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Characterization of mouse bone marrow-derived macrophages differentiated in L929 cell conditioned medium and colony stimulating factor-1 in *Listeria monocytogenes* infection

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ABSTRACT

Mouse bone marrow-derived macrophages (BMDM) have been identified as an important host cell model for studying mammalian macrophage functions during pathogen infection. Whereas colony stimulating factor (CSF)-1 is required for BMDM differentiation, the commercial CSF-1 is expensive. Therefore, L929 cell-conditioned medium (LCM) has been reported to be used as a source of CSF-1 in many recent studies. However, whether BMDM differentiated by commercial CSF-1 and LCM had any functional differences in bacterial infection, particularly in Listeria monocytogenes (LM) infection, has remained a question. This study was aimed to examine the morphology of macrophages differentiated from CSF-1 and LCM as well as the phagocytic function of these macrophages in LM infection. Mouse bone marrow cells were differentiated in CSF-1 or LCM through six days before being infected by LM. Phagocytotic roles of BMDM were evaluated through the capability of macrophages to take up the bacteria, as shown by infection assay and immunofluorescence microscope. The results showed that macrophages grown in CSF-1 and LCM were similar in morphology and phagocytic functions during LM infection. Macrophages from LCM-supplemented media were homogenous and obtained CD11b and F4/80 surface markers. Immunofluorescence images demonstrated that bone marrow-derived macrophages were able to successfully take up LM at 0.5 hour but unable to control intracellular bacterial replication by 4 hours after infection.

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1 INTRODUCTION

Macrophages are key effectors for both the innate and adaptive immunity, as they participate in different biological processes, including antigen presentation and processing, pathogen eradication, cytokine secretion, and clearance of dead cells (Taylor *el al.*, 2005; Gordon, 2007). Therefore, mouse macrophages have been an important host cell model for studying mammalian macrophage functions. In infectious diseases, various murine macrophages cell lines have been utilized to study pathogen biology and host-pathogen interaction, such as J774A.1, RAW264.7 and P388D1. However, these immortalized cells can behave differently from primary macrophages and continual subculture of these cells *in vitro* can result in mutations that affect macrophage

functions. As a result of that, using primary macrophages isolated from mice is usually preferable in immunological studies. There are three types of primary mouse macrophages, including peritoneal macrophages, alveolar macrophages and BMDM. Although resident peritoneal macrophages and alveolar macrophages can be readily isolated from the mouse, the collected cell number is low, and the cell function can be varied among sanitary conditions of the animal facility. In contrast, BMDM has fully differentiated *in vitro* from bone marrow (BM) stem cells, hence, their functions were stable regardless of the health conditions of the experimental mice. Moreover, BM can give a high yield of a homogeneous population of BMDM. Therefore, using BMDM was more favored in most immunological studies (Marim *et al.*, 2010).

To generate BMDM from BM stem cells, the cell medium must be supplemented with CSF-1 which is required for macrophage differentiation and survival (Pyonteck *et al.*, 2013). Whereas CSF-1 was indispensable during BMDM differentiation, it was quite expensive to use commercial CSF-1 for macrophage generation. An alternative source of CSF-1, which is derived from L929 cell-conditioned medium (LCM), has been used in a number of studies. Macrophages generated from CSF-1 and LCM have shown comparative functions in cell biological studies (Lin and Gordon, 1978; Stuart *et al.*, 1990).

However, whether BMDM produced by commercial CSF-1 and LCM behaved similarly in LM infection has been unknown. In this study, the morphology of macrophages differentiated in CSF-1 and LCM was examined. Additionally, the expression of macrophage surface markers was quantitatively measured. Furthermore, phagocytotic functions of BMDM from these two conditions were evaluated by infecting them with the professional bacteria LM.

2 METHODOLOGY

2.1 Generation of mouse bone marrow-derived macrophages from bone marrow stem cells

Collection of BM cells from bone marrow

Seven- to thirteen-week old C57BL/6 mice (Animal Resources Centre, Perth, WA, Australia) were housed under specific pathogen-free conditions at the Translational Research Institute (TRI, Brisbane, Australia). To isolate BM cells, muscles and tissues surrounding femurs and tibias were removed. Bones were decontaminated with 70-80% ethanol and then rinsed in sterile phosphate-buffered saline (PBS). The isolation of BM cells was performed under sterile conditions. For each femur or tibia, the bone ends were cut and BM cells were flushed into 15 mL tubes with 5 mL Dulbecco's modified Eagle medium (DMEM), using a 25 g needle (Thermo). Tubes containing BM cells were then centrifuged at 340 x g for 10 minutes at 4°C. Cell pellets were resuspended and grown for 6 days in 25 mL macrophage differentiation medium using 150 mm x 15 mm bacteriological petri dishes (Falcon, FAL351058).

Differentiation of BM cells in LCM or CSF-1

Macrophage differentiation medium can be either made up of LCM or CSF-1. Cell pellets from one bone (tibia or femur) were differentiated in 25 mL complete DMEM supplemented with 20% LCM (Moore and Matlashewski, 1994; Weischenfeldt, 2008) for 6 days. Fresh medium (5 mL) was added to the cells every second day. On the sixth day, the supernatant was removed and adherent cells were washed once with 10 mL warm PBS. Ice-cold Ethylenediaminetetraacetic acid (EDTA)/PBS at 1 mM (10 mL) was added to the cells and incubated for 10 minutes at 4°C. After 10 minutes, 10 mL of ice-cold PBS was added, cells were collected in a 50 mL tube and centrifuged at 244 x g for 5 minutes at 4°C. The cell pellet was resuspended in 10 mL of macrophage maintenance medium (complete DMEM supplemented with 10% LCM). Cells were enumerated and transferred to a 24-well plate or 48-well plate prior to infection.

Alternatively, BMDM can be differentiated with recombinant CSF-1. BM cells were differentiated in complete DMEM supplemented with CSF-1 (MACS Miltenyi Biotec, 200 U/mL or 10 ng/ml). Complete DMEM contains 10% heat-inactivated Fetal Bovine Serum (FBS) (Bovagen), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco) and 10 mM HEPES (Gibco). On the fourth day, the media was removed and replaced with fresh media, and cells were collected on the sixth day.

2.2 Flow cytometry

Cells were harvested and incubated with medium containing 10% LCM for 12-16 hours before flow cytometry analysis. Cell viability was examined by staining cells with fluorescent reactive dye (LIVE/DEAD fixable Aqua Dead cell staining kit, Life Technologies) (dilution 1:500 in PBS) for 0.5 hour in the dark at 4°C, followed by surface staining. Cell surface Fc receptors were blocked with anti-CD16/32 (BD Pharmingen) (5µg/mL) for 0.5 hour at 4°C. Cells were labelled with antibodies for macrophage surface markers including CD11b (PE, 5 µg/mL, clone M1/70, BD Pharmingen) and F4/80 (FITC, 5 µg/mL, clone BM8, eBioscience) for 1 hour at 4°C in the dark. All antibodies were diluted in Flow Cytometry Staining buffer (FACS) buffer

(3% newborn calf serum in PBS). Cells were incubated for 1 hour at 4°C in the dark. All antibodies were diluted in FACS buffer. Samples were acquired using the LSRII (Beckton Dickinson) and analyzed using FlowJo (Tree Star, lnc).

2.3 Listeria monocytogenes infection

2.3.1 Bacteria preparation

Three to four single bacterial colonies of LM strains (wild type 10403S, GFP-expressing LM (10403S) (Shen and Higgins, 2005) were added to 5 mL of Brain Heart Infusion/BHI broth, incubated at 37°C overnight in a rocking shaker at 250 rpm, 37°C to obtain an OD_{600nm} between 1.4 to 1.6. The bacterial culture was then diluted 1/100 in 10 mL of BHI broth, and further shaken in the rocking shaker for 2 hours to obtain an OD_{600nm} from 0.05 to 0.1 (Myers *et al.,* 2003). Bacteria were centrifuged at 3273 x g at 4°C for 10 minutes. The pellet was resuspended and diluted in pre-warmed cell culture medium to obtain the desired multiplicity of infection (MOI) before adding to cells. For MOI check, LM inoculum was plated as 10-fold serial dilutions (10^{-3}, 10^{-4}, 10^{-5}) on BHI agar plates. These plates were incubated at 37°C from 24 to 48 hours, and colonies counted to determine colony forming units (CFU).

