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DIFFERENTIAL EXPRESSION OF CYTOKINES AND ARGININE METABOLIZING ENZYMES DURING *in vitro* MATURATION OF BOVINE MONOCYTE DERIVED MACROPHAGES

Renjith Sebastian, M. Sravanthi, K. Ganesh, V. Umapathi, K. Narayanan, M. Priyanka, H.J. Dechamma, A. Sanyal, G.R. Reddy*

Indian Veterinary Research Institute, Hebbal, Bangalore 560024, India

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Monocyte-derived macrophages

Inflammatory cytokines

Interferons

Inducible nitric oxide synthase

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ABSTRACT

Macrophages represent a major class of innate immune cells mainly derived from peripheral blood monocytes. Monocytes migrate into extra-vascular space in response to wide range of stimuli and differentiate into macrophages. The differentiation of monocytes into macrophages involves morphological and functional changes through the co-ordinated regulation of numerous genes. The functional changes, especially changes in cytokine profile and arginine metabolism during differentiation of monocytes into macrophages is largely unknown. In this study, the difference in the expression of major cytokines and arginine metabolizing enzymes during *in vitro* differentiation of bovine monocytes to macrophages was investigated. Monocytes were obtained from the whole blood of indigenous cattle by immunomagnetic separation, and differentiated into monocyte-derived macrophages (MDM) using M-CSF. There was a significant down regulation of pro-inflammatory cytokines with concomitant up regulation of anti-inflammatory cytokines in the MDM as compared to primary monocytes. Further, the expression of Type I and II interferons was significantly down regulated in MDM during *in vitro* maturation. To understand the potential changes in arginine metabolism during *in vitro* maturation of monocytes to MDM, the expression of inducible nitric oxide synthase (iNOS) and arginase 1 (ARG1) was also studied. A significant up regulation of iNOS by 3-folds and down regulation of ARG1 by 2-folds in MDM suggested that the differentiation of monocytes into MDM is associated with induction of NO production from arginine.

* Corresponding author
E-mail: drreddygr@gmail.com (G.R. Reddy)

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1 Introduction

Monocytes and macrophages are the major constituents of mononuclear phagocyte system (MPS). Monocytes constitute around 5-10% of the total blood leukocyte population and are considered as immature macrophages in blood (Tizard, 2013). When monocytes migrate into tissues, they become macrophages including Kupffer cells in the liver, Langerhans cells in the skin, microglia in the brain, histiocytes in connective tissues, splenic red pulp macrophages, lung alveolar macrophages, adipose tissue macrophages and bone osteoclasts, to name a few (Gautier et al., 2012; Davies et al., 2013). Monocytes initiate the innate immune responses against microbial pathogens by secreting inflammatory cytokines, phagocytosis and killing the pathogens by reactive nitrogen and oxygen intermediates such as nitric oxide and superoxide free radical (Serbina et al., 2008). Monocytes are recruited into tissues in response to a wide range of stimuli including microbial products, damaged cells, cytokines or by the activation of the complements or clotting cascades which release bioactive peptides such as C5a (Sallusto & Baggiolini, 2008). Tissue-resident macrophages are relatively long-lived cells, replacing themselves at a rate of about 1% per day unless activated by inflammation or tissue damage (Tizard, 2013).

Macrophages represent an important component of the innate immune system which plays a pivotal role in the host defense mechanism. Activated macrophages not only have a marked ability to engulf and destroy a wide variety of microorganisms (Rigden et al., 2002; Lee & Jeon 2005), but also secrete a mixture of cytokines and interferons that promote both innate and adaptive immune responses (Cavaillon, 1994). In addition, macrophages contribute directly to the maintenance of tissue homeostasis by clearing dead and damaged cells and assist in tissue repair after inflammation (Duffield et al., 2005; Mantovani et al., 2013). Pathogen infected macrophages can produce cytokines and nitric oxide (NO) to limit the replication of the intracellular microorganisms (Lee & Jeon 2005; Denis et al., 2007). L-arginine metabolism plays a key role in the activation and regulation of immune responses in mononuclear phagocytic cells. Nitric oxide, a key component of antimicrobial defense in monocytes and macrophages, is produced from arginine by inducible nitric oxide synthase (iNOS) (Gross & Wolin, 1995). This enzyme acts on L-arginine using NADPH and oxygen to produce large amounts of NO and citrulline. The sustained production of NO permits macrophages to kill the pathogens. In contrast, arginase-1, another major enzyme of arginine metabolism, degrades L-arginine and down regulates the NO production by reducing the availability of substrate (Gross & Wolin, 1995).

As the maturation and differentiation of monocytes into macrophages involve concerted regulation of numerous genes, we hypothesized that the major cytokines such as TNFα, IL12, IL10 and IFNs are differentially expressed in bovine primary monocytes and monocyte derived macrophages. Hence, in this study, we investigated the expression of major pro-and anti-inflammatory cytokines, interferons and the expression of enzymes related to L-arginine metabolism (iNOS and arginase-1) during the differentiation of bovine monocytes to macrophages.

2 Materials and Methods

2.1 Animals

Male calves (8-12 months age, n=3) of Hallikar breed (*Bos indicus*) were used for blood collection and monocyte separation. All procedures were performed in accordance with the guidelines and protocols of the Institutional Animal Ethics Committee, IVRI, Bengaluru *vide* F.8-56-Vol.II/RCSS/2018-19/2. Blood samples were drawn from the jugular vein into heparin-coated vacutainer tubes (BD, USA) and processed within 2 hrs of collection.

2.2 Isolation of monocytes from peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation method using Histopaque-1077 (Sigma Aldrich, USA). Briefly, the buffy coat was separated from the whole blood by centrifugation at 1000×g for 20 min at room temperature. The diluted buffy coat was layered over Histopaque-1077 and centrifuged at 1200×g for 30 min at room temperature. The separated PBMC band was collected and washed twice with PBS before proceeding to monocyte separation. Monocytes were separated from freshly isolated PBMCs using magnetically labelled CD14 Micro Beads and MACS LS Columns (Miltenyi Biotec, Germany) in a MACS separator (Miltenyi Biotec, Germany) according to the manufacturer's instructions. A fraction of isolated monocytes was used for total RNA extraction and cDNA preparation while the remaining cells were cultured for producing monocyte derived macrophage.

2.3 Generation of bovine monocyte-derived macrophages (MDM)

Isolated bovine monocytes were resuspended in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, UK), 100 U/mL of penicillin, 100 ug/mL of streptomycin (Sigma-Aldrich, USA) and 40 ng/mL of macrophage-colony stimulating factor (M-CSF) (Sigma-Aldrich, USA). To differentiate bovine peripheral monocytes into macrophages, 1×10^6 monocytes/mL were plated in poly-D-lysine (Merck Millipore, USA) coated 12 well tissue culture plates (Corning, USA) and grown for 7 days at 37°C in a humidified incubator with 5% CO_2. Fresh growth media was added on alternate days, supplemented with the same concentration of M-CSF. Macrophages (MDM) were harvested after day 7 for RNA extraction and cDNA preparation for qRT-PCR.

2.4 RNA isolation and preparation of cDNA

Total RNA was extracted from freshly isolated monocytes and MDM after day 7 using HiPurA™ Total RNA Miniprep Purification Kit (Himedia, India) as described by the manufacturer. The quality and quantity of isolated RNA samples were analyzed on NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA samples with 1.8-2.0 value for A_{260}/A_{280} and 2.0-2.2 for A_{260}/A_{230} were considered as pure and used for cDNA preparation. One microgram of the total RNA from each sample was then reverse transcribed into first strand cDNA using oligo dT primers and RevertAid Reverse Transcriptase enzyme (Thermo Scientific, USA) according to the manufacturer's instructions.

2.5 Real-time PCR

The relative expression of major cytokines such as TNFα, IL12, IL10, IFNα, IFNβ, IFNγ and IFNλ3 and arginine metabolizing enzymes such as iNOS and ARG1 were analyzed by SYBR Green based quantitative RT-PCR using ABI 7300 Real-time PCR system (Applied Biosystems, USA). The oligonucleotide sequences used in real-time PCR assay are given in Table. 1. After an initial incubation of 10 µL reaction mix (5 µL SYBR Green master mix, 200 nM of both forward and reverse primers and 1 µL of the template cDNA) for 10 min at 95°C, amplification was performed for 40 cycles consisting of 15 sec denaturation at 95°C and 1 min annealing and extension at 60°C. GAPDH was used as an endogenous control and the mRNA expression of target genes were normalized to GAPDH to generate ΔCt. The relative fold change, $2^{-\Delta\Delta Ct}$, was calculated using monocyte as calibrator group as per Livak's method (Livak & Schmittgen, 2001).

2.6 Statistical analysis

Results are presented as a mean ± standard error of the mean (SEM). Each experiment was repeated thrice with triplicates. One-sample t-test was used to find the statistical difference with the hypothetical mean of one. Significance was set at 95%. GraphPad Prism 5.0 software (San Diego, CA, USA) was used for statistical analysis and preparation of bar charts.

3 Results

3.1 Morphological changes during maturation.

Recombinant human M-CSF, which has reactivity with multiple species including bovine, was used, for differentiating bovine CD14+ monocytes into MDM *in vitro*. Figure 1 shows the typical morphological changes during the maturation process of bovine peripheral monocytes into macrophages *in vitro*. MDM are elongated and enlarged with small filopodia-like projections. However, complete differentiation of monocytes into macrophages required 7 days (Figure 1A and 1C). Under the influence of M-CSF (40ng/mL), 90% of the monocytes differentiated into MDM based on microscopic morphology.

3.2 Expression of pro and anti-inflammatory cytokines in bovine monocytes and monocyte derived macrophages

Bovine monocytes and MDM were analyzed by qRT-PCR to investigate any difference in the expression of selected pro- and anti-inflammatory cytokines during *in vitro* maturation (Figure 2). As shown in Figure 2A and 2B, a significant ($P<0.01$) down regulation in the expression of TNFα and IL12 by 1.8 and 2.5 folds, respectively was observed in MDM compared to monocytes. In contrast, IL10 showed 1.9-fold up regulation in MDM, which was statistically significant ($P<0.01$) as compared to monocytes (Figure 2C). Taken together, monocyte derived macrophages expressed significantly ($P<0.01$) lower levels of pro-inflammatory cytokines and increased anti-inflammatory cytokine than primary monocytes (Figure 2), indicating that differentiation of monocytes into MDM resulted in decreased inflammatory profile.

Table 1 Details of primers used in this study

Gene	Forward primer	Reverse primer	Reference/Accession Number
GAPDH	5'gatggtgaaggtcggagtgaac 3'	5'gtcattgatggcgacgatgt 3'	Yang et al., 2017
TNFα	5'caaaagcatgatccgggatg 3'	5'ttctcggagagcacctcctc 3'	Puech et al., 2015
IL12	5'tttatgacaaccctgtgccttaga 3'	5'gcctgcatcagctcagcaata 3'	NM_174355.2
IL10	5'gagatgcgagcaccctgtct 3'	5'ggctggttggcaagtggata 3'	Talukder et al., 2018
IFNα	5'gtgaggaaatacttccacagactcact3'	5'tgaggaagagaaggctctcatga 3'	Weng et al., 2015
IFNβ	5'gatgcctgaggagatgaagc 3'	5'ggtgagaatgccgaagatgt 3'	Weiner et al., 2012
IFNγ	5'cagagccaaattgtctccttc 3'	5'atccaccggaatttgaatcag 3'	Puech et al., 2015
IFNλ3	5'actcatccctgggccaca 3'	5'gcttggagtggatgttctgca 3'	Díaz-San Segundo et al., 2011
iNOS	5' cgaggaacaggtggaggactatt3'	5'ggcagcagctttaacttctgtgg 3'	NM_001076799.1
ARG1	5'atggaagtgaatccgtctctgg 3'	5'tggtgggctaaggtaatcaatagg3'	NM_001046154.1

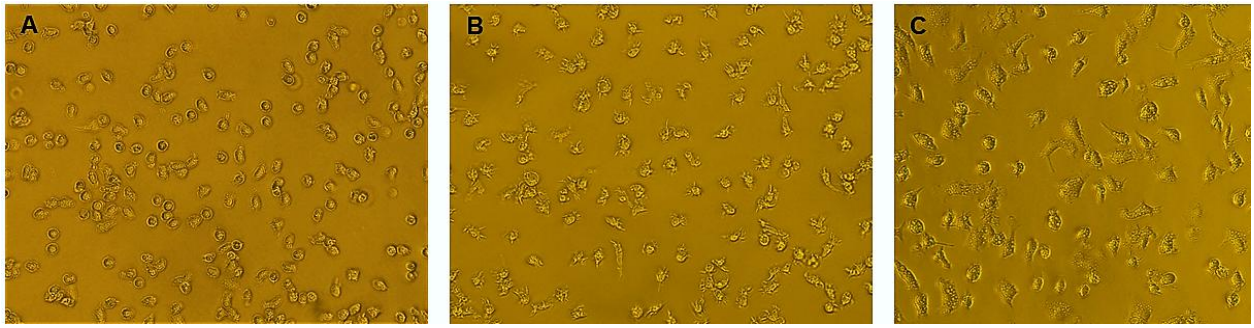

Figure 1 Generation of bovine monocyte-derived macrophages (MDM) from peripheral blood monocytes *in vitro* (Representative micrographs show the morphological changes on day 1, 4 and 7 post-seeding (200X). (A) Bovine peripheral monocytes on day 1 (B) immature MDM on day 4 and (C) differentiated MDM showing clusters of veiled cells with filopodia-like extensions on day 7)

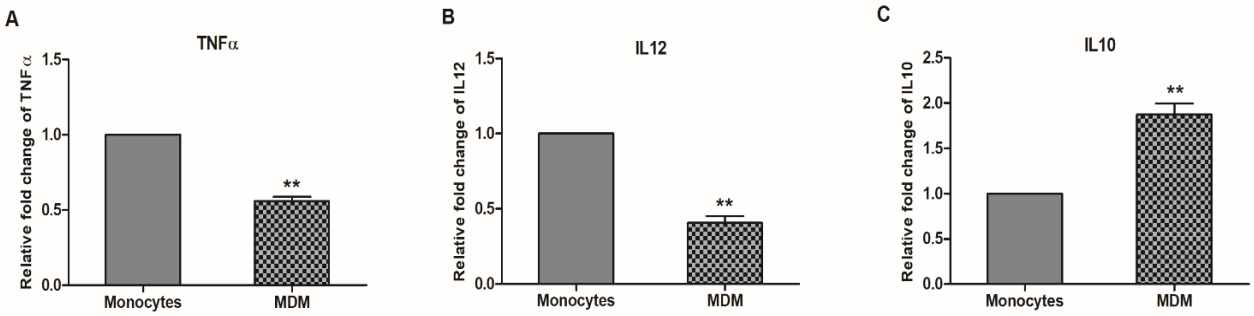

Figure 2 Pro and anti-inflammatory cytokine mRNA expression in bovine monocytes and monocyte-derived macrophages (MDM)

3.3 Expression of interferons in primary monocytes and monocyte derived macrophages

Expression of different interferons were investigated during *in vitro* maturation of bovine monocyte to MDM (Figure 3). In general, Type I (IFNα and β) and Type II (IFNγ) interferon mRNAs were significantly ($P<0.01$) down regulated in MDM as compared to monocytes. The expression of IFNα and β in MDM were down regulated by 2.2 and 1.4 folds, respectively, compared to monocytes (Figure 3A & B). Similarly, a 1.3 fold decrease in the expression of IFNγ was also detected in MDM (Figure 3C). However, the expression of Type III IFN (IFNλ3) in MDM was not modulated ($P>0.05$) and was comparable with monocyte control (Figure 3D). Overall, the maturation of monocytes to MDM was associated with down regulation of Type I and II IFNs with marked effect on IFNα (Figure 3).

3.4 Expression of arginine metabolizing enzymes during *in vitro* maturation of bovine monocyte derived macrophages

The mRNA expression of iNOS and ARG1 in monocytes and MDM was also evaluated (Figure 4). Interestingly, the maturation of bovine monocytes to MDM was associated with a significant ($P<0.01$) up regulation of the enzyme iNOS and down regulation of ARG1. The iNOS mRNA levels in MDM were nearly 3-fold higher than those of primary monocytes (Figure 4A). Whereas, the expression of ARG1 was significantly decreased by 2-fold in MDM compared to monocytes (Figure 4B). Figure 5 shows the amplification plot and dissociation curve of different genes used in this study.

4 Discussion

Macrophages are considered as the most potent phagocytic cells and are essential for both innate and adaptive immune responses. Monocytes can be differentiated into macrophages (MDM) using M-CSF or GM-CSF under *in vitro* conditions (Lacey et al., 2012). In the present study, bovine peripheral monocytes were isolated by immunomagnetic separation technique and induced their differentiation into MDM using M-CSF as reported in human studies (Ciborowski et al., 2007; Rey-Giraud et al., 2012). MDM displays an increased cellular size and elongated morphology compared to monocytes which are primarily small, round cells (Figure 1 A-C). The maturation and differentiation of monocytes into macrophages was associated with several changes such as increased cell size, organelles and increased phagocytic ability (Arango & Descoteaux, 2014).

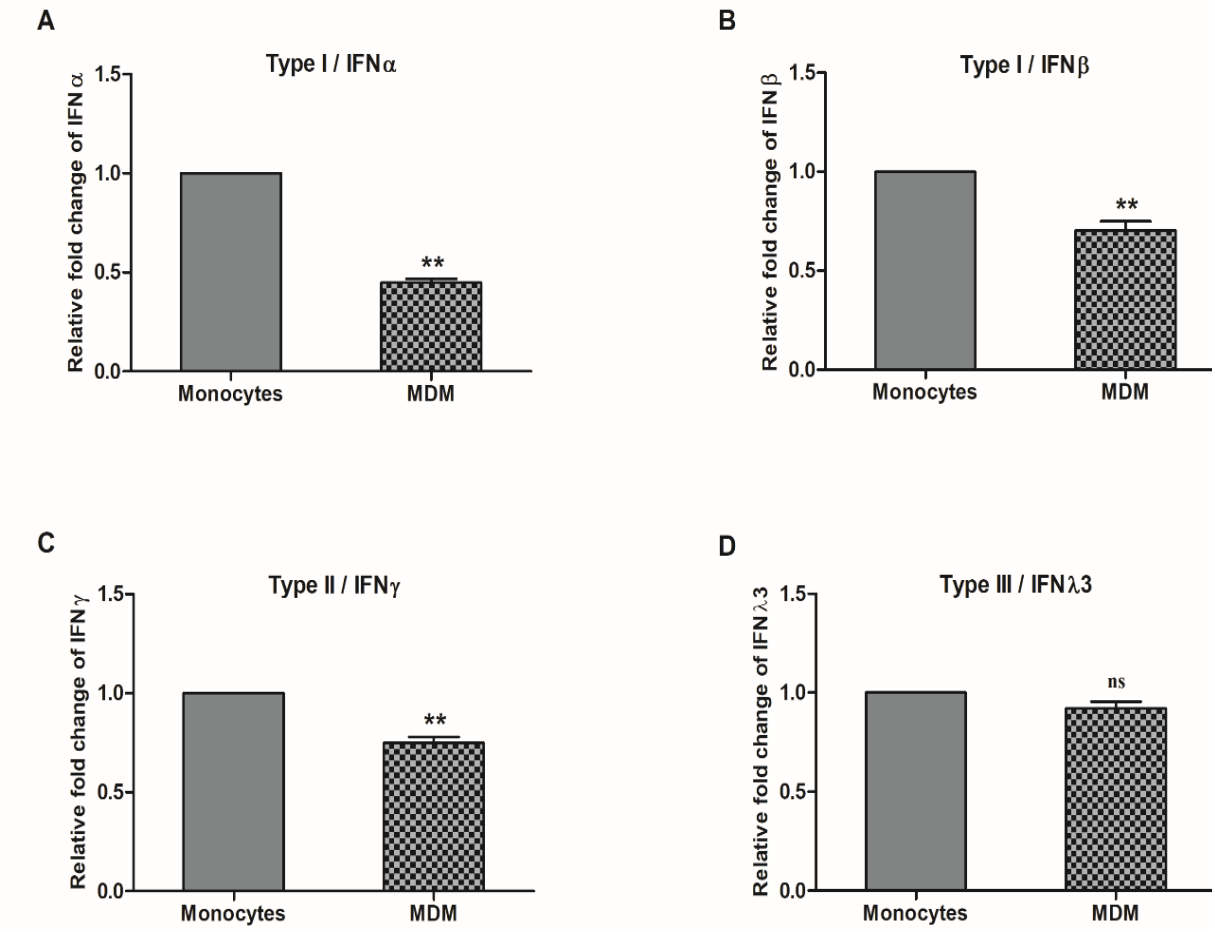

Figure 3 The expression of interferons in bovine monocytes and monocyte-derived macrophages (MDM)

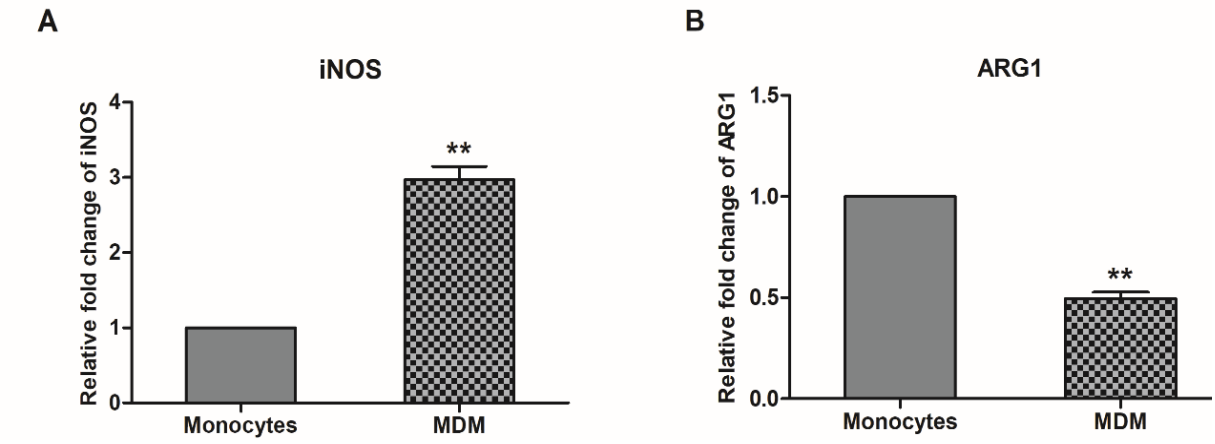

Figure 4 Expression of key arginine metabolizing enzymes in monocytes and MDM

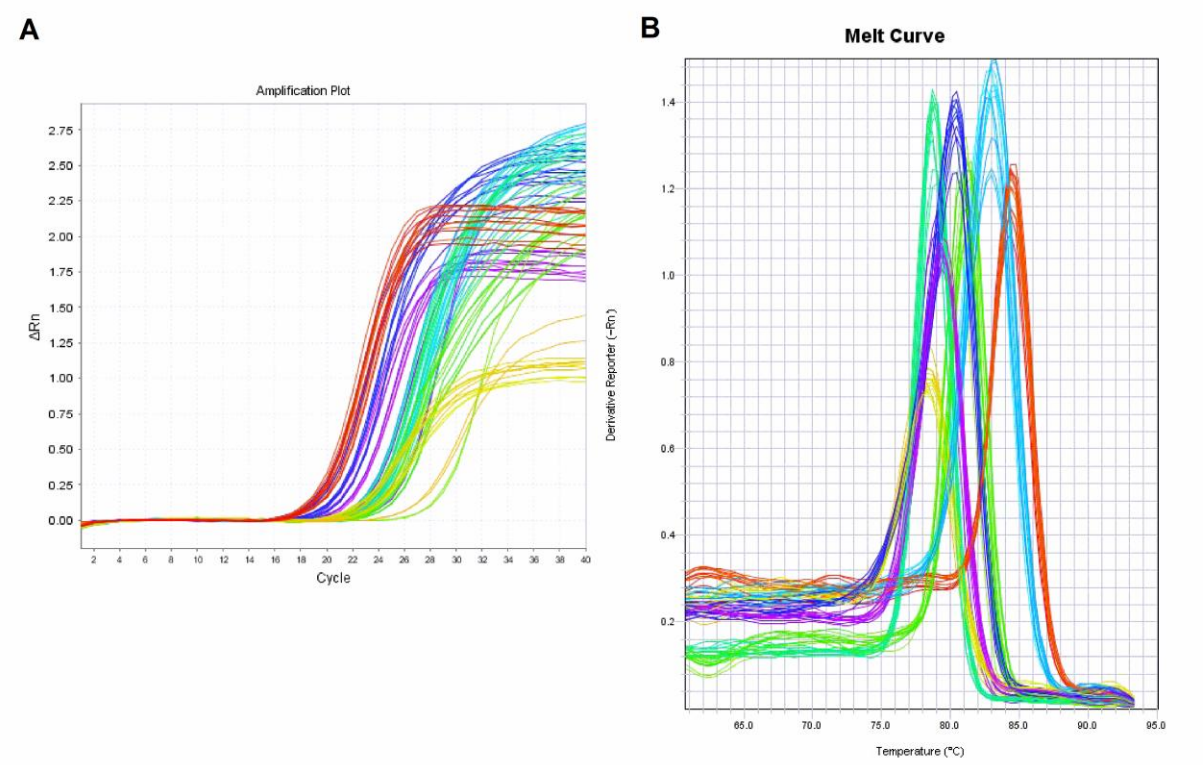

Figure 5 (A) Amplification plot and (B) Dissociation curve of different genes used in the study.

Cytokines produced by macrophages plays a central role in inflammation and other immune responses against microorganisms. Activated macrophages secrete different cytokines which regulates the host immune responses (Cheung et al., 2002; Kumagai et al., 2007). In the current study, it was reported that cytokine profile of un-stimulated bovine monocytes and MDM are distinctly different. Up regulation of anti-inflammatory cytokine IL10 and the down regulation of pro-inflammatory cytokines such as TNFα and IL12 as well as type I and II interferons were notable in MDM (Figure 2 and 3). The inflammatory/interferon response is beneficial for the host system when the aforementioned cytokines are released in appropriate level, but fatal when secreted in an unregulated fashion (Beutler, 1999). Macrophages can respond to various stimuli that are rapidly generated following infection or tissue damage. These stimuli can activate macrophages and up regulate the production of cytokines to limit the replication of the intracellular microorganisms (Standiford et al., 1996). In the current study, MDM can be equated to naïve macrophages as they are not exposed to any inflammatory stimuli or environment. The non-stimulated, naïve macrophages are characterized by limited cytokine production and activity compared to M1 or M2 differentiated macrophages (Italiani & Boraschi, 2014).

To investigate the potential changes in arginine metabolism during *in vitro* maturation of monocytes to MDM, the expression of key enzymes of arginine metabolism (iNOS and ARG1) was also analyzed in this study. Up regulation of iNOS and down regulation of ARG1 in the MDM (Figure 4) suggest basal production of NO even when the cells are non-stimulated. The induction of NO production through iNOS activation indicates the antimicrobial capacity of mononuclear phagocytic cells (Mosser & Edwards, 2008). On the other hand, the activation of the enzyme, arginase 1, degrades the substrate L-arginine and down regulates the NO production in phagocytic cells (Morris, 2007). In this study, arginase 1 expression is significantly decreased in MDM compared to monocytes (Figure 4B), indicating a shift in arginine metabolism toward NO production during *in vitro* maturation of MDM.

Conclusion

M-CSF can induce differentiation of bovine peripheral monocytes into MDM in 7 days of culture. Down regulation of inflammatory cytokines and interferons was hallmark feature of MDM. Up regulation of iNOS suggests that the maturation process is associated with a shift in arginine metabolism toward NO production. However, further studies on estimating the production

of cytokines and interferons in the supernatant will enhance our understanding on cytokine differences during maturation of monocyte-derived macrophages.

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Conflict of Interest

The authors declare no potential conflicts of interest concerning the research, authorship, publication of this article, and/or financial and personal relationships that could inappropriately influence this work.

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