Short communication

A method to differentiate chicken monocytes into macrophages with proinflammatory properties

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A B S T R A C T

Macrophages are part of the first line of defense against invading pathogens. In mammals, the *in vitro* culture of macrophages from blood monocytes or bone marrow cells is well established, including culturing conditions to differentiate them towards M1 or M2-like macrophages. In chicken, monocyte-derived macrophages have been used in several studies, but there is no uniform protocol or actual characterization of these cells. Therefore, to generate proinflammatory M1-like macrophages, in this study blood monocytes were differentiated using GM-CSF for 4 days and characterized based on cell morphology, surface marker expression and cytokine expression response to TLRs stimulation at each (daily) time point. Cell morphology showed that one-day-cultured cells contained a mixture of cell populations, while the homogenous population of cells on day 3 and day 4 were flat and had a 'fried-egg' like shape, similar to human M1 macrophages. In addition, cell surface marker staining showed that 3 and 4- days-cultured cells expressed a high level of MRC1L-B (KUL01) and MHC-II. Furthermore, LPS stimulation of the cultured cells induced gene expression of the proinflammatory cytokines IL-1β, IL-6 and IL-8 after 3 days of culture. Finally, it was shown that day 3 macrophages were able to phagocytose avian pathogenic *E. coli* (APEC) and respond by nitric oxide production. Overall, our systematic characterization of the monocyte derived cells from blood showed that a 3-days culture was optimal to obtain pro-inflammatory M1 like macrophages, increasing our knowledge about chicken macrophage polarization and providing useful information for studies on chicken macrophage phenotypes.

1. Introduction

Macrophages play an important role in the innate immune system against invading pathogens. They are actively involved in phagocytosis and subsequent killing of pathogenic microorganisms. In addition, they are also key regulatory cells of the immune system by the production of an inflammatory or anti-inflammatory response upon stimulation. These immune responses are initiated by specialized pathogen recognition receptors, including the toll-like receptor (TLR) family. TLR4 and TLR7 are important to recognize components of bacteria (LPS) and viruses (RNA), respectively (Akira 2003). In mammals, macrophages have different phenotypes, such as M1, M2, M (Hb), Mox, and M4. However, all these phenotypes are nowadays considered differentiated 'extreme-states' of a macrophage and depending on environmental factors macrophages can convert and cover the whole spectrum between these different states (Porcheray et al. 2005; Lee et al., 2011).

In recent years, M1 and M2 macrophages have been studied the most and are linked to different macrophage functions. In a simplified view, M1 macrophages are usually considered pro-inflammatory macrophages that play a role in killing intracellular pathogens, while M2 macrophages are 'tolerant' anti-inflammatory macrophages important for wound healing and tissue repair (Italiani and Boraschi 2014). *In vitro*, M1-like and M2-like macrophages can be grown by applying different supplements (Mantovani et al. 2004; Martinez and Gordon 2014). Morphologically M1-like macrophages differentiate into so-called "fried-egg" shaped cells that express inducible nitric oxide synthase (iNOS) and produce pro-inflammatory cytokines such as IL-6 and TNF-α in response to stimulation. M2-like macrophages are stretched, spindle-like cells that express arginase and produce anti-inflammatory cytokines such as IL-10 in response to stimulation (Verreck et al. 2004; Fleetwood et al. 2007; Gao et al. 2018). Besides morphology, M1 macrophages can also be distinguished from M2 by their different expression of surface

markers (Mantovani et al. 2004; Martinez and Gordon 2014). For instance, MHC-II is expressed by both subsets and high MRC1 is a characteristic of M2 macrophages. These different phenotypes of macrophages provide a very useful tool for understanding the function and especially potential of macrophages *in vivo*.

Unlike *in vitro*-cultured mammalian macrophages that have well described distinct phenotypes under specific conditions, chicken macrophages have been poorly described or standardized. Development of such an avian macrophage culturing system would especially benefit infection studies of avian specific pathogens such as avian pathogenic Escheria (*E.) coli* or *Salmonella Gallinarum*. In addition it would enable a better comparison between mammalian and avian macrophage responses towards interaction with pathogens.

