



Research Article

Effect of Dexamethasone, Lipopolysaccharide or Interferon-Gamma on the Recovery of Viable *Mycobacterium avium* Subspecies *paratuberculosis* from *In Vitro*-Infected Primary Bovine Macrophages

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Abstract

Study background: The study was designed to evaluate if the addition of Dexamethasone, IFN-g, or LPS into culture media of primary bovine Monocyte-Derived Macrophages (MDMs), could support *in vitro* infection with *Mycobacterium avium* subspecies *paratuberculosis*.

Methods: Primary bovine Monocyte-Derived Macrophages (MDMs) were infected *in vitro* with a reference strain of Map at 5:1 MOI for 2h. Map-infected MDMs were stimulated with IFN-g, LPS, Dexamethasone or medium alone for 24h. At 0, 6, 72 and 120h of culture, it was

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evaluated the presence of Map by bacterial culture and amplification of the IS900 fragment by real-time PCR. The function of Map-infected MDM was evaluated by measurement of TNF- α , IL-6, IL-8, IL-10, IL-12, IP-10, and CCL3 in culture supernatants by Luminex. Data were analyzed by Kruskal-Wallis test.

Results: The IS900 segment was amplified in samples of Map-infected MDM from all stimuli. The growth of Map in bacterial culture was observed at each time-point evaluated without statistically significant differences between groups. Map-infected-MDMs stimulated with Dexamethasone significantly reduced cytokine production compared with control, excepting for IP-10 production from 6 to 120h ($P < 0.01$). Overall cytokine production at 72h was significantly higher in Map-infected MDM treated with LPS ($P < 0.01$) excepting for IP-10 and CCL3 production at 120h. IL-8 and IL-12 production at 72h and IP-10 production at 120h were significantly higher in Map-infected MDM treated with IFN-g ($P < 0.01$).

Conclusion: Primary bovine MDM obtained from peripheral blood mononuclear cells could be used for growth of Map *in vitro*. The addition of LPS or IFN-gamma reduced the capability of MDM for sustaining the growth of Map until 120h post-infection, although Dexamethasone sustained the recovery of viable Map until 120h in culture.

Keywords: Chemokines; Cytokines; Intracellular Pathogens; Monocyte-Derived Macrophages

Abbreviations

- JD: Johne's Disease
Map: *Mycobacterium avium* subspecies *paratuberculosis*
IFN-g: Interferon gamma
IL-10: Interleukin 10
TGF- β : Transforming Growth Factor type beta
Th1: Type 1 CD4+ T helper cells
Th2: Type 2 CD4+ T helper cells
CTL: Cytotoxic T cells
LAK: Lymphokine-Activated T cells
MDM: Monocyte-Derived Macrophages
LPS: Lipopolysaccharide
IS900: Insertion sequence 900, used as a target for the identification of *Mycobacterium avium* subsp. *paratuberculosis*
MoAb: Monoclonal Antibody
MOI: Multiplicity of Infection
ATCC: American Type Culture Collection
PBMC: Peripheral Blood Mononuclear Cells
TNF- α : Tumor Necrosis Factor alpha
CCL3: CC Chemokine Ligand 3
IP-10: Interferon-induced Protein 10
Mycobactin-j: Iron-chelated substance used as a growth factor for the isolation of Mycobactin-dependent *Mycobacterium*
BCG: Bacillus Calmette-Guérin (vaccine)

Introduction

Mycobacterium avium subspecies *paratuberculosis* (Map) causes paratuberculosis or Johne's Disease (JD), which is a chronic

granulomatous disease affecting the intestine of domestic and wild ruminants [1]. The Map is an intracellular pathogen that infects resident and circulating macrophages [2]. Once into cells of the phagocytic mononuclear system, Map reaches the lamina propria of intestinal mucosae, where it proliferates in a low-rate manner and causes the typical chronic granulomatous enteropathy found in clinical stages of JD [3]. In susceptible animals, two subclinical and two clinical phases of JD occurs [4]: In subclinical phases I and II, there are no clinical signs of the disease, IFN-g is the predominant cytokine in tissues where Map is present, and there is no evidence of Map proliferation into macrophages [5,6]. Infected animals cannot be detected by conventional methods, although elimination of Map in feces could occur. In intermittent clinical (Phase III) and terminal clinical (Phase IV) phases, infected bovines exhibit clinical signs such as intermittent to chronic diarrhea and emaciation. Also, IL-10 and TGF- β are the cytokines predominantly found in the lamina propria [4], and Map is actively proliferating into macrophages. During phase IV of JD Map-infected circulating or resident macrophages are detected in peripheral blood [2], milk [7], and mesenteric and mediastinal lymph nodes, respectively, and infected macrophages are detected in the spleen [2,7]. Under natural and experimental infections, Map persists into the infected macrophages during extended periods of time, although the mechanisms responsible for Map proliferation after its reactivation and its ability to evade the host's immune response remains unclear [8]. Once pathogenic Map strains are located into the macrophages' phagolysosome, Map activates the transcription of genes responsible for blocking its destruction into the phagolysosome [9].

Interestingly, it appears that production of Th1 cytokines is required for an efficient elimination of Map and Map-infected macrophages by activation of genes [10,11] mediating the interaction of the infected macrophage with Cytotoxic T (CTL) cells and Lymphokine-Activated Killer (LAK) cells. In this work, we hypothesized that modulating the conditions of macrophages cultured *in vitro*, with Th1 or Th2 cytokines or the immunomodulatory agent Dexamethasone, could affect macrophages' ability to support Map infection and proliferation. Cytokine and chemokine production by the infected-MDM as well as Map survival and proliferation were considered as indicators of MDM functionality in our system. The study was designed to evaluate if primary bovine MDM could be used for the intracellular replication and further recovery of Map under the addition of Dexamethasone, IFN-g, or LPS *in vitro*.

Materials and Methods

The Institutional Board on Animal Experimentation from the University of Antioquia approved the study (Act # 37, June 7, 2007), and graded the project as "Minimal risk" for animals used in the study. Donor cows were cared and handled under responsible handling and respect for animal rights by the veterinarians, according to the Colombian law for animal protection and the Vancouver statement on animal experimentation. The same veterinarian researcher sampled the cows always.

Selection of cows for isolation of primary Monocyte-Derived-Macrophages (MDM)

Map-free lactating Holstein cows (*Bos taurus*) were selected as blood donors for MDM isolation. Holstein cows were from a dairy located in the Northern Region of Antioquia State (Colombia), on

a humid subtropical forest, 2450 meters over sea level, an average 2600 mm/year rainfall, and 85% relative humidity. The region is one of the most important dairy regions in Colombia [12,13]. Fecal samples were taken from JD-free lactating Holstein cows (n=17) and were tested for amplification of the Insertion Sequence 900 (IS900) of Map, using real-time PCR [14]. Cows were checked for two years for detection of clinical signs compatible with JD. The clinical outcome included evaluation of progressive weight loss, body condition score, and the presence of chronic or intermittent diarrhea. Seven out of 17 cows were discarded because of clinical problems not related to JD (respiratory disease, laminitis, eosinophilic enteritis, and liver abscesses). The remaining ten cows were negative for Map excretion in feces as evidenced by consecutive negative real-time PCR tests. Finally, one out of the 10 Map-negative cows exhibiting the best conditions for *in vitro* culture in MDM (e.g., cell morphology, viability and longtime persistence in culture) was selected as a donor of MDM for *in vitro* infection.

Collection of fecal and blood samples

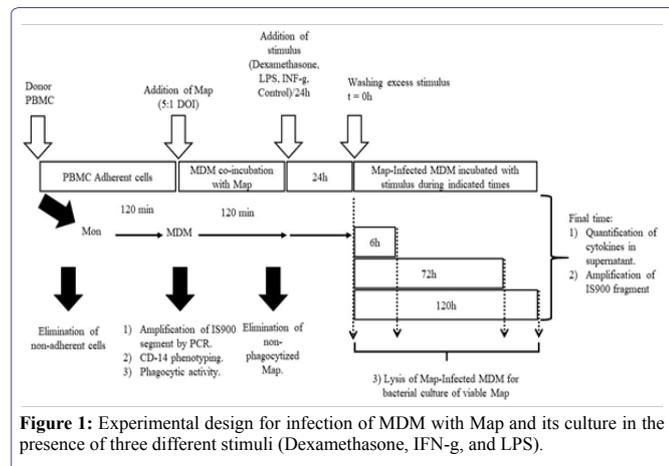
Fecal samples from 17 clinical healthy lactating cows were collected from the rectal ampulla with disposable sterile obstetric gloves. Samples were labeled and transported at 4°C to the laboratory within 4h after collection and later on were frozen at -80°C until processing. Blood samples from the donor cow were collected from the jugular vein with a 14 mm venous catheter after aseptically cleaning of the jugular area and put into a pearl-containing sterile glass 100 mL Erlenmeyer. Samples were transported to the laboratory at 4°C.

DNA extraction from fecal samples

Total DNA was extracted from fecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. DNA from Map-infected MDM was extracted using the DNeasy® Blood and tissue kit (QIAGEN, Texas USA) according to the manufacturer's instructions. DNA purity and concentration was calculated by the 280/260 nm ratio using a Nanodrop® ND1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The extracted DNA samples were stored in aliquots at -80°C until processing. In all cases, DNA integrity was evaluated using 1.8% agarose gel electrophoresis (Agarose I Amresco® Ohio, MI, USA). The gel was run in a Biorat Universal Hood II (Laboratory Segrate, Milan, Italia) at 110 V for 50min.

Isolation and culture of MDM, infection with Map *in vitro* and its functional evaluation

In figure 1, we presented the scheme of the experimental protocol used in the study. Bovine monocytes were obtained after gradient centrifugation on Fycoll Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). Peripheral Blood Mononuclear Cells (PBMC) were incubated in 10% Fetal Bovine Serum (FBS) (Gibco/Invitrogen, Miