POLARIZATION AND FUNCTIONAL CHARACTERIZATION OF CHICKEN MACROPHAGES

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ABSTRACT

Macrophages may exist in different functional states or phenotypes, regarded as macrophage polarization. The two major extreme phenotypes are M1 and M2. Where M1 macrophage phenotypes may be activated by LPS and IFN-γ and are pro-inflammatory in nature, the anti-inflammatory M2 phenotypes may be activated by IL-4, IL-13, and cAMP, and partake in wound healing and tissue remodelling. The concept of macrophage polarization has been well-established in mammalian species and fish. However, in chicken, the study of the polarization of macrophages is still in its infancy. Macrophage polarization forms an important immune mechanism; therefore, we aimed to investigate its existence in chickens in an *in vitro* study using the chicken macrophage cell line, HD11. The HD11 cells were cultured in a 75cm^2 culture flask, seeded into 96-well and 6-well culture plates and incubated overnight at 41°C, with LPS (2.5 and 5 µg/mL), IFN-γ (6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL), and IL-4 (100 ng/mL) or IL-13 (100 ng/mL) with cyclic cAMP (1 mM). Supernatants from the incubated cells were harvested for the NO assay while the cell lysates were harvested for the Arginase assay. RNA was also isolated from the cell lysates and used for RT-qPCR analysis. The results showed that IFN-γ primed the HD11 cells to produce more NO in the presence of LPS as co-stimulant, but stimulation with only IFN-γ did not elicit dose response at the different concentrations of IFN-γ and showed no significant difference ($p \leq 0.05$) from the untreated cells. Furthermore, IL-13 did not inhibit LPS-induced NO production as IL-4. Moreso, the results consistently showed pronounced Arginase activities in all LPS-treated cells, either in individual stimulation or in co-stimulation with other stimulants. Finally, NOS2 (iNOS) was significantly up-regulated and Arginase 2 down-regulated in LPS-stimulated HD11 cells, which was reversed when IL-13 + cAMP + LPS co-stimulation. Our study showed that HD11 can polarize into cells with an M1 or M2 phenotype upon stimulation with LPS, IFN-γ, IL-4, and IL-13 with cAMP. This study may be found useful in the development of effective vaccines and other immunotherapeutic measures to control infectious diseases in the poultry industry, and for enhancing the quality and safety of poultry meat. Further *in vitro* studies with primary cells as well as *in vivo* studies are needed to establish the existence of macrophage polarization in chicken.

Key Words: Macrophage polarization, chicken, HD11, nitric oxide, Arginase, pro-inflammatory, anti-inflammatory, M1, M2.

CHAPTER ONE: LITERATURE REVIEW

1. Macrophages

Macrophages are important cells of the innate immune system. In vertebrates, macrophages are part of the mononuclear phagocytic system, the same leukocyte system to which monocytes and dendritic cells (DC) belong (Lawrence & Natoli, 2011). Macrophages, such as tissue-resident macrophages, come from the progenitor cells that are produced in the yolk sac. Other macrophages, the monocyte-derived macrophages, originate from the haematopoietic stem cells of the bone marrow (Martinez & Gordon, 2014; Wang et al., 2019). The biological functions of macrophages cannot be over-emphasised as they play central roles in the immune system.

Macrophages confer protection on their vertebrate hosts in four major ways that summarize the functions of macrophages. The first is their ability to monitor their host's internal environment to detect senescent or damaged cells and remove them by phagocytosis without harming the host (Martinez & Gordon, 2014; Mills & Ley, 2014). The second function centres on the antimicrobial property of macrophages. This macrophage function could be mediated by the production of nitric oxide (NO) and phagocytic activity (van den Biggelaar et al., 2020), as well as pro-inflammatory cytokine-induced T-cell activation (Truong et al., 2020).

The third function of macrophages includes their ability to initiate wound healing and tissue remodelling. This is notably mediated via the arginine/ornithine pathway and the production of other anti-inflammatory molecules such as the transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) (Medina-Buelvas et al., 2021; Mills & Ley, 2014; Rath et al., 2014; Wynn & Vannella, 2016). The fourth macrophage function is their ability to act as antigen presenting cells (APCs). Although dendritic cells (DCs) are the major APCs, macrophages also do present antigens to B and T lymphocytes (Mills & Ley, 2014). This is one of the functions of macrophages that directly link innate immune response to adaptive immunity. Hence, Mills (2015) posited that it is innate immunity that directs adaptive immunity, and not the other way round. To fulfil all these functions, macrophages have a high plasticity that enables them to change their phenotypes. The most extreme phenotypes are the M1 and M2 macrophages.

2. M1/M2 macrophage paradigm

The M1/M2 macrophage paradigm is well studied in the mammalian species in a variety of contexts, ranging from pathogenic infection (Medina-Buelvas et al., 2021) to cancer (Ries et al., 2014; Troiani et al., 2020), autoimmune disease (Funes et al., 2018), and other vascular diseases (Fan et al., 2021). In relation to infectious diseases, studies show that, upon infection, M1 macrophages are recruited to the site of infection to eliminate the pathogen. On the other hand, M2 macrophages may be more prominent in promoting tissue repair and limiting inflammation in chronic infections or autoimmune diseases (Atri et al., 2018; Medina-Buelvas et al., 2021). This explains the reason for the dominance of M1 macrophages at the early phase of infection compared to the M2 macrophages (Medina-Buelvas et al., 2021). In cancer, M1 macrophages can be induced to regress tumour by the release of cytotoxic molecules and activation of T cells, while M2 macrophages may promote tumour growth and progression by suppressing the immune response and promoting angiogenesis (Liu et al., 2021; Ries et al., 2014). Therefore, the M1/M2 macrophage paradigm is an important framework for understanding the complex role of macrophages in the immune response and in various disease states in mammalian species, and it is currently being explored in other vertebrate species.

In fish, M1/M2 macrophages have been identified and characterized in some species, including rainbow trout (Braun-Nesje et al., 1982), zebrafish (Nguyen-Chi et al., 2015; Wiegertjes & Elks,

2022), carp (Joerink et al., 2006; Wentzel et al., 2020), and other species. As in the mammalian macrophages, M1 macrophages in fish are associated with pro-inflammatory cytokines, phagocytosis, and reactive oxygen species (ROS) production, while M2 macrophages are associated with anti-inflammatory cytokines and tissue repair (Hodgkinson et al., 2017; Wentzel et al., 2020; Wiegertjes & Elks, 2022).

In chicken, M1/M2 macrophages are currently being identified and characterized. However, the study of chicken macrophage polarization is still in its infancy. Recent studies show the M1/M2 paradigm or polarisation in chicken (Chaudhari et al., 2018; He et al., 2011). Chaudhari et al demonstrated this M1/M2 paradigm with the regulatory activity of chicken interleukin-4 (chIL-4). In their study, they showed that chIL-4 could inhibit NO production induced by LPS, when HD-11 cells are co-stimulated with chIL-4 and LPS. IL-4 is associated with M2-mediated arginase activity while NO production is associated with M1-mediated iNOS activity (Rath et al., 2014). Generally, the M1/M2 macrophage paradigm appears to be conserved across different species, including chicken. Hence, identifying, and characterising M1/M2 macrophages in chicken may provide insights into the evolution of the immune system and the role of macrophages in various disease states.

3. Macrophage activation, polarization, and (intermediate) phenotypes

Macrophages are activated in response to specific signals in their microenvironment (Cui et al., 2021). Thus, such macrophages assume a specific phenotype characterised with specific function. This unique ability of macrophages to change phenotypes as they respond to signals from their microenvironment is known as polarization or plasticity (Funes et al., 2018). Orliaguet et al. (2020) nicely reviewed a detailed overview of the mechanisms and pathways involved in macrophage polarization with respect to the macrophage phenotypes.