As a common initial step, chicken macrophages are cultured from monocytes purified from peripheral blood or bone marrow by adherence to glass or plastic. After that methodologies diverge and differentiation of monocytes to macrophages occurs without external stimulation, but usually in the presence of chicken serum, or is stimulated through addition of (G)M-CSF (Feng et al. 2016; Johnston et al., 2016 Kappala et al. 2018). Interestingly, a recent manuscript used IL-4 to stimulate differentiation into a more M2-like phenotype (Chaudhari et al., 2018). Besides differences in culturing conditions, there is also no consensus in literature on the time of culturing required to obtain macrophages with times ranging from 1- 6 days. This lack of standardization of culturing (blood monocyte derived) macrophages affects reproducibility and comparability of different studies. Although macrophage cell line (HD11) have been shown to take up and kill avian pathogenic *E. coli* (APEC) (Peng et al. 2018), the role of primary macrophages in controlling APEC is less studied due to the lack of standardization of culturing and characterization of macrophages.

In this study, a thorough characterization of the effect of time of culturing was performed to optimize culture conditions of chicken blood monocyte derived macrophages in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF). The morphology, expression of cell surface markers and immune responses upon TLRs agonists stimulation during cell differentiation were determined. Finally, the optimized macrophage culture was functionally assessed for phagocytosis of avian pathogenic *E. coli* and nitric oxide (NO) production.

2. Methods and materials

2.1. Bacterial strains

Avian pathogenic *E. coli* (APEC, O78) was isolated from chicken (Cuperus et al. 2016) and cultured in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) at 37 °C. Green fluorescent protein (GFP) labeling of the APEC strain was performed as described previously (Peng et al. 2018).

2.2. Cell isolation, culture and cell surface marker staining

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of 76-week-old healthy chickens using Ficoll gradient and cryopreserved until use. PBMCs (1×10^7 cells) were seeded in a 24-wells plate containing 1 mL RPMI-1640 + glutamax media with 10% FCS and penicillin (100 U/mL)/streptomycin (100 µg/mL) (P/S) and incubated at 41 °C. After overnight incubation, all non-attached cells were removed and attached cells were maintained in RPMI-1640 + glutamax media with 10% FCS and P/S supplemented with chicken GM-CSF for another 3 days at 41 °C. Cell morphology was microscopically examined at each day. In addition, cells were harvested after 1, 2, 3 and 4 days in PBS containing 0.5 mM EDTA (hereafter referred to as day 1 - day 4 cells). After centrifugation and washing steps, cells were stained for the chicken Mannose receptor C-type 1-like-B (MRC1L-B) using the KUL01-FITC antibody (clone KUL01, isotype IgG1; Southern Biotech, Birmingham, AL, USA) and MHC-II (MHC-II-PE, 'clone 2G11'; isotype IgG1;

Southern Biotech) in FACS buffer (0.5% bovine serum albumine [BSA] in PBS) at 4 °C for 30 min. Afterwards, cells were washed and analyzed using flow cytometry (FACSCanto-II, BD Biosciences, CA, USA) and FlowJo Software v. 10.5 (FlowJo LCC, Ashland, OR, USA).

2.3. LPS and R848 stimulation of monocyte derived macrophages

Cells were cultured as described above. At each given time point (1, 2, 3 and 4 days), 100 ng/mL ultrapure LPS *E. coli* O111:B4 (InVivoGen, San Diego, CA, USA) or 10 µg/mL R848 (InVivoGen, San Diego, CA, USA) diluted in RPMI-1640 + glutamax media with 10% FCS, was added to the cells for 4 h at 41 °C. Afterwards, cells were washed and lysed in Trizol (Ambion, Carlsbad, CA) stored in -20 °C for RNA isolation.

2.4. Quantitative real-time PCR (qPCR)

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Primers and TaqMan probes were designed and produced by Eurogentec (Seraing, Belgium) (Peng et al. 2018). Quantitative real time PCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the housekeeping gene GAPDH.

