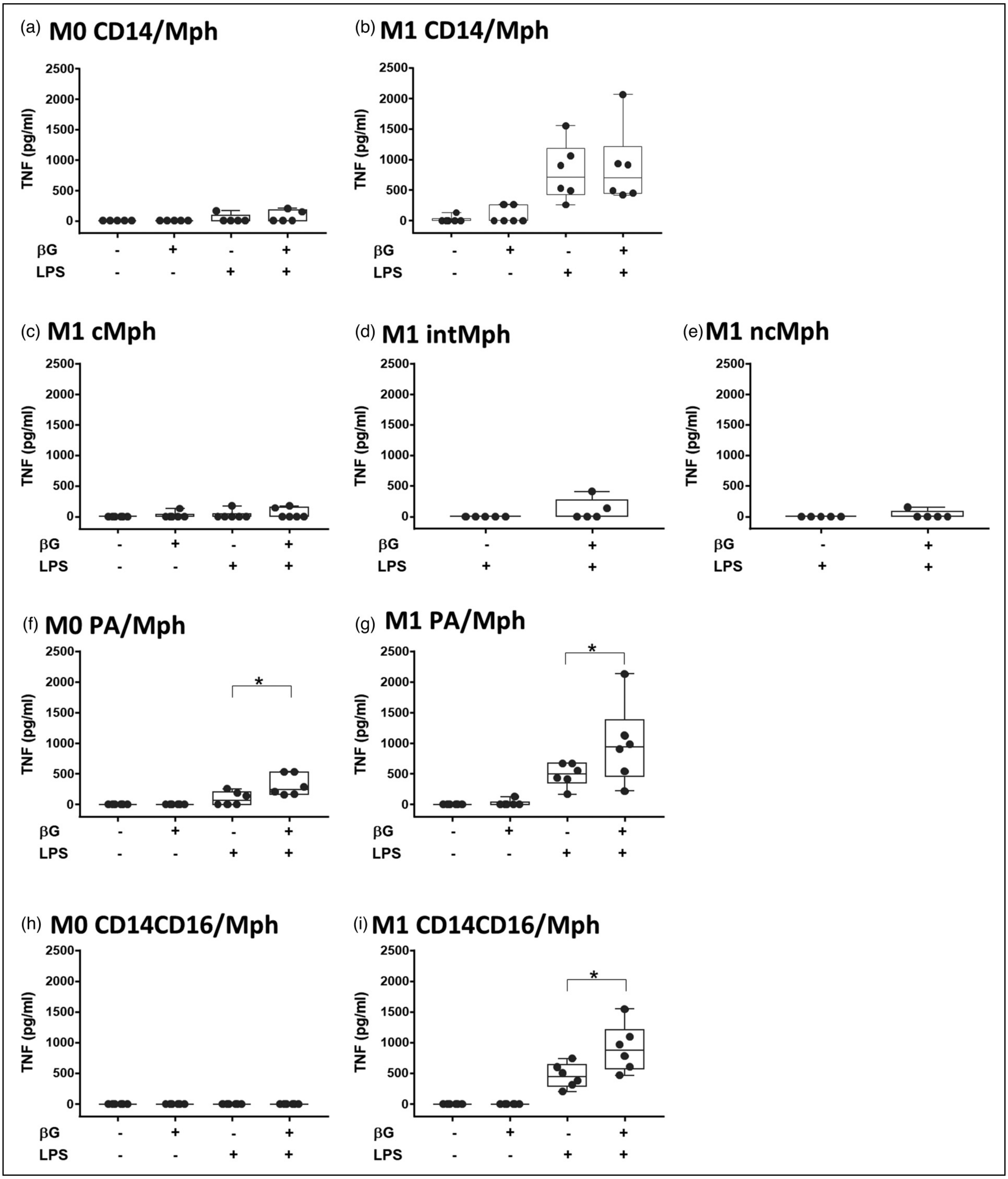

Figure 6. TNF secretion of bovine macrophages. Monocytes were selected as CD14/M (a, b), single monocyte subsets (c, d, e), PA/M (f, g) or CD14CD16/M (h, i) from six different animals and submitted to the protocol seen in Figure 1. Briefly, cells were conditioned with 10 µg of βG and then stimulated with 10 ng of LPS, with a 48 h-break in between. Daily supplementation with GM-CSF and IFN γ occurred in b, c, d, e, g and i to polarize monocytes into M1 Mph. TNF amounts were measured by ELISA in the supernatants at the end of the experiment. Statistical differences are indicated (*$P < 0.05$). PA/Mph: Plastic adherent macrophages derived from PA/M, CD14/Mph: Macrophages derived from CD14/M, CD14CD16/Mph: Macrophages derived from CD14CD16/M, cMph: Macrophages derived from classical monocytes, intMph: Macrophages derived from intermediate monocytes, ncMph: Macrophages derived from non classical monocytes, βG: β glucan, LPS: Lipopolysaccharide, Mph: Macrophages, GM-CSF: Granulocyte macrophage colony stimulating factor, IFN-γ: Interferon γ, ELISA: Enzyme-linked immunosorbent assay, TNF: Tumor necrosis factor.

Table 1. Trainability of different macrophage preparations.

Macrophage population	Seeded monocyte subpopulation	Macrophage polarization	Trainability
PA/Mph	cM, intM, ncM	M0	Yes
		M1	Yes
CD14/Mph	cM, intM	M0	No
		M1	No
cMph	cM	M1	No
intMph	intM	M1	No
ncMph	ncM	M1	No
CD14CD16/ Mph	cM, intM, ncM	M0	No
		M1	Yes

of *C. albicans* and *S. cervevisiae* βG sources for the receptor (Dectin-1) expressed on monocytes. A recent study underlined the importance of the kind of βG, especially with regard to the potential to not only bind but also to activate Dectin-1.[20]

The adaption of the commonly used strategy to train MACS-separated monocytes proved to be insufficient to induce training in bovine CD14+ monocytes (Figure 6a and b). Whether this was the consequence of an antibody-mediated pre-activation of cells after positive MACS separation could not be resolved since, to the best of our knowledge, negative selection of bovine monocytes is not possible at the moment. However, the proof that positively selected whole bovine monocytes populations (CD14CD16M, see below) can be trained, argues against a mere technical reason for the inability to train bovine CD14+ monocytes with βG.

Such positively selected bovine CD14+ monocytes contained both classical and intermediate monocytes (Figure 2b), whereas negatively selected human monocytes were composed of classical monocytes only.[17] Interestingly, classical human monocytes also could not be trained by βG (*C. albicans*), which supports our finding that in vitro models with single monocyte subsets may be insufficient to analyze innate immune training. Other in vitro models took advantage of mixed monocytes populations. In the human system these are, for instance monocyte preparations obtained after negative selection of monocytes from PBMC after depletion of CD3-, CD19- and CD56-expressing cells without characterizing the obtained monocyte subpopulations regarding their CD14 and CD16 expression.[18] Using such monocyte preparations, putatively containing all monocytes subpopulations, their trainability could be demonstrated.[18]

Indirectly, we could demonstrate the trainability of mixed/complete monocyte subpopulations with macrophages generated from plastic-adherent monocytes (PA/M) (Figure 6f and g). The approach to use plastic adherent cells from PBMC preparations was used in several human monocyte studies,[5,16] although the experimental details differed. We were not able to prove that bovine cM, intM, and ncM adhered to the same extent to the plastic and that differentiated Mph developed from all subsets. Moreover, this approach resulted in an insufficient purity of Mph, with contaminating lymphoid cells still present on day 5 (Figure 2c). Indirect evidence, that these contaminating lymphocytes play no decisive role for the βG-induced memory effect was the lack of correlation between memory effects and individually different fractions of CD2+ lymphoid cells at the time of PBMC seeding (Table S1). In contrast, however, the fraction of non-classical monocytes among all monocytes in the PBMC fraction correlated strongly and significant with the M_e (Table S1). This could serve as a strong indication that the presence and the amount of bovine ncM is crucial for the induction of trained in immunity in bovine macrophages.

To analyze this further and since we could not fully rule out a potential role of contaminating lymphocytes among PA/Mph and their potentially secreted training-regulating mediators, we established a MACS-based separation protocol for all monocyte subpopulations by using CD14- and CD16-specific para-magnetically-labeled antibodies simultaneously. The positively selected cell populations were indeed composed of cM, intM, and ncM (Figure 2b), of which intM and ncM showed the highest variation between individuals, and did not contain CD16+/CD335 + natural killer cells (Figure S2). The trainability of this monocyte preparation (CD14CD16/M) by βG further strengthened the hypothesis that non-classical monocytes are necessary to achieve trained immunity in cattle. We hypothesize that different monocyte subsets have to interact directly or indirectly with each other to achieve training as we could not detect training when seeding single monocyte subsets.

The different trained phenotypes of bovine Mph were not due to a differential viability-modulating effect of βG conditioning (Figure 3) but depended on monocyte-macrophage differentiation conditions: With the exception of M0 PA/MPh (Figure 6f) we only noted a trained or enhanced trained phenotype (Figure 6g) when monocytes were cultured in the presence of M1-polarzing cytokines (Figure 6i).

An enhanced MHC-II expression is one feature of M1 macrophages[21] and we noted this kind of enhanced expression on macrophages after addition of M1-polarizing cytokines to PA/M, CD14/M, and CD14CD16M (Figure 4 ACD versus BDF). The expression level of MHC-II molecules/cell differed between bovine Mph populations. Notably, M1 CD14CD16/Mph expressed only half of the amount compared to M1 PA/Mph or M1 CD14/Mph. This may suggest that different bovine monocyte preparations respond differently to GM-CSF and IFN-γ, probably regulated by mediators secreted from contaminating cells. We tested the hypothesis, that there is endogenous production of IFN-γ by seeded cells, but did not found consistent levels of IFN-γ 24 h after addition of cytokines regardless of the seeded cell population (Table S2). However, the magnitude of MHC-II expression after bovine macrophage differentiation in vitro does not seem to correlate with a functional trained phenotype (compare Figure 4 and 5). We hypothesized, that the decrease in MHC-II expression after LPS and βG stimulation may have been mediated by IL-10. LPS and βG have been shown to induce IL-10 in bovine monocytes,[12,22] and IL-10 was shown to counteract an IFN-γ-induced upregulation of MHC-II on human macrophages.[23] In line with those results, we noticed enhanced levels of IL-10 in the supernatants of βG-treated M1-macrophages of all kinds, whereas LPS did not lead to a stronger secretion of IL-10 in our settings (Figure S3).

Overall, we found evidence for a contribution of non-classical monocytes in the context of bovine monocyte/macrophage trained immunity. The task for the future is a deeper characterization of crucial interactions with other monocyte subsets, the identification of the actual cellular subset secreting higher TNF amounts after secondary stimulation of complex macrophage populations, and the unraveling of underlying mechanistic events.

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Data availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon reasonable request.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

This study was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (33.19-42502-05-17A176). All procedures involving animals were carried out in accordance with German legislation on animal welfare.

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Supplemental material

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