2.3.2 Listeria monocytogenes infection of macrophages

LM infection of BMDM was performed as described previously (Tilney and Portnoy, 1989). Cells were seeded into 48-well plates at 1.5×10^5 cells per well overnight in complete DMEM. Cells were infected with LM at MOI 5 and centrifuged at 335 x g for 2 minutes at room temperature. Infected cells were incubated at 37°C and 5% CO_2. At 0.5 hour post infection (p.i.), cells were washed twice with 50 µg/mL gentamicin diluted in DMEM to kill extracellular LM (Kuhn *et al*., 1988). Cells were washed twice with warm PBS and further incubated with complete DMEM supplemented with 5 µg/mL gentamicin to prevent continual reinfection of macrophages by LM released from dying cells. At different periods after infection, cells were washed once with 0.5 mL warm PBS and lysed in 1 mL of sterile 0.1% Triton X-100 in PBS. Numbers of viable intracellular LM were determined by performing 10-fold serial dilutions and plating on BHI agar plates. Aliquots of 20 µL of undiluted, 10^{-1}, 10^{-2}, 10^{-3} diluted lysate in PBS were spread on BHI agar plates (1.5% agar). Plates were incubated at 37°C from 24 to 48 hours and colony forming units (CFU) were counted.

2.4 Immunofluorescence

Sterile glass coverslips 15 mm ϕ (G420-15, ProSciTech) were put into each well of a 24 well plate. Macrophages were seeded at 2 x 10^5 cells per well in 350 µL complete medium one day prior to infection. Macrophages were infected with LM at MOI 3 (1 macrophage: 3 LM). At 0.5 hour p.i., cells were washed twice with 50 µg/mL gentamicin diluted in DMEM to kill extracellular LM. Cells were washed twice with warm PBS and further incubated with complete DMEM supplemented with 5 µg/mL gentamicin. At different time points after infection, cells were fixed by 4% paraformaldehyde diluted in PBS for 15 minutes at room temperature. Cell nuclear was stained with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride / DAPI (1 µg/mL, Molecular Probes) for 1 hour in the dark. After staining, the coverslips were washed three times with PBS and one time with distilled water to remove residual salts. Coverslips were mounted with ProLong Gold Antifade Mountant (Life Technologies), and coverslip edges were sealed on microscope slides (S21102A Menzel) using clear nail polish. Microscopy slides were examined using an Olympus Epifluorescence inverted microscope IX73 (60x magnification). Scale bars represent 10 µm.

2.5 Statistics

GraphPad Prism 7.0 software was employed for statistical analysis and graphing.

3 RESULT AND DISCUSSION

3.1 Morphology of bone marrow-derived macrophages differentiated in LCM and CSF-1

Prior to the subculture, the density of BMDM in LCM was slightly higher than BMDM in CSF-1 when growing in large tissue culture dishes (**Figure 1**. A-B). This can be attributed to the protocol difference in changing media. In LCM supplemented media, the old media was not removed but added with fresh media once per two days. However, in CSF-1 supplemented media, on the fourth day of differentiation, old media was removed and new media was added to the cell culture. At the fourth day of differentiation, there were some viable suspended cells, which were pre-macrophages but not ready to adhere to the petri dish. Therefore, the replacement of media on the fourth day in CSF-1 conditions may also lead to a slight loss of BMDM eventually. After 6 days of being differentiated in LCM or CSF-1, BMDM from large culture dishes were collected and sub-cultured into 24-well plate on the seventh day (**Figure 1**. C-D). At the same concentration of 2 x 10^5 cells per well on the seventh day, macrophages generated in LCM and CSF-1

have similar morphology of adherent macrophages. These macrophages were amoeboid, elongated spindle-like or spherical depending on their lamellipodial extensions (Lee *et al.*, 2013).

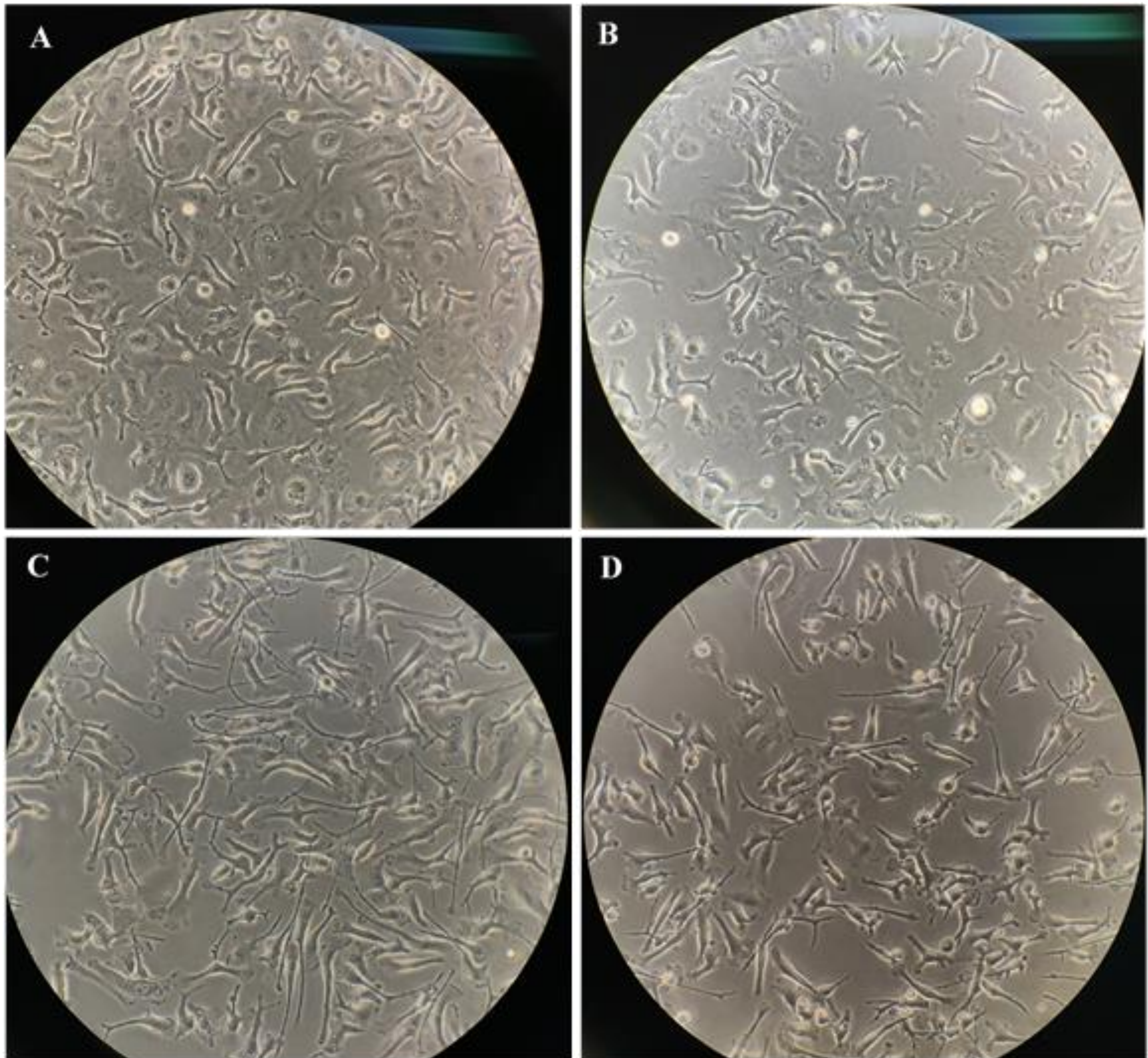

Fig. 1: Macrophages were grown on the day sixth (A, B) and day seventh (C, D) of differentiation in LCM and CSF-1 (Samples were visualized on the Olympus microscope at the magnification of 20)

3.2 Functions of bone marrow-derived macrophages in the phagocytosis and intracellular control of the bacteria Listeria monocytogenes

LM is a Gram-positive bacterium that causes acute infection in immunocompromised people, pregnant women and newborns (Birmingham et al., 2008). Studying pathogenesis of LM during its intracellular invasion of host cells has provided useful knowledge of host-pathogen interaction in immunology (Becattini et al., 2017). Next, the phagocytosis and control of intracellular LM by BMDM grown in LCM and CSF-1 were investigated. At 0.5 hour p.i., when macrophages have successfully internalized LM (Myers et al., 2003), cells were washed with gentamicin at a high concentration to kill extracellular bacteria. Quantification of intracellular bacteria was determined by plating cell lysates for colony forming unit (CFU) determination at 0.5 hour p.i. to examine bacterial uptake by macrophages. Intracellular viable bacteria were then analyzed at 4 hours p.i. In both LCM and CSF-1 conditions, the differentiated BMDM were able to phagocytose LM at 0.5 hour p.i. but could not control LM by 4 hours. This can be seen as a slight increase of intracellular bacterial burden at 4 hours p.i (Figure

2). This was consistent with the replication of intracellular LM reported by many independent studies (Stockinger et al., 2002; Henry et al., 2006; Mitchell et al., 2015). The results demonstrated that BMDMs grown in LCM and CSF-1 were functional during the phagocytosis and intracellular control of the bacteria LM.

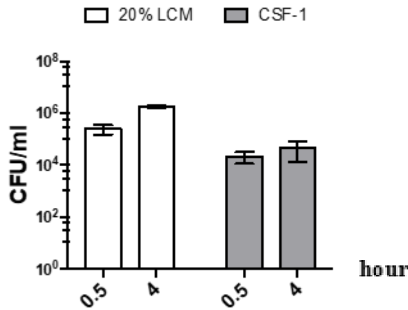

Fig. 2: Intracellular bacteria number in LM infected macrophages (one representative of two independent experiments, each performed in triplicates)

3.3 Examination of the heterogeneity of macrophages differentiated in LCM

The characterization of BMDM differentiated in LCM was examined by using flow cytometry (**Figure 3**). At the day sixth after differentiation, BMDM were stained with Aqua Live/Dead, F4/80 and CD11b to examine macrophage viability and surface marker expression. Flow cytometric analysis showed that the differentiated cells obtained a high homogeneity of the $CD11b^{+}F4/80^{+}$ population. There were 96.75% ± 0.806 SEM of cells acquiring CD11b and F4/80, which are basic macrophage surface markers. This result is consistent with the high purity of macrophages obtained from Ying *et al.* (2013), in which 95% to 99% of CD11b+F4/80+ cells were obtained at the day seventh of the culture. Taken together, these data demonstrated that BM cells differentiated in LCM produced a highly homogenous population of BMDM.

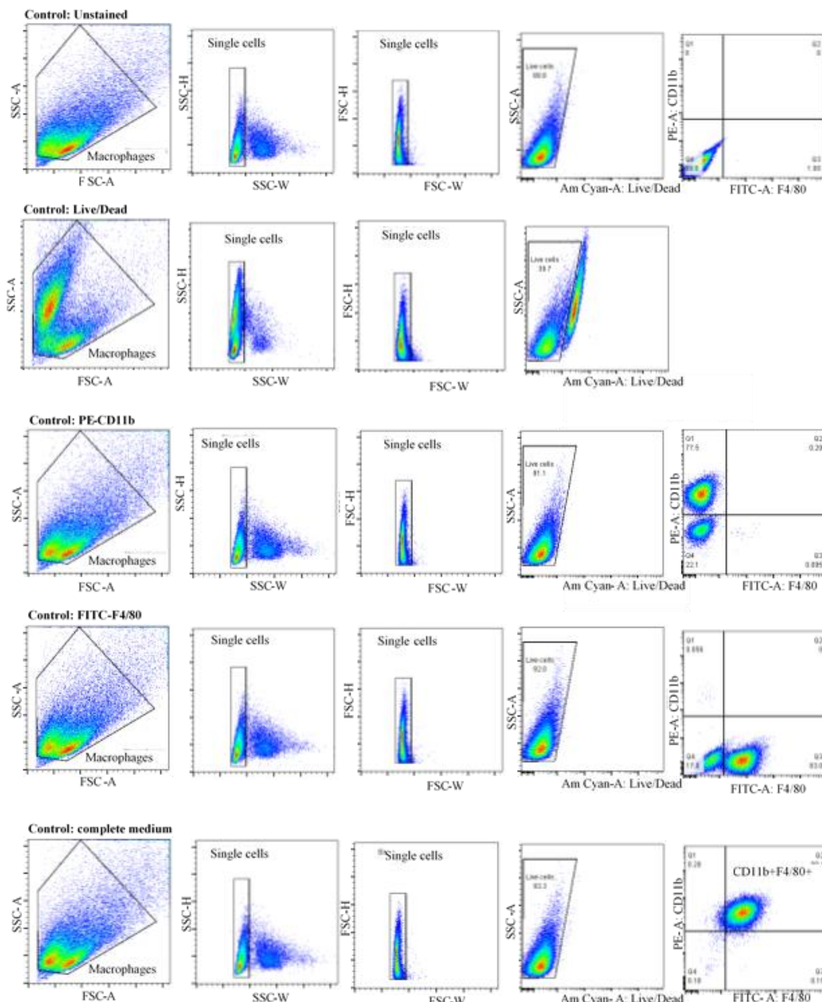

Fig. 3: Flow cytometric analysis of CD11+F4/80+ macrophage population (a representative data from four independent experiments)