2.5. APEC infection in monocyte derived macrophages

Day 3 macrophages were used in this experiment. Aliquots of 0.5 mL of bacterial suspensions (1×10^6 CFU/mL) were added to each well with three replicate wells in a 24-well plate format and incubated for 3 h at 41 °C. After 3 h, bacterial suspensions were removed and cells were washed three times with RPMI-1640 + glutamax media with 10 % FCS. Then, RPMI-1640 + glutamax containing 500 µg/mL gentamicin was added to cells in order to kill all extracellular, non-phagocytosed bacteria, followed by 1 h incubation at 41 °C. After that, cells were incubated again at 41 °C. At each time point, infected cells were washed three times with RPMI-1640 + glutamax and lysed by 0.5 mL 0.5% Triton X-100. After lysis, dilution series of cells were plated on tryptone soya agar plates and incubated at 37 °C for 24 h to quantify viable bacteria.

2.6. Confocal microscopy

PBMCs (0.5×10^7 cells) were seeded on a 12 mm glass coverslip in a 24-wells plate and incubated for 3 days as described above. Then, cells were infected with GFP-APEC (1×10^6 CFU/mL) for 3 h at 41 °C as described above. After three wash steps with RPMI-1640 + glutamax, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). Subsequently, cells were incubated with 50 mM NH_4Cl in PBS for 10 min at RT and blocked with 3% BSA in PBS for 30 min. Then, cells were stained with anti-*E. coli* rabbit serum (1:500) (Cuperus et al. 2016) for 1 h. After the wash steps with PBS, cells were incubated with Donkey anti-Rabbit Alexa 647 (Jackson ImmunoResearch, West Grove, PA, USA) (1:100) for 1 h. Finally, cells were washed with PBS and water and mounted in FluoroSave. Slides were observed on a Leica SPE-II DMI4000 microscope with LAS-AF software (Leica, Wetzlar, Germany) using a $63 \times$ HCX PLAN APO OIL CS objective.

2.7. NO production assay

Nitrite, a stable metabolite of NO, was measured by the Griess assay (Green et al. 1982). PBMCs were seeded in a 24-wells plate and incubated as described above for 3 days. Then, cells were incubated with

bacteria at 41 °C for 3 h and treated with 500 μg/mL gentamicin for 15 h. After 18 h incubation, supernatants were collected and nitrite was determined as described previously (Peng et al. 2018).

2.8. Statistical analysis

Data are represented as mean ± SEM of three independent experiments for each group (n = 3) and were analyzed by a T-test for two groups or by one-way ANOVA with Tukey's multiple comparisons test for more than two groups. Bio-Rad CFX Manager 3.0 software was used for qPCR data analysis. All the graphs were made using GraphPad Prism® 8.0.

3. Results and discussion

3.1. Alteration of cell morphology and surface marker expression during cell differentiation

Monocytes were isolated from PBMCs of 76-week-old healthy chickens and cultured for 1, 2, 3 and 4 days in the presence of chicken GM-CSF to differentiate monocytes into macrophages. The cultures were inspected over time for changes in macrophage morphology and purity. At one day post-isolation, adherent cells were monocytes (Fig. 1A, black arrows), but the floating cells were a mixed population of other cells including lymphocytes and heterophils. Non-adherent cells were removed and did not contain monocytes (FACS analysis, data not shown). Cells became flat after two days of culture (Fig. 1B, dashed arrow). At day three and four, heterophils were lost from the culture and monocyte-derived macrophages remained, as indicated by the' fried eggs-like' shape of the cells (Fig. 1C and D, dashed arrows), similar to classic mammalian M1-like macrophages (Gao et al. 2018). Next, we used flow cytometry to characterize these cells. Macrophages were determined based on FSC (cell size) and SSC (granularity). Increasing amounts of these cells were detected up to 3 days and then leveled between day 3 and 4 (Fig. 2A). The presumed macrophages on different days were MRC1-B and MHC-II positive cells as shown in Fig. 2B. Then, the expression of MRC1L-B and MHC-II were quantified by the geometric mean fluorescence intensity (gMFI). High surface expression of MRC1L-B was detected at day 1 (Fig. 2C), reflecting that monocytes in the blood have a high expression of MRC1L-B. Subsequent monocyte differentiation and proliferation resulted in higher numbers of cells that were identified as macrophages by our FACS gating strategy, but with a tendency (although not statistically different from day 1) to lower average MRC1L-B expression on day 2. However, MRC1L-B expression increased on day 3 reflecting maturation of the newly differentiated macrophages. The expression of MHC-II increased from day 1 to 3 and then stabilized between day 3 and day 4 (Fig. 2C), indicating the proliferation and maturation of macrophages. In general, these results indicate that most of the differentiation of monocytes into macrophages was reached at 3 days post-incubation and then remained stable for at least 1 day.