The functional role of a macrophage depends on its phenotype. Extremely, two main macrophage phenotypes have been identified. These include the pro-inflammatory M1 phenotype, often referred to as the classically activated macrophages, and the anti-inflammatory M2 phenotype, otherwise called the alternatively activated macrophages (Funes et al., 2018). Macrophages polarize to M1 phenotype when stimulated with interferon-gamma (IFN-γ) and microbial components such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan, and other Toll-like receptor (TLR) agonists. On the other hand, macrophages polarize to the M2 phenotypes upon stimulation with cytokines such as interleukin-4 (IL-4), interleukin-13 (IL-13), and interleukin-10 (IL-10), or with cyclic adenosine monophosphate (cAMP) (Funes et al., 2018; He et al., 2011; Martinez & Gordon, 2014; Wentzel et al., 2020). However, there is the likelihood that stimulation with IL-4 and IL-13 may need the co-stimulating effect of cAMP to effectively polarize macrophages to the M2 phenotypes, especially in an *in vivo* situation (Polumuri et al., 2021). This may be due to the role of intracellular cAMP in activating protein kinase A (PKA) in macrophages. The activated PKA subsequently phosphorylates other transcription factors, thereby directing further cellular responses (Figure 1).

Although the M1 and M2 polarization states were initially described *in vitro*, studies have shown that both states can be observed *in vivo* in both healthy and diseased tissues and in response to various stimuli (Liu et al., 2016; Mosser & Edwards, 2008; Murray et al., 2014). However, due to the heterogeneity of the *in vivo* setting, it could be quite challenging to characterise macrophages *in vivo* compared to *in vitro* methods, except with the application of modern technology such as single-cell RNA sequencing (Marino et al., 2015; Mattila et al., 2013; Russell et al., 2019). Further evidence supporting the *in vivo* characterisation of macrophages can be found in the Review of Russell et al.

(2019). Moreover, recent research suggests that macrophages can exhibit a spectrum of polarization states rather than a strict binary classification as M1 or M2 (Atri et al., 2018).

Interestingly, other intermediate macrophage phenotypes have been identified, and prominent among these intermediate phenotypes are the M2a, b, c, and d. Each intermediate phenotype has activation signal and characteristic function specific to it. For example, M2a is activated by IL-4, and it is important for elimination of parasites as well as for encapsulating extracellular parasites. On the other hand, M2b is generated by the activation signals induced by Fc receptors and immune complexes, and M2b is crucial for the activation of Th2. While M2c is activated by glucocorticoids, M2d intermediate phenotype is a product of the adenosine-dependent switching of M1. Functionally, M2c plays a role in matrix deposition, and M2d in the induction of angiogenesis (Anders et al., 2019; Atri et al., 2018; Funes et al., 2018; Medina-Buelvas et al., 2021).

4. Macrophage effector functions and relevant immune response pathways

Macrophages play a key role in both innate immune responses and subsequent adaptive immunity, making them crucial innate immune effectors against microbial infections. Innate immune effector cells are activated by pathogen-associated molecular patterns (PAMPs), such as elements of bacterial cell walls or viral nucleic acids, by means of pattern recognition receptors (PRRs). Hence, macrophages recognize microbial PAMPs by their PRRs (Sun et al., 2021; van den Biggelaar et al., 2020). Subsequent immune responses ensue after the recognition of PAMPs and macrophage activation to mount defence against pathogens and their molecules. These immune responses may range from pro-inflammatory responses to phagocytosis, apoptosis, and anti-inflammatory responses, depending on the activation signal and the macrophage phenotype activated (Sun et al., 2021).

As earlier mentioned, the M1 macrophages are typically associated with pro-inflammatory responses. They are characterized by the expression of cytokines such as interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-alpha), and interleukin-12 (IL-12), and the production of reactive oxygen species (ROS) and nitric oxide (NO). M2 macrophages, on the other hand, are typically associated with anti-inflammatory and tissue-repairing responses, and are characterized by the expression of cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-beta), and the production of arginase and other factors involved in tissue remodelling (Avolio et al., 2022; Ballerini et al., 2017; Muñoz et al., 2020). Fundamentally, the effector functions of the M1 and M2 macrophage phenotypes strongly depends on their enzymatic metabolism of the protein, Arginine (Rath et al., 2014). Therefore, macrophage biology is substantially influenced by the arginine metabolic pathway that predominates in a developing or on-going immune response.

Macrophages metabolize Arginine via two major pathways: the nitric oxide (NO) and the Ornithine pathways by the M1 and M2 phenotypes, respectively (Muñoz et al., 2020). M1 macrophage phenotypes express the enzyme inducible nitric oxide synthase (iNOS/NOS2) that converts Arginine to NO and Citrulline. While Citrulline can potentially be recycled for effective NO synthesis via the Citrulline-NO cycle, NO can be converted to additional downstream reactive nitrogen species. On the other hand, M2 macrophages express the enzyme Arginase, with which they hydrolyse Arginine to Ornithine and urea. The Arginase route restricts the amount of Arginine that may be used to synthesize NO, whereas, Ornithine can as well enter into the Polyamine and Proline synthesis pathways. These Polyamine and Proline synthesis pathways are crucial downstream processes relevant for proliferating cells and tissue regeneration (Furukawa et al., 2021; Muñoz et al., 2020; Rath et al., 2014; Wang et al., 2020). Hence, NO production and Arginase activity characterise the main effector functions associated with macrophage polarization in vertebrate species, including chicken.

The enzyme Arginase has two isoforms – Arginase 1 (Arg1) and Arginase 2 (Arg2) that are identified in mammals. These isoforms are involved in the urea cycle and the metabolism of arginine (Joerink et al., 2006; Kasmi et al., 2008; Li et al., 2022). Arg1 is primarily expressed in the liver and is involved in the conversion of arginine to urea and ornithine, while Arg2 is expressed in many tissues, including the liver, kidney, and heart, and is involved in the regulation of Arginine metabolism and mitochondrial function (Clemente et al., 2020; Joerink et al., 2006; Murray et al., 2014). Crosstalk exists between Arginase and iNOS enzymes. For instance, although iNOS and Arg2 are co-expressed in LPS-stimulated macrophages, upregulation of NO production often down-regulates the expression of Arginase (Joerink et al., 2006). In birds, including chicken, iNOS (NOS2) and Arg2 are the representative markers for M1 and M2 macrophage phenotypes, respectively (Cui et al., 2021), while Arg1 is yet to be identified in chicken. Interestingly, Arg2 is known to be up-regulated in response to LPS stimulation in chicken, therefore competing with iNOS for Arginine (Verwoolde et al., 2021).

5. Macrophage cell lines and macrophage primary cells: Advantages and disadvantages

Macrophage cell lines and primary cells are commonly used in research to study macrophage biology and immune responses. Each type of cell has its advantages and disadvantages, depending on the specific research question, experimental design, and resources available. For example, one advantage of macrophage cell lines is that they are easy to obtain and culture (Avolio et al., 2022). That is, macrophage cell lines can be obtained from various sources, such as commercial repositories or established in-house from a tissue biopsy. Once established, macrophage cell lines can be easily cultured *in vitro* without ethical issues, making them readily available for experiments (Avolio et al., 2022; Feier et al., 2022). Another important advantage of macrophage cell lines is that they are homogeneous. Macrophage cell lines are clonal populations of cells that have identical genetic backgrounds and phenotypes. This homogeneity allows for better reproducibility of experiments and reduces inter-individual variability (Wilkesmann et al., 2019). Finally, cell lines have high proliferation rates, as cell lines can undergo unlimited cell divisions that can be scaled up to large quantities for experiments that require many cells (Dillon et al., 2012; Feier et al., 2022).

However, macrophage cell lines may not represent the *in vivo* environment due to genetic and phenotypic alterations that do not represent the physiological state of macrophages *in vivo* (Murray & Wynn, 2011). Also, macrophage cell lines may become contaminated with other cell lines or microorganisms during culture and may sometimes become unstable due to genetic drift, thereby affecting the reproducibility of experiments. Moreover, macrophage cell lines may have altered signalling pathways and responses to stimuli due to genetic and phenotypic alterations, which may not accurately reflect the response of primary macrophages *in vivo* (Murray et al., 2014; Murray & Wynn, 2011; Wang et al., 2020).

On the other hand, primary cells closely imitates what happens in an *in vivo* environment despite being cultured *ex vivo*, as primary cells are derived directly from tissues or organs (Czekanska et al., 2014; Geuna et al., 2016), and thus represent the physiological state of macrophages *in vivo*. This entails one of the major advantages of primary cells over cell lines. Furthermore, primary cells have intact signalling pathways and authentic responses to stimuli, which may provide a more accurate representation of the *in vivo* response than macrophage cell lines (Feier et al., 2022). Also, macrophage primary cells can be obtained from patients and used for personalized medicine, such as drug screening or cell-based therapies (Feier et al., 2022).

However, most primary cells are difficult to obtain and culture since they require specialized techniques for isolation and culture and may have limited proliferation rates and lifespans (Pamies et al., 2018). Related to this disadvantage is the ethical, legal, and logistic constraints that may limit the

availability and use of primary lines, particularly in large-scale experiments (Feier et al., 2022; Pamies et al., 2018; Thasler et al., 2003). Finally, macrophage primary cells are heterogeneous in nature and may, therefore, have inter-individual and intra-individual variability due to genetic and environmental factors (Murray & Wynn, 2011). This may affect the reproducibility of experiments.

6. Relevance and application of macrophage polarization in chicken research and poultry industry

Macrophage polarization is an important immune phenomenon in animals, including chicken. Therefore, the relevance and application of macrophage polarization in chicken research and poultry industry cannot be over-emphasised. Firstly, macrophage polarization influences the immune response to infections. Macrophages form major part of the first immune cells to respond to infections. Where the M1 phenotypes mediate the inflammatory processes that eliminate the invading pathogens, the M2 phenotypes mediate the processes that lead to the resolution of inflammation, tissue repair, and regulation of immune responses (Funes et al., 2018). Hence, understanding the mechanisms of macrophage polarization in chicken infections can help to develop strategies for effective disease control and prevention.

Secondly, macrophage polarization could be relevant to the development of vaccine. Macrophages are important antigen-presenting cells (APCs) (Mills & Ley, 2014) that play a crucial role in initiating and regulating immune responses to vaccines. The polarization of macrophages towards M1 or M2 phenotypes can influence the efficacy of vaccines. For example, M1 macrophages are associated with Th1 immune responses that are essential for the clearance of intracellular pathogens, while M2 macrophages promote Th2 responses that are important for humoral immunity and allergic reactions (Shapouri-Moghaddam et al., 2018; Sun et al., 2021). Therefore, understanding the polarization of macrophages in response to vaccines can help to design vaccines that induce appropriate immune responses.

Finally, application of the macrophage polarization in the poultry industry can enhance the quality and safety of meat. Macrophages are involved in tissue repair and regeneration (Wynn & Vannella, 2016), which are important factors that affect meat quality. The activation of M2 macrophages is associated with tissue repair and regeneration, while the polarization towards M1 phenotype is associated with tissue damage and inflammation. In poultry, studies have shown that the modulation of macrophage polarization can affect meat quality and safety. For example, using natural polysaccharides as dietary supplements can polarize macrophages towards M2 phenotype (Li & Bratlie, 2021), which improves meat quality and reduces the incidence of bacterial contamination. Hence, understanding the mechanisms of macrophage polarization can help to develop strategies for controlling diseases, designing vaccines, and improving meat quality and safety in the poultry industry.

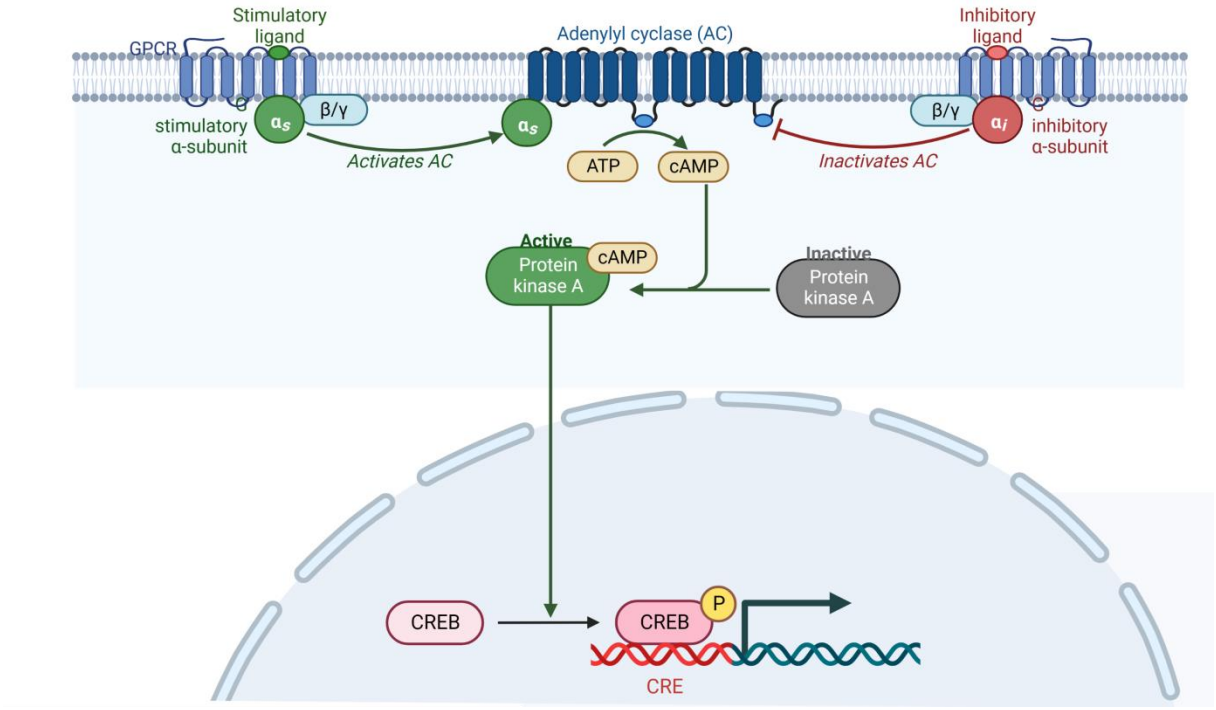


Figure 1: cAMP and the signalling pathways in macrophages (Created with BioRender.com). *Stimulated GPCRs (G protein-coupled receptors) produces intracellular cAMP (cyclic adenosine monophosphate) from ATP (adenosine triphosphate). cAMP activates PKA (protein kinase A), and the activated PKA phosphorylates CREB (cAMP-response element binding protein), a transcription factor that binds to CRE (cAMP response element) and thus stimulates further transcription and cellular responses (Ghigo & Mika, 2019; Polumuri et al., 2021; Rosenberg et al., 2002; Veremeyko et al., 2018).*

CHAPTER TWO: REPORT

1. Introduction

Macrophages are essential immune cells of the innate immune system which are derived from the vertebrates mononuclear phagocytic system comprising of monocytes, tissue macrophages as well as monocyte-derived dendritic cells (Lawrence & Natoli, 2011). Macrophages are known for their active involvement in phagocytosis, inflammation, and other biological processes such as immunoregulation, wound healing and tissue remodeling (Barros et al., 2013; Rath et al., 2014; Wang et al., 2019). One outstanding feature of macrophages is their plasticity, that is, their ability to polarize. Macrophage polarization is the phenomenon where macrophages assume a specific phenotype, and consequently, a specific functional role, dependent on the activation signals they receive at a particular space and time (Murray, 2017; Orecchioni et al., 2019). In vertebrates, such as mammals and fish, macrophages can polarize into M1 and M2 macrophages, depending on the activation signal. The M1 phenotype, also known as classically activated macrophage, polarizes in response to intracellular pathogens, cell components such as bacterial cell lipopolysaccharide (LPS), and Th1-associated cytokines such as interferon-gamma (IFN-γ) (Medina-Buelvas et al., 2021). In contrast, the M2 macrophages, regarded as alternatively activated macrophages, are activated by the Th2-associated cytokines such as interleukin-4 (IL-4) or interleukin-13 (IL-13) (Chaudhari et al., 2018; Evans et al., 2021; Murray, 2017).

The M1/M2 polarized macrophage phenotypes perform significant roles in immune responses against infectious agents and diseases (Medina-Buelvas et al., 2021; Wang et al., 2019). For example, M1 macrophages elicit pro-inflammatory immune responses with which they combat and eliminate infectious agents. These pro-inflammatory responses involve the production of pro-inflammatory cytokines such as IL-1β, IL-6, TNF, and IL-12 and expression of the enzyme iNOS (inducible nitric oxide synthase) that mediates the production of NO (nitric oxide). NO is an important effector molecule involved in immune regulation and the killing of pathogens. On the other hand, M2 macrophages are opposite to M1 as they mediate anti-inflammatory immune responses, maintain immune homeostasis and are as well responsible for wound healing and tissue remodeling. For M2, anti-inflammatory responses include production of anti-inflammatory cytokines like TGF-β and IL-10 and the production of arginase (Cabrera-Fuentes et al., 2015; Chaudhari et al., 2018). Arginase plays crucial role in the metabolism of arginine to produce urea and ornithine, and ornithine mediates tissue repair activities and proliferation of cells (Mills & Ley, 2014; Rath et al., 2014).