3.4 Observation of macrophage during phagocytosis of *Listeria monocytogenes*

It was verified that BMDM generated in LCM were able to phagocytose the bacteria LM using fluorescence microscope. BMDM were infected with GFP-expressing LM (MOI 3). After 0.5 hour, cells were washed by gentamicin 50 µg/mL to remove extracellular bacteria and media was added with fresh medium containing gentamicin 5 µg/mL. At 0.5 hour and 2 hours p.i., infected cells were washed and fixed with 4% paraformaldehyde. Cell nuclear was stained with DAPI. The staining showed a number of LM was taken up by macrophages at 0.5 hour (**Figure 4**). At 2 hours p.i., bacteria number sharply increased inside host cells. This indicated that after 2 hours p.i., the pathogen was successfully replicated inside the host cells, as reported by Stockinger *et al.* (2002), Henry *et al.* (2006) and Mitchell *et al.* (2015). This increase of bacterial burden is also consistent with the CFU data in **Figure 2**, which show a rise of intracellular LM by 4 hours.

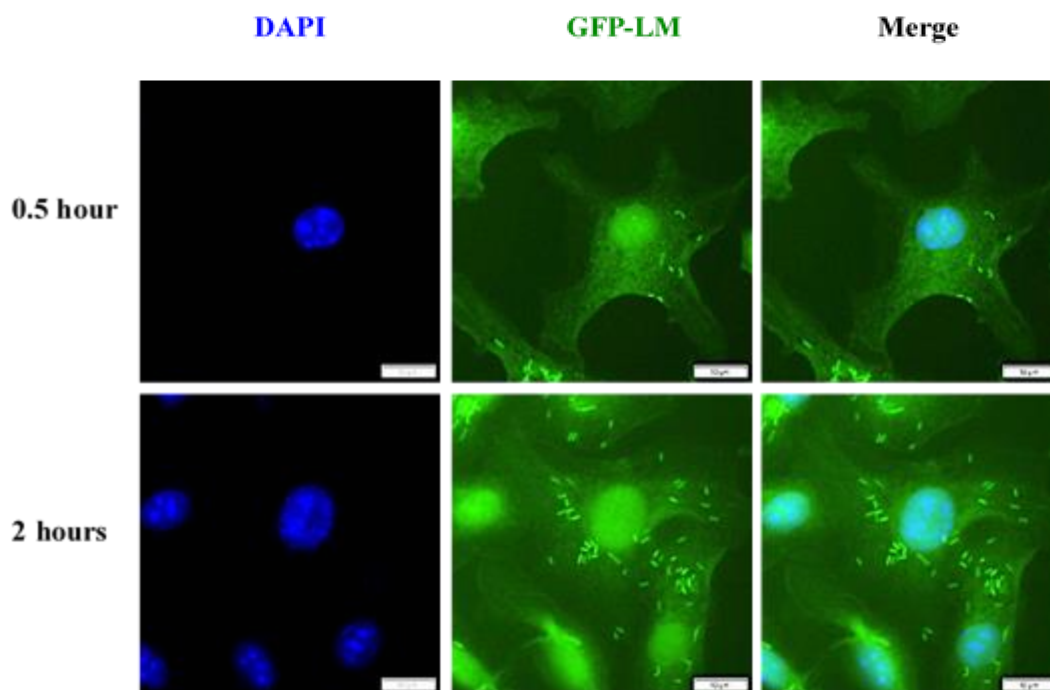

Fig. 4: Visualization of intracellular LM in infected BMDM at 0.5 hour and 2 hours after infection (Images were taken by epifluorescence microscope at the magnification of 60x. Scale bars represent 10 µm)

4 CONCLUSIONS

In this study, the phenotype and functions of BMDM generated in LCM and CSF-1 in the context of LM infection characterized were. BM cells growing in LCM and CSF-1 produced a homogenous population of macrophages, which are able to phagocytose the professional bacteria LM. Due to high cost of the commercial CSF-1, LCM is more common used to generate *in vitro* BMDM for infection assays. In LM infection, BMDM are able to take up the bacteria as early as 0.5 hour p.i. From 2 hours to 4 hours p.i., the number of intracellular bacteria significantly increased, indicating that macrophages cannot resist to this professional bacterium *in vitro* infection.

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