Based on presumed homology of chicken monocyte-derived macrophages with mammalian cells, expression of the cell surface markers (MRC1L-B and MHC-II) and morphology seem good indicators for
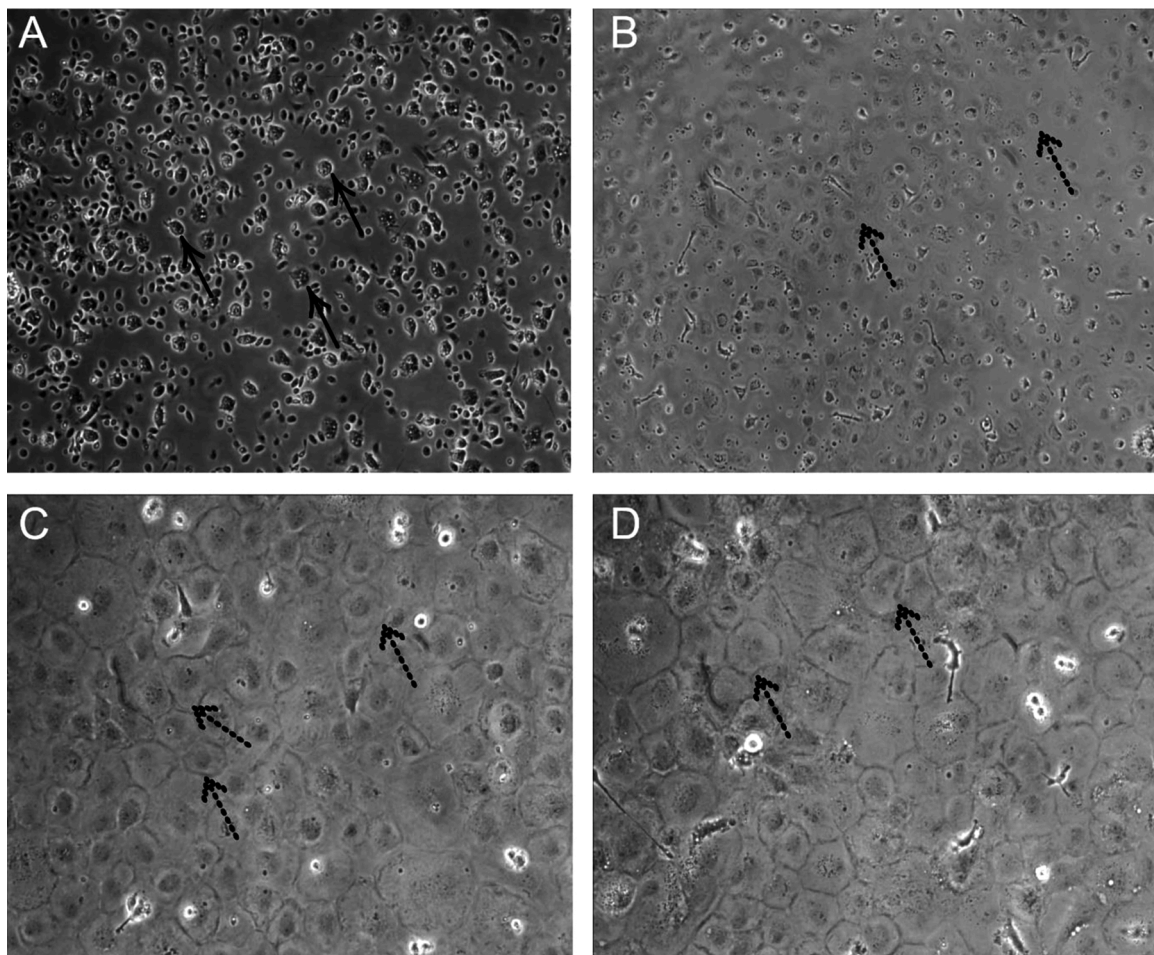