The fundamental knowledge of the different chicken macrophage phenotypes and their specific roles in the immune system is highly important for effective immunotherapeutic measures to control infectious diseases in the poultry industry. However, the study of polarization of macrophages in chicken is still in its infancy. This paucity of information makes this current study of obvious importance in order to contribute meaningfully to the body of knowledge on macrophage polarization in chicken. The main objective of this study was to investigate whether the chicken macrophage cell line, HD11, can polarize into cells with an M1 or M2 phenotype upon stimulation with specific cytokines and Toll-like receptor (TLR) ligands. To test the occurrence of polarization, we aimed to evaluate the characteristic functions and gene expression of some marker genes. We set the following research questions to address study objective:

1. Do macrophages polarize differently upon stimulation with different molecules or pathogens?
2. How do chicken macrophages respond to stimulation with IFN-γ + LPS, IL-4 + cAMP, IL-13 + cAMP, and/or other combinations of these stimulants?
3. What are the relevant and easy-to-measure biomarkers for polarized chicken macrophages?

Therefore, we hypothesized that parameters such as nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) gene expression would be up-regulated for the M1 macrophage phenotype, while the up-regulation of Arginase activity and Arginase gene expression would characterise the M2 phenotype macrophages (Figure 1).

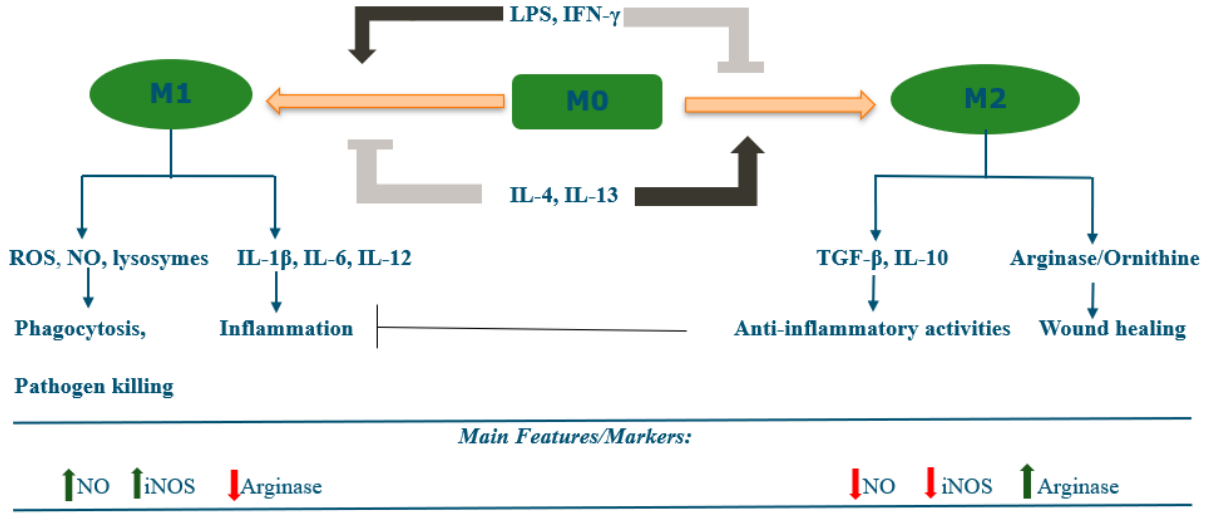


Figure 2: Schematic representation of M1/M2 macrophage polarization. *Green thick arrows shown in the main features/markers indicate up-regulation, while red thick arrows indicate down-regulation.*

2. Materials and methods

2.1. Cell culture

The chicken macrophage HD11 cell line was the *in vitro* experimental model for this study. The HD11 cell line is a macrophage-like cell line obtained from the bone marrow of chicken and transformed with MC29, a type of bird myelocytomatosis virus (Beug et al., 1979). The HD11 cells for this experiment were provided by the laboratory of Adaptation and Physiology Group, Wageningen University and Research. These cells were already cryopreserved at -150^{o}C in liquid nitrogen, with dimethyl sulphide (DMSO) as cryoprotectant. HD11 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Life Technologies Europe B.V. Bleijswijk, Netherlands) containing 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Life Technologies Europe B.V. Bleijswijk, Netherlands) supplemented with 100 U penicillin/mL and 100 µg streptomycin/mL antibiotics (cat. No. 15140122; Gibco, Fisher Scientific B.V., Netherlands). Cells were maintained at 41°C in a 5% CO_2 humidified air incubator. The cells were passaged after 2-3 days to subculture them at about 60-80% confluent in a 75cm^2 culture flask (Greiner Bio-One B.V. Alphen aan den Rijn). All experimental procedures with the cells were carried out in an aseptic manner, to avoid contamination of the cells with microorganisms.

2.2. Cell counting, seeding and stimulation

The cultured HD11 cells were retrieved from the incubator and washed twice with about 10 mL of phosphate buffer saline (PBS) (Gibco, Life Technologies Europe B.V. Bleijswijk, Netherlands) per time. Cells were trypsinized with 1.5-2 mL of 10× Trypsin/EDTA or 5x Trypsin (Gibco, Life Technologies Europe B.V. Bleijswijk, Netherlands) and incubated for 5-10 minutes for the cells to detach. After detaching the cells, an appropriate volume of culture medium was added to about 10 mL of cell suspension. The number of cells in suspension was determined using a Burker-Turk counting chamber viewed under a light microscope at 10x magnification. Approximately 3.5×10^4 cells/well were seeded into a 96-well culture plate (Greiner Bio-One B.V. Alphen aan den Rijn) and 1.0×10^6 cells/well seeded into a 6-well culture plate (Greiner Bio-One B.V. Alphen aan den Rijn). Plates were incubated overnight at 41°C in a 5% CO_2 humidified air incubator, for the cells to attach.

To polarize HD11 cells to M1/M2 phenotypes, the incubated plates were stimulated with lipopolysaccharide (LPS; 2.5 and 5 µg/mL) (*Escherichia coli* O55:B5; Sigma-Aldrich, Merck Life Science NV, Amsterdam), interferon-gamma (IFN-γ; 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL) (Yeast-derived Recombinant Protein; Kinfisher Biotech, Inc. via ITK diagnostics BV. Uithoorn, Netherlands), interleukin-4 (IL-4; 100 ng/mL) (Yeast-derived Recombinant Protein; Kinfisher Biotech, Inc. via ITK diagnostics BV. Uithoorn, Netherlands), interleukin-13 (IL-13; 100 ng/mL) (Yeast-derived Recombinant Protein; Kinfisher Biotech, Inc. via ITK diagnostics BV. Uithoorn, Netherlands), and cyclic adenosine monophosphate (cAMP; 1 mM) (cat. No D0627-100MG; Sigma-Aldrich, Merck Life Science NV, Amsterdam). Stimulated cells were incubated overnight for a 24-hour timepoint.

2.3. Cell harvesting and lysis

The cultured HD11 cells were retrieved from the incubator and 100 µL of cell supernatants were pipetted (in triplicate) into a new 96-well microplate for NO assay. Cells for Arginase assay and RNA

isolation were harvested in a similar manner from 6-well plates. Cells for Arginase assay were lysed at room temperature with 100 µL of lysis buffer, which is 10mM Tris-HCL, pH 7.4, containing 1 µM pepstatin A, 1 µM leupeptin, and 0.4% (w/v) Triton X-100. The lysis buffer for RNA isolation was prepared by adding 10 µL of β-mercaptoethanol (2-mercaptoethanol) to 1 mL of Buffer RLT (supplied in the RNA extraction kit) (RNeasy Plus Mini Kit; Qiagen, Venl, Netherlands). Cells for RNA isolation were then lysed with 350 µL of the prepared lysis buffer and preserved at -20°C for RNA synthesis.

2.4. Nitric oxide and arginase assays

Part of the effector functions of macrophages was determined by quantifying the activity of inducible nitric oxide synthase (iNOS) by measuring the concentration of NO in the culture supernatant using Griess reagents. The Griess reagents comprise of Griess reagent A (0.2% N-(1-naphthyl) ethylenediamine in MilliQ water) and Griess reagent B (2% sulphanilamide in 5% H3PO4), mixed in ratio 1:1. In this NO assay, 50 µL of Griess reagent solution was added to 50 µL of cell supernatant from culture plate transferred in replicates to a 96-well microtiter plate. After 10 minutes of incubation (in the dark) at room temperature, the nitrite concentration was assessed by measuring absorbance at 540 nm with a microplate reader (Multiscan™, Thermo Fisher Scientific, Waltham, MA, USA). A standard curve generated from the serial dilution of sodium nitrite in milliQ water was used to determine the amount of NO that was produced.

Arginase assay was run to determine the Arginase activity of the stimulated cells. First, all reagents (Arginase Activity Assay Kit; Sigma-Aldrich, Merck Life Science NV, Amsterdam) were brought to room temperature except for Arginine buffer that was pre-heated to 37°C. 40 µL of the prepared cell lysate was transferred in duplicate to a 96-well microtiter plate. Where the first set represented the sample wells, the second set were the sample blank wells. 50 µL of diluted urea standard and 50 µL of water were added to separate wells on the same plate. 10 µL of 5× substrate buffer – comprising 8 µL of the pre-warmed Arginine buffer and 2 µL of Manganese solution – was added to the sample wells only and incubated for 2 hours at 37°C. The sample blank wells were incubated at 37°C for 2 hours without the 5× substrate buffer which was later added after stopping the Arginase reaction.

To stop the Arginase reaction, 200 µL of urea reagent was added to all wells: sample, sample blank, urea standard and water blank wells. The mixture was incubated at room temperature for 60 minutes. The absorbance was measured at 430 nm with the aid of a microplate reader (Multiscan™, Thermo Fisher Scientific, Waltham, MA, USA). For each sample, the arginase activity was estimated and expressed as units/L using the formula stated below:

$$Arginase\ Activity\ = \frac{(A_{430})sample - (A_{430})blank}{(A_{430})standard - (A_{430})water} \times \frac{(1\ mM\ \times 50\ \times 10^3)}{(V\ \times T)}$$

Where:

A_{430} = Absorbance read at 430 nm for sample, sample blank, standard, and water blank wells.

T = Reaction time.

V = Volume (µL) of sample added to the well.

1 mM = Concentration of urea standard.

50 = Reaction volume (µL).

10^3 = mM to µM conversion.

2.5. Gene expression

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify the expression levels of marker genes linked with M1 and M2 macrophages, based on their mRNA levels. In this experiment, HD11 cells were stimulated with 2.5 µg/mL LPS, 100 ng/mL IL-13, IL-13 + 1mM cAMP, IL-13 + LPS, and IL-13 + cAMP + LPS, for 24 hours timepoint. The Control group comprises the non-stimulated cells, that is, cells treated with only medium for the same timepoint. RNeasy Mini kit (Qiagen,Venl, Netherlands) was used to extract total RNA from the cell lysate, and NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop™, Fisher Scientific B.V. , Netherlands) was used to quantify the RNA. In a 20 µL reaction volume, the cDNA synthesis was performed using the cDNA Reverse Transcriptase kit (Thermo Scientific™ RevertAid First Strand cDNA Synthesis Kit; Fisher Scientific B.V., Netherlands).

The gene expression of NOS2, TNF-α, and Arg2 (primer sequences not available) was quantified using the SensiMix Mastermix (SensiMix SYBR Low-ROX Kit, GC Biotech BV., Waddinxveen, The Netherlands). With QuantStudio™ 5 Real-Time PCR System (96-well; Applied Biosystems™ ThermoFisher Scientitic, The Netherlands), DNA was amplified and measured under the PCR conditions of 10 minutes initial denaturation at 95ºC and 40 cycles in three steps of 95ºC for 15 seconds, 60ºC for 1 minute and 72ºC for 15 seconds. The QuantStudio™ 5 system software was used to analyze the melt curves for each sample, and the melt curves were regarded usable in instance of just one visible peak. β-actin was used as the endogenous control gene to determine the mean change in cycle threshold for each primer set. Finally, the relative quantification (gene expression) of each primer set was determined in reference to the Control sample, using the $2^{-\Delta\Delta CT}$method (Livak & Schmittgen, 2001).

2.6. Statistical analysis

The obtained data was expressed in the form of mean ± standard deviation (SD). The statistical difference among variables was determined by student t-test using Excel spreadsheet, with the level of significance set at $p \leq 0.05$.

3. Results

3.1. IFN-γ-stimulated HD11 cells influence NO production and Arginase activity

We hypothesized that IFN-γ primes the stimulated HD11 cells to produce more NO in the presence of LPS. To test this hypothesis, the relationship between the concentrations of both stimulants was investigated. HD11 cells were stimulated with 5 µg/mL and 2.5 µg/mL LPS and 100 ng/mL and 50 ng/mL IFN-γ, respectively, both as individual stimulants and as co-stimulants, and incubated for 24 hours. Cells stimulated with 5 µg/mL LPS produced more NO than 2.5 µg/mL LPS by about 2 µM (data not shown). There was no significant difference between the untreated cells (control) and cells treated with only IFN- γ of either concentration. However, when co-stimulated with LPS, there were higher values of NO concentration, which was more for 2.5 µg/mL LPS + 100 ng/mL IFN-γ co-stimulation. Furthermore, we hypothesized that higher concentrations of IFN-γ enhances the NO production of a fixed LPS concentration. We tested this hypothesis by treating HD11 cells with 2.5 µg/mL LPS and a serial concentration of 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL IFN-γ, both as individual stimulants and as co-stimulants (Figure 3). The result showed that 100, 200, and 400 ng/mL IFN-γ primed LPS for increased NO production in cells treated with LPS + IFN-γ co-stimulants, which peaked at 200 ng/mL IFN-γ (Figure 3).

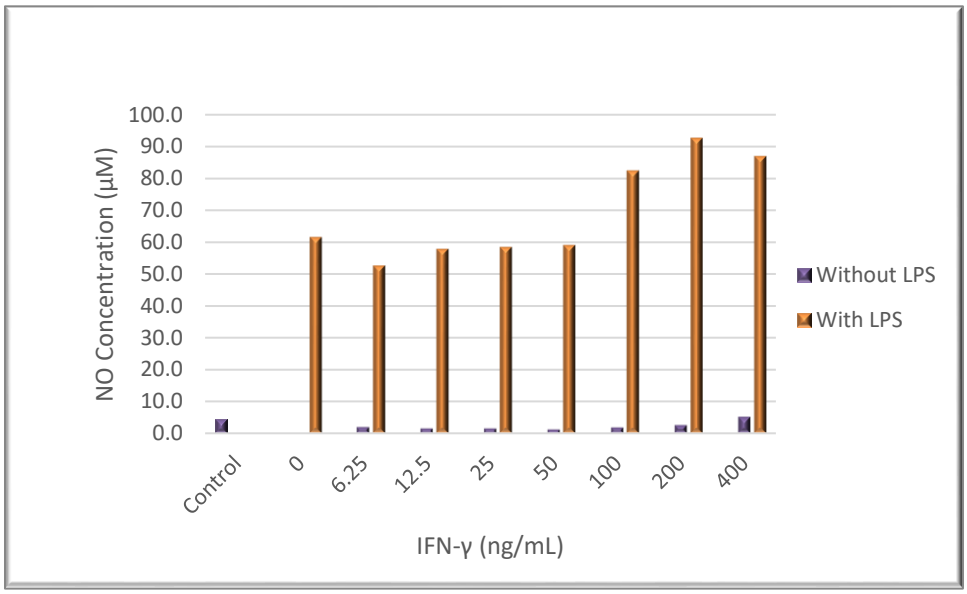
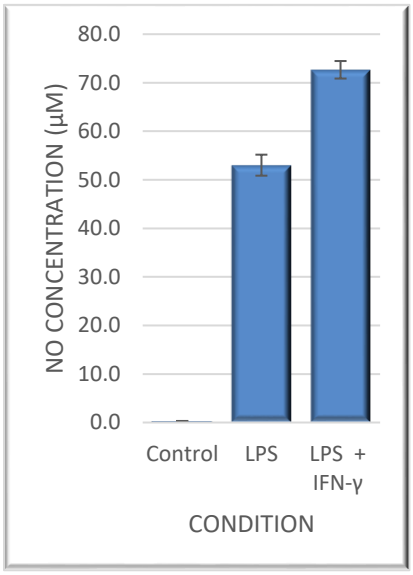


Figure 3: Dose response of the effect of IFN-γ on LPS stimulated HD-11 cells. *Approximately 3.5 x 10^4 HD11 cells were seeded in a 96 well plate and incubated for 24 h at 41^0C. HD11 cells were then stimulated with a fixed LPS concentration of 2.5 µg/mL and/or serial concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL IFN-γ. Cell supernatants were taken in triplicates for each condition to measure NO production. Figure 3 shows NO production in µM for the various conditions.*

We also hypothesized that IFN-γ and LPS-stimulated HD11 cells result in the M1 macrophage phenotype with induced NO production and little or no Arginase activity. To test this hypothesis, HD11 cells were treated with 5 µg/mL LPS and 100 ng/mL IFN-γ and incubated for 24 hours. NO assay indicated that NO production was up-regulated when IFN-γ was added as a co-stimulant with LPS, thus priming LPS for more NO production by about 20 µM (Figure 4A). Corresponding Arginase assay revealed that the treated cells produced considerable amount of Arginase compared to non-treated cells, and that this Arginase activity was numerically higher in cells treated with LPS alone compared to LPS + IFN-γ co-stimulation (Figure 4B), but this was not tested statistically.

A. NO assay



B. Arginase assay

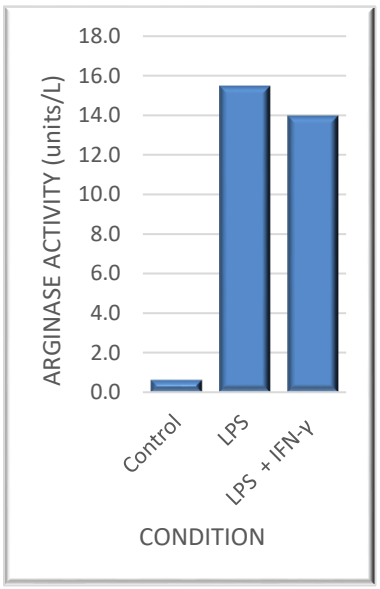


Figure 4: Effect of IFN-γ as co-stimulant with LPS for HD11 cells. *Approximately 1 x 10^6 HD11 cells were seeded in a 6 well plate and incubated for 24 h at 41^0C. The HD11 cells were subsequently stimulated with 5 µg/mL LPS and/or 100 ng/mL IFN-γ. Each condition for the NO measurement was tested in triplicate and arginase in unity.* ***Figure A*** *shows the production of NO in µM.* ***Figure B*** *shows the arginase activity in units/L.*

3.2. Comparison between NO production by IL-4-stimulated and IL-13-stimulated HD11 cells in relation to other stimulants

Comparing the NO production between IL-4-stimulated and IL-13-stimulated HD11 cells, we hypothesized that IL-13 inhibits LPS-induced NO production as IL-4. HD11 cells were stimulated with respective stimulants and incubated for 24 hours. NO Assay showed a considerable reduction in NO production by cells treated with IL-4 + LPS co-stimulant by a difference of about 26 µM in reference to LPS treatment (Figure 5A). The NO production was further inhibited in the presence of cAMP as co-stimulant. On the other hand, almost no reduction (just about 3 µM) occurred in the NO production by cells treated with IL-13 + LPS co-stimulant, although there was a significant reduction when cAMP was added as co-stimulant (Figure 5B).

A. **B.**

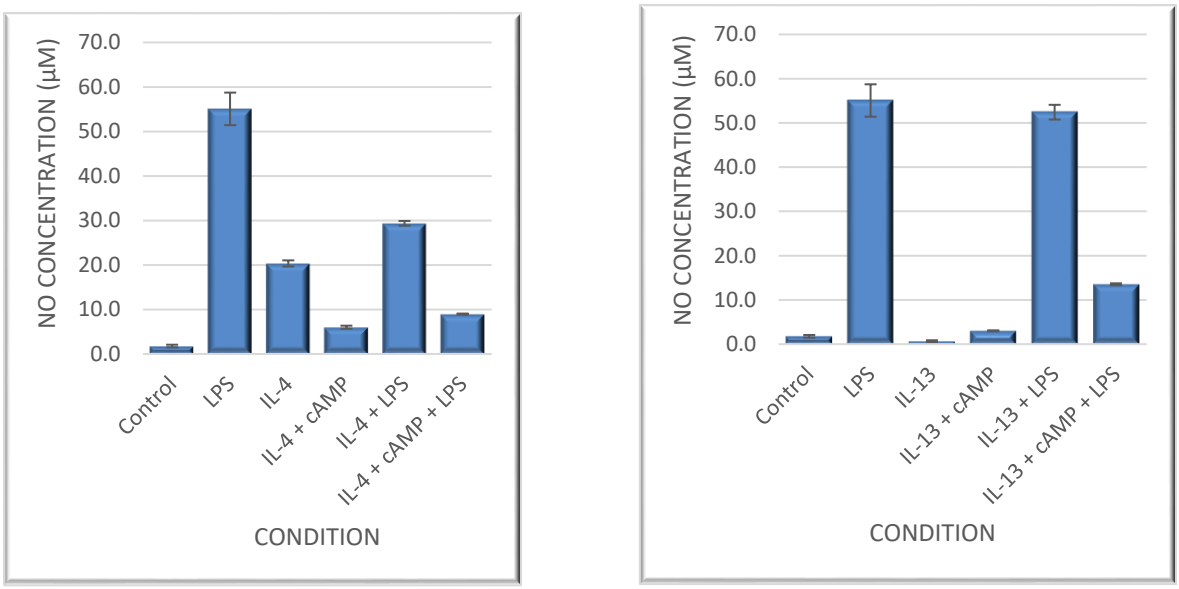


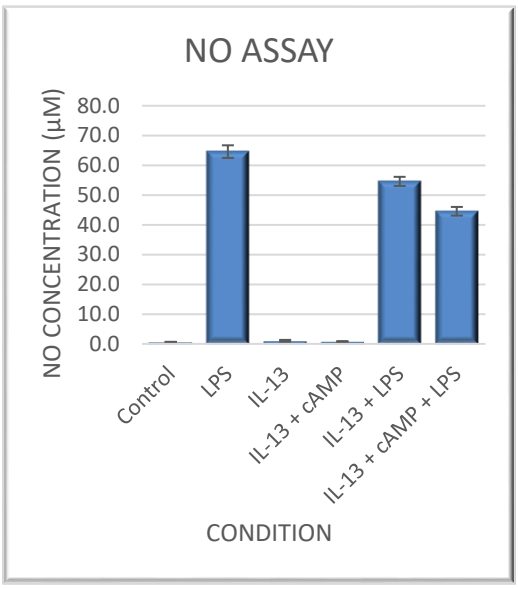
Figure 5: Effects of IL-4 and IL-13 on LPS-induced NO production. *Approximately 1 x 10^6 HD11 cells were seeded in a 6 well plate and incubated for 24 h at 41^0C. The HD11 cells were subsequently stimulated with 2.5 µg/mL LPS, 100 ng/mL IL-4, 100 ng/mL IL-13 and 1mM cAMP. NO production was measured in triplicates for each condition.* ***Figure A*** *shows the NO production in µM by IL-4-stimulated cells, with significant reduction when LPS was added as a co-stimulant.* ***Figure B*** *shows the NO production in µM by IL-13-stimulated cells, and there was no significant reduction in NO production when LPS was added as co-stimulant.*

3.3. Response of IL-13 to LPS co-stimulation

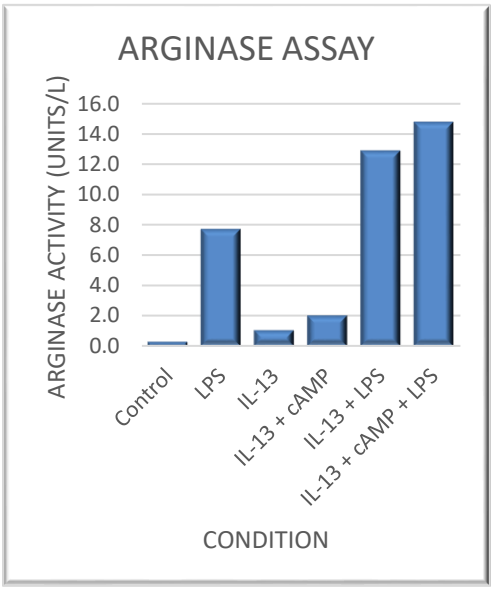
We were curious about the response of IL-13 to LPS as macrophage co-stimulating agent. Therefore, we investigated this relationship further through different assays (Figure 6). NO assay confirmed that IL-13 weakly inhibits LPS-induced NO production by IL-13-treated cells, except in the presence of cAMP as a co-stimulant (Figure 6A) that earlier showed a stronger inhibition (Figure 5B). Strikingly, LPS-treated cells produced a considerable amount of Arginase of about 7.8 units/L (Figure 6B), and IL-13-treated cells exhibited increased Arginase activity in the presence of LPS as a co-stimulant. Arginase activity was even higher when cAMP was added as additional co-stimulant (Figure 6B). We also compared the values obtained from the NO and Arginase assays by potting a graph of NO/Arginase ratio (Figure 6C). This graph showed the highest value (8.3 units) for the HD11 cells treated with only LPS. The value was considerably low for IL-13-treated cells, except when treated with LPS as a co-stimulant (Figure 6C). A corresponding schematic overview of the NO/Arginase ratio (Figure 7) shows the balance in relation to M1/M2 macrophage phenotypes.

In addition, real time quantitative polymerase chain reaction (RT-qPCR) analysis showed the expression of relevant gene markers by the stimulated HD11 cells (Figure 6D). Arginase-2 (ARG-2) gene was significantly expressed in cells co-stimulated with IL-13, cAMP and LPS. Although nitric oxide synthase-2 (NOS-2) gene (or iNOS – inducible nitric oxide synthase) had the highest expression in cells treated with only LPS, NOS-2 gene was significantly expressed in other IL-13-treated cells when LPS was added as a co-stimulant. There was increased expression of NOS-2 in IL-13-treated cells with both cAMP and LPS as co-stimulants compared to IL-13-treated cells with only LPS as co-stimulant. TNF-α showed little or no expression in all samples – treated and untreated cells.

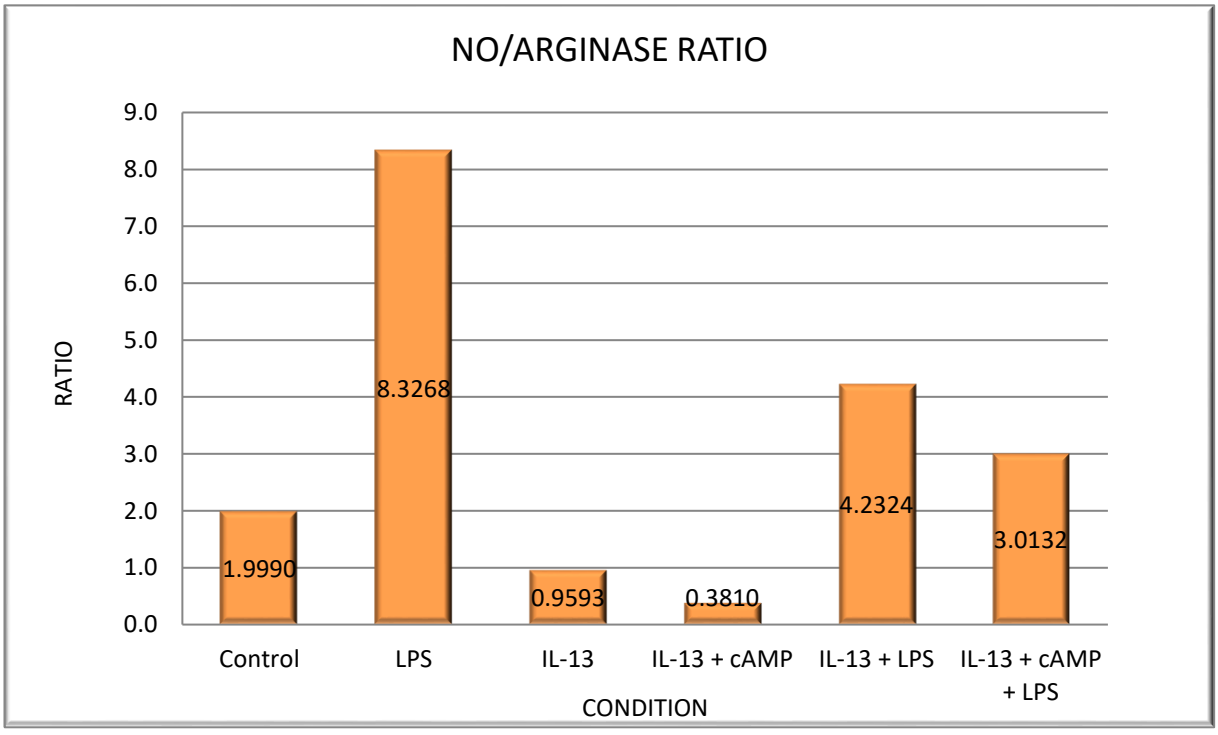
A.


NO ASSAY
NO CONCENTRATION (µM)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Control
LPS
IL-13
IL-13 + cAMP
IL-13 + LPS
IL-13 + cAMP + LPS
CONDITION

B.


ARGINASE ASSAY
ARGINASE ACTIVITY (UNITS/L)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
LPS
IL-13
IL-13 + cAMP
IL-13 + LPS
IL-13 + cAMP + LPS
CONDITION

C.


NO/ARGINASE RATIO
RATIO
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1.9990
8.3268
0.9593
0.3810
4.2324
3.0132
Control
LPS
IL-13
IL-13 + cAMP
IL-13 + LPS
IL-13 + cAMP + LPS
CONDITION

D.

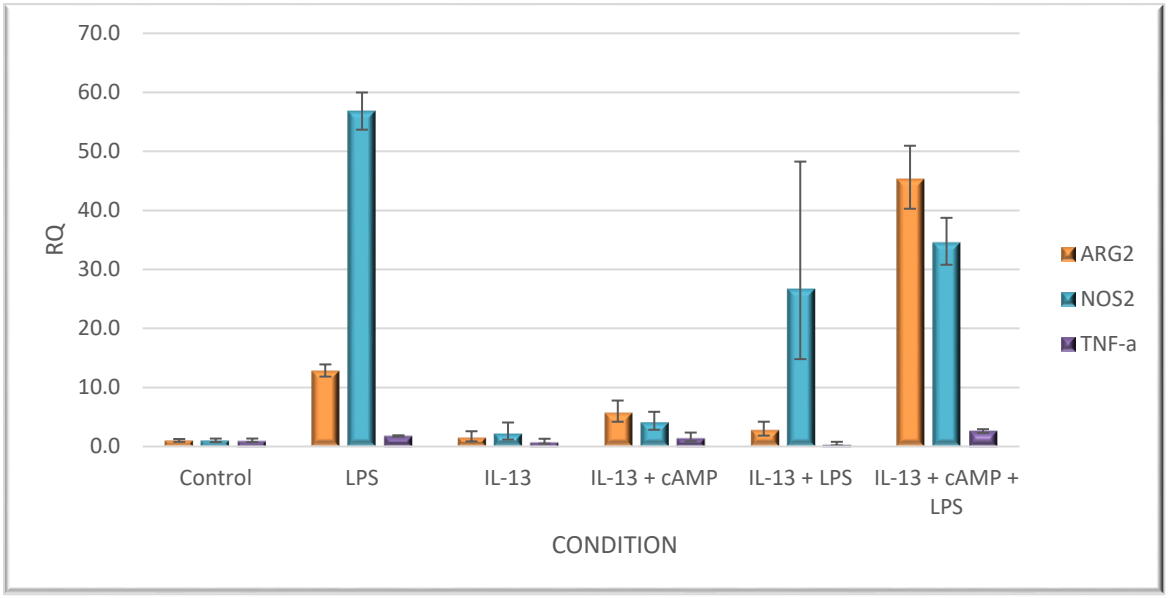


Figure 6: Indicators of effector functions and marker genes expression by IL-13-stimulated HD11 cells. *About 1 x 10^6 HD11 cells were seeded in a 6 well plate, incubated for 24 h at 41^0C, and stimulated with 2.5 µg/mL LPS, 100 ng/mL IL-13 and 1 mM cAMP. NO production was measured in triplicates, and Arginase in unity, per condition. Figure A shows the NO production in µM by IL-13-stimulated cells, which shows no significant reduction in the presence of LPS as a co-stimulant. Figure B shows LPS-induced Arginase activity (units/L) by IL-13-stimulated cells. Figure C shows the NO/Arginase ratio plot, with the highest value in cells stimulated with only LPS. Figure D shows the RT-qPCR analysis of the marker genes expressed by the IL-13-stimulated HD11 cells. NOS-2 and ARG-2 shows the highest expression in cells stimulated with only LPS and IL-13-cAMP-LPS co-stimulants, respectively.*

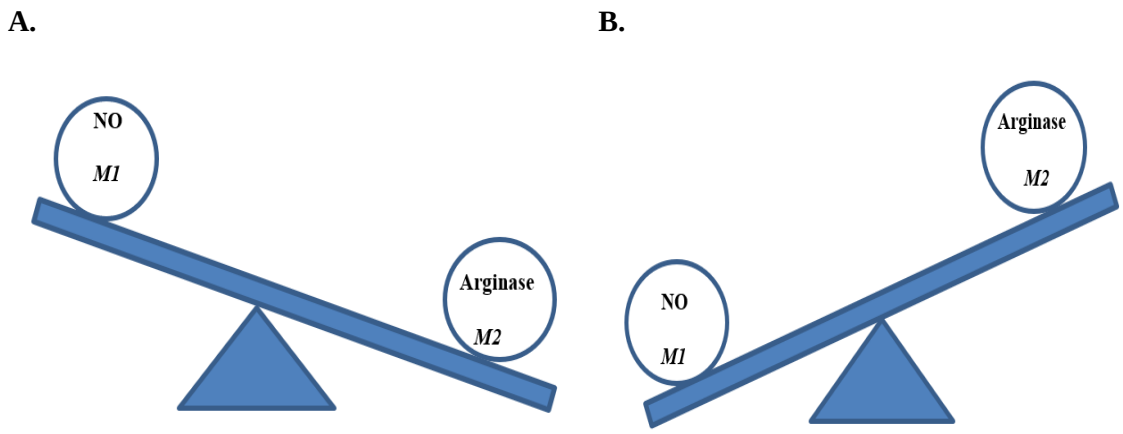


Figure 7: Schematic representation of NO/Arginase ratio in relation to M1/M2 macrophages. *Higher values depict high NO production with low Arginase activity, typical of M1 phenotype (A). Lower values depict low NO production with high Arginase activity, typical of M2 phenotype (B).*

4. Discussion

The HD11 cell line is a macrophage-like cell line derived from the bone marrow of chicken and transformed with the bird myelocytomatosis virus, MC29 (Beug et al., 1979). Many *in vitro* studies use the HD11 cell line to characterize the immune functions of chicken macrophages (Bai et al., 2021; He et al., 2018; Hong et al., 2020; Lee et al., 2021; van den Biggelaar et al., 2020). Our study investigated the polarization of HD11 macrophage cells into the M1 or M2 phenotype upon stimulation with LPS, IFN-γ, IL-4, and IL-13 with cAMP. We evaluated the response of chicken macrophages to stimulant combinations such as IFN-γ + LPS, IL-4 + cAMP, and IL-13 + cAMP, among other combinations. We have also assessed the effector functions of the polarized macrophages as determined by NO production, Arginase activities, and characteristic gene expressions.

Our results showed that the HD11 cells stimulated with only IFN-γ showed no significant difference from the control (untreated cells), and that the IFN-γ primed the HD11 cells to produce more NO in the presence of LPS as co-stimulant (Figures 3 and 4A). These results are in accord with the study by He et al. (2011) who proved that co-stimulation of HD11 cells with IFN-γ and TLR agonists such as LPS, LTA, and other agonists, significantly increased the production of NO. However, in the present study, IFN-γ did not show dose response at different concentrations (Figures 3), thus not consistent with the finding of He et al. (2011) that demonstrated IFN-γ-induced dose-dependent NO production. Overall, our results further substantiate the fact that a PAMP is required for NO production (Balan & Babu, 2017), since IFN-γ itself is not potent enhancer of NO production. The present results indicate also that the IFN-γ itself does not contain LPS or any other NO-stimulating molecule.

Furthermore, we demonstrated that IL-4 inhibits NO production induced by LPS (Figure 5A, about 47% inhibition). This agrees with the work of Chaudhari et al. (2018), who showed that chIL-4 considerably inhibited LPS-induced NO production (about 85% inhibition), inhibited iNOS gene expression, and enhanced Arginase activity, when HD-11 cells were co-stimulated with chIL-4 and LPS. According to Chaudhari et al. (2018), it appears that chIL-4 overrules the effect of LPS on chicken macrophages through arginase-dependent metabolic pathway of arginine. Hence, Arginase activity (M2) could be more pronounced than iNOS activity (M1) in chIL-4 + LPS co-stimulation. However, comparing the response of IL-4 with IL-13 in this present study showed that IL-13 weakly inhibited LPS-induced NO production, when IL-13 was used as a co-stimulant with LPS. However, further study is needed to confirm this trend for IL-13, because IL-4 and IL-13 are both known to polarize macrophages to the M2 phenotype (Gonzalez et al., 2022; Hodgkinson et al., 2017; Wynn & Vannella, 2016).

Moreover, since LPS stimulation is a characteristic feature of M1 macrophages (Funes et al., 2018; He et al., 2011; Martinez & Gordon, 2014; Wentzel et al., 2020), we hypothesized little or no Arginase activities in the LPS-stimulated HD11 cells. Interestingly, our results consistently showed pronounced Arginase activities in all LPS-stimulated cells, either in individual stimulation or in co-stimulation with other stimulants (Figure 4B, 5, and 6B). Although the possible cause of this outcome is not yet fully known, it could be due to the expression of Arg2 by the LPS-stimulated cells, similarly identified by Verwoolde et al. (2021). Arg2 is known to be activated in pro-inflammatory environments. Since Arg1 is absent in chicken, Arg2 strongly competes with iNOS for Arginine metabolism in chicken (Cui et al., 2021; Verwoolde et al., 2021). Thus Arg2 drives more pro-inflammatory responses than anti-inflammatory responses when co-expressed with iNOS (Li et al., 2022). Finally, the result of the gene expression showed that NOS2 (iNOS) was significantly up-regulated and Arg2 down-regulated

in LPS-stimulated HD11 cells, which was strikingly reversed when IL-13 and cAMP were added as co-stimulants with LPS. A similar response of Arg2 to cAMP stimulation was earlier found in fish (Joerink et al., 2006).

One major weakness of this study was that some results, such as the Arginase activities for IL-4 and IL-13, did not show consistent outcomes after repeated experiments, thereby making it difficult to draw valuable conclusions from them. This necessitates more future experiments with consistent outcomes.

To further investigate the existence of macrophage polarization in chicken, we recommend that the study may include checking for intermediate phenotypes, functional tests such as wound healing, and phagocytic activities of the polarized macrophages. Further studies may also include different timepoints, concentration (dose) effects of stimulants, as well as testing for polarization on other macrophage cell lines. Finally, since cell lines may not duly represent the *in vivo* environment of macrophages (Murray & Wynn, 2011), we recommend that further study of macrophage polarization in chicken may include *in vivo* experiments, such as single-cell RNA sequencing. Also, future *in vitro* studies may include the use of chicken primary macrophage cells for comparison with HD11 cell line or other chicken macrophage cell lines.

5. Conclusions

Our study showed that HD11 can polarize into cells with an M1 or M2 phenotype upon stimulation with LPS, IFN-γ, IL-4, and IL-13 with cAMP. NO production and the expression of NOS2 (iNOS) are indicators for the presence of the M1 macrophage phenotype, while the Arginase activities and the expression of Arg2 are indications for the M2 phenotype. The application of the findings from this study may help in the development of effective vaccines and other immunotherapeutic measures to control infectious diseases in the poultry industry. The study may also be useful in enhancing the quality and safety of poultry meat. Further *in vitro* studies with primary cells as well as *in vivo* studies are needed to establish the existence of macrophage polarization in chicken.

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