Fig. 1. Morphology changes during cell differentiation. Representative microscopic images of monocyte-derived macrophages after (A) one-day-culture, (B) two-days-culture, (C) three-days-culture, and (D) four-days-culture. The black arrows show monocytes and dashed arrows show flat macrophages. All images are at 20 × magnification.
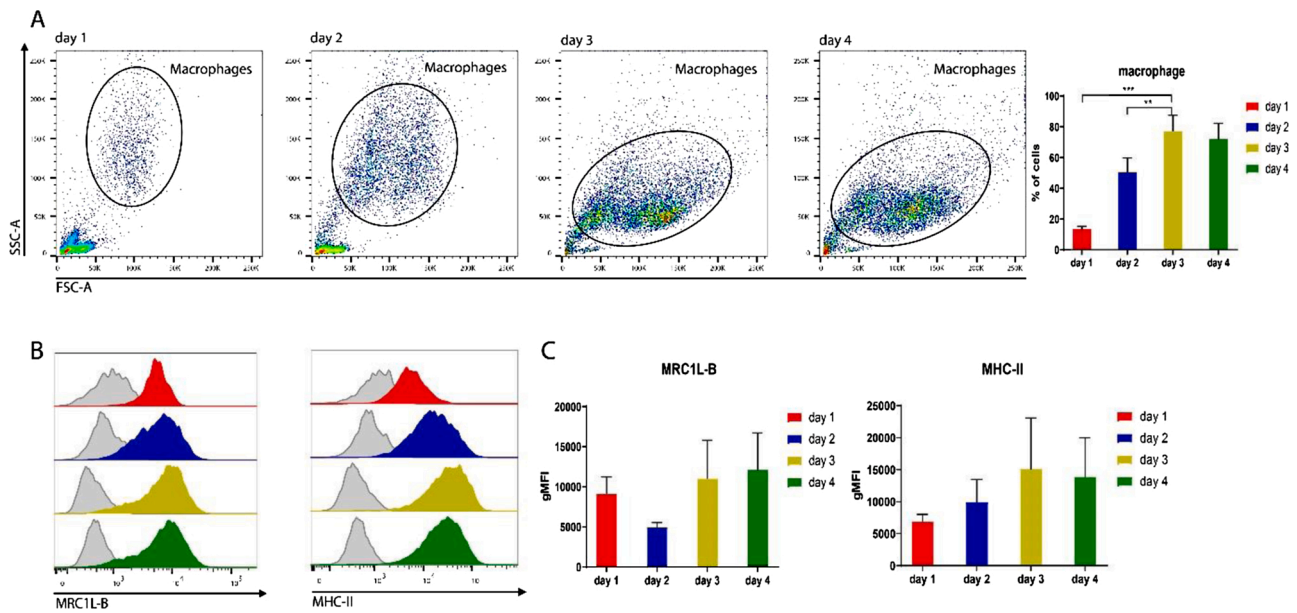
Fig. 2. Expression of cell surface marker during cell differentiation. Chicken PBMCs were cultured for 1-4 days in the presence of GM-CSF. Then, cells were analyzed by flow cytometry. (A) percentage of macrophages based on FSC (cell size) and SSC (granularity). (B) Representative histograms of MRC1L-B and MHC-II expression. Histograms in grey show unstained controls. (C) The expression of MRC1L-B and MHC-II on the macrophages at different culture times were quantified by the geometric mean fluorescence intensity (gMFI). Data are represented as mean ± SEM of three independent experiments with three samples per experiment in the bar graphs. For data analysis, one-way ANOVA with Tukey's multiple comparisons test was used. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005.

chicken monocyte-to-macrophage differentiation. Although the expression of MRC1L-B and MHC-II were used to characterize macrophages, their expression is not limited to macrophages. MHC-II was highly expressed on chicken *in vitro* bone marrow-derived dendritic cells (DCs) (Wu et al. 2010) and also highly expressed by B cells and DCs *in vivo* (Manh et al. 2014). The monoclonal antibody KUL01 recognizes a mannose receptor (also known as CD206 in mammals) and was first used to characterize macrophages by Mast et al in different tissues including spleen and gut (Mast et al. 1998). A previous study has described that chicken actually have five paralogous genes of mannose receptor (MRC1L-A to MRC1L-E), contrary to mammals, and KUL01 only recognizes MRC1L-B (Staines et al. 2014). KUL01 does not exclusively bind to macrophages, since it also identified Langerhans cells in the chicken skin

(Mast et al. 1998). Interestingly, in mammalian macrophages, MRC1 expression is considered to be connected to M2-like macrophages. However, for MRC1L-B, which is only one of the orthologs of MRC1 in chicken (Staines et al. 2014), it is unclear if mammalian data can be extrapolated.

3.2. LPS- or R848-induced cytokines during cell differentiation

The abilities of the macrophages to induce an immune response upon stimulation with TLR4 agonist LPS and TLR7 agonist R848 was tested. R848 induced only low expression levels of IFN-β, IL-1β, IL-6, IL-8 and IL-10 (Fig. 3), and no significant effect of different culturing times on cytokine expression was observed. On the other hand, LPS induced a
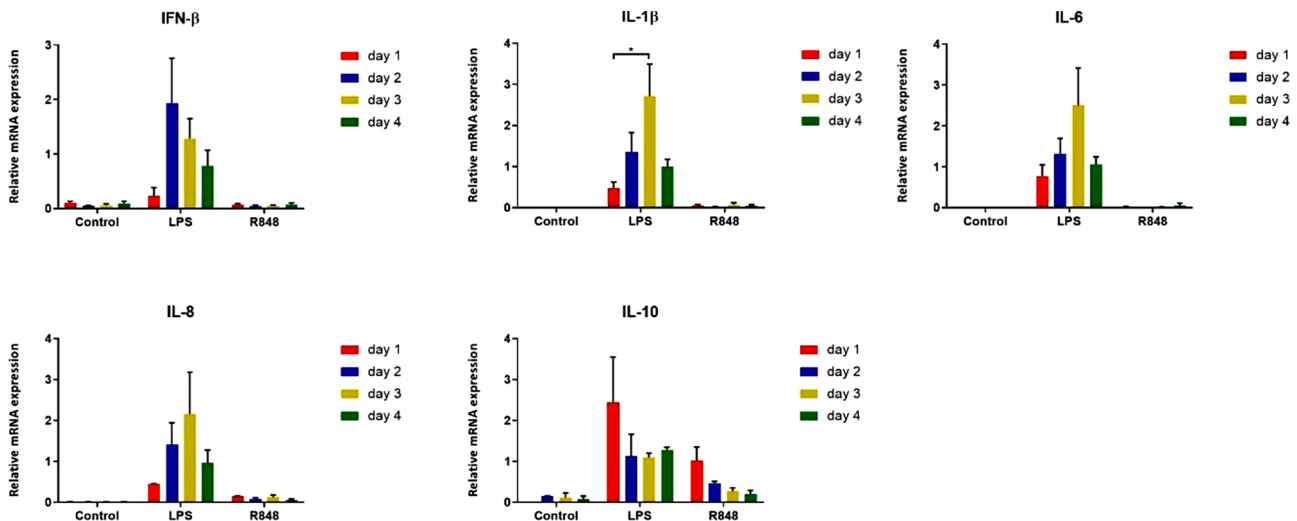

Fig. 3. TLR ligands-induced cytokines expression during cell differentiation. Chicken PBMCs were cultured for 1-4 days in the presence of GM-CSF, and subsequently stimulated with LPS or R848 for 4 h. Cells were lysed and mRNA was isolated. Finally, qPCR was used to detect gene expression of cytokines. Relative gene expression levels were normalized against the expression levels of the housekeeping gene GAPDH. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. For data analysis, one-way ANOVA with Tukey's multiple comparisons test was used. *P ≤ 0.05.

strong expression of these cytokines (Fig. 3). In addition, expression of all pro-inflammatory cytokines significantly increased from Day 1 to Day 3 cultured cells, while IL-10 did not significantly change. Interestingly the gene expression of cytokines upon LPS stimulation decreased again on day 4 of culture indicating that there is not a lasting linear correlation between culture time and (pro)-inflammatory response.

The macrophage response upon TLR stimulation is related to their differentiation state. For example, in mammals, M1 macrophages produce pro-inflammatory cytokines, whereas M2-like macrophages produce anti-inflammatory cytokines in response to TLR stimulation (Fleetwood et al. 2007; Gao et al. 2018). The production of these pro-inflammatory cytokines plays an important role in macrophages. IL-1β is an important pro-inflammatory cytokine for host defense against infection (Jayaraman et al. 2013) and has been used as an immunoadjuvant to improve vaccination efficacy (Deryabin et al. 2014). IL-6 has multiple functions including stimulating differentiation of monocytes to macrophages (Chomarat et al. 2000) and IL-8 is chemotactic for heterophils. This study showed that 3-days-cultured macrophages have an M1 pro-inflammatory differentiation state based on high expression of IL-1β, IL-6 and IL-8. Therefore, high expression of these cytokines upon stimulation indicate that macrophages were most responsive to LPS stimulation at day 3.

Unlike LPS stimulation, R848 did not induce any differences of the immune response in these macrophages. R848 has been reported to induce cytokines expression including TNF-α, IL-6 and IL-12 in mouse macrophages (Lee et al. 2003; Hemmi et al. 2002). It also induced gene expression of IL-1β and IL-6 in chicken TLR7^{+} macrophage-like HD11 cell line (Philbin et al. 2005) but only low expression was detected, which is similar to our observation that low gene expression of IL-1β, IL-6 and IL-8 was induced upon R848 stimulation. A recent study also showed that low gene expression of IFN-β and IL-1β was induced upon R848 4 h stimulation in chicken PBMCs (Ramakrishnan et al. 2015). These results indicate that chicken macrophages might not sensitivein response to TLR7 agonists compared with mammalian macrophages.

3.3. Avian pathogenic E. coli (APEC)-induced activation of macrophages

To assess the function of day 3 macrophages, cells were incubated with APEC, one of the major bacterial pathogens for chicken. After 4 h, intracellular bacteria were detected (Fig. 4A) and after 6 h and 8 h, the number of bacteria in the cells was significantly decreased compared to 4 h initially intracellular bacteria (Fig. 4A), indicating that these macrophages are capable of phagocytosing and subsequent killing of APEC. To confirm that bacteria were taken up by macrophages, confocal microscopy was used to distinguish intracellular from extracellular bacteria. Macrophages were infected with GFP-APEC, after which bacteria were stained with anti-*E.coli* rabbit serum. Since the macrophages were not permeabilized in the procedure, only extracellular GFP-APEC were labeled with antibody and thus double-labelled (Fig. 4B, yellow bacteria), while intracellular bacteria were only positive for GFP fluorescence (Fig. 4B, green bacteria). A decreased number of bacteria over time and distinction between intra- and extra- cellular bacteria in macrophages are similar to observations that APEC were taken up by HD11 cells (Peng et al. 2018).

Phagocytosis is an important function of macrophages and in the current study it was shown that cultured primary macrophages phagocytosed and killed APEC. A number of *in vitro* studies have shown phagocytosis by chicken macrophage cell lines challenged with different bacterial strains (Wisner et al., 2011; He et al. 2012; Jarvis et al. 2016; Lavrič et al. 2008). *In vivo*, increased numbers of macrophages have been detected in the lung and air sacs after chicken infection with APEC (Matthijs et al. 2009), indicating that macrophages play an important role in controlling APEC infection.

Finally, APEC-induced NO was determined. NO is an important mediator for host defense against microorganisms (Eisenstein 2001) and is mainly produced by activated pro-inflammatory M1 macrophages. APEC induced NO production in 3-days-cultured macrophages (Fig. 4C) although to a lower extend than previously observed for HD11 cells, which is actually known for producing high amounts of NO (Peng et al. 2018). Similarly, LPS has been shown to induce NO in other macrophage cell lines such as MQ-NCSU and chicken monocytes (He et al. 2006; Alkie et al. 2017; Dil and Qureshi 2002), showing that NO production is a substantial contribution to the TLR4 induced immune response.

4. Conclusion

This study describes an *in vitro* chicken monocyte-derived macrophage culture in the presence of GM-CSF over time. Our systematic characterization showed that a 3-day culture was optimal to obtain pro-inflammatory M1 like macrophages. This provides a tool for further studies on host-pathogens interactions on macrophages, in which plasticity and diversity of macrophage subsets are taken into account, in line with current studies on mammalian macrophages.
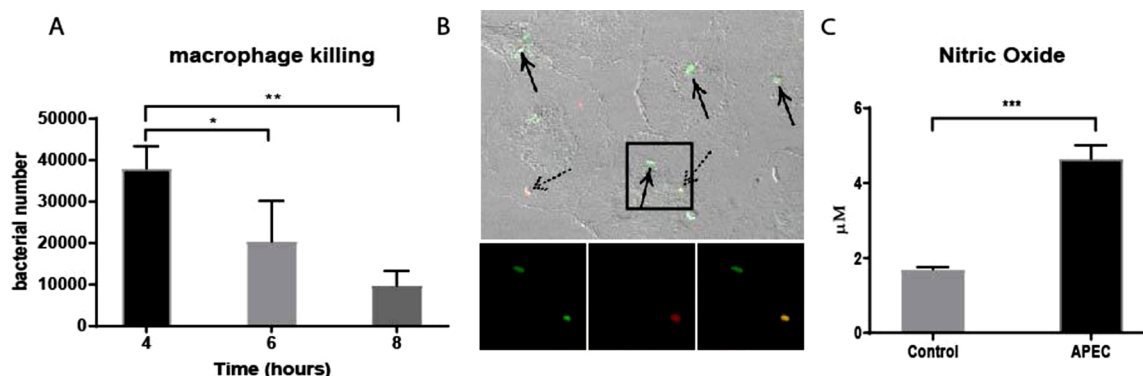

Fig. 4. APEC-induced activation of macrophages. (A) Day 3 cultured cells were infected with APEC for 3 h, then non-adherent bacteria were removed and gentamicin was added to kill extracellular bacteria. Intracellular bacteria were quantified at each time point by plating out dilution series of cells on TSA plates. (B) Day 3 cultured cells were infected with GFP-APEC for 3 h, then macrophages were fixed but not permeabilized. Extracellular APEC were stained with rabbit anti-*E. coli* rabbit serum and Donkey anti-Rabbit Alexa 647 (red). Macrophages were visualized with differential interference contrast (DIC) microscopy. Extracellular bacteria were yellow (double labelled green + red, dashed arrows) and intracellular bacterial were only labelled green (solid arrows). (C) Day 3 macrophages were infected with APEC for 3 h, then bacteria were removed,extracellular bacteria were killed with gentamicin for 1 h and subsequently culturing of macrophages was continued 14 h. NO was measured in the supernatant by the Griess assay. Data are represented as mean ± SEM of three independent experiments with three samples per experiment. For data analysis, a T-test was used in two groups in figure C and one-way ANOVA with Tukey's multiple comparisons test was used in more than two groups in figure A. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Lianci Peng: Methodology, Conceptualization, Validation, Formal analysis, Writing - original draft, Visualization. **Robin H.G.A van den Biggelaar:** Writing - review & editing, Methodology. **Christine A. Jansen:** Writing - review & editing, Methodology. **Henk P. Haagsman:** Writing - review & editing, Conceptualization. **Edwin J.A. Veldhuizen:** Writing - review & editing, Conceptualization, Supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2020.152004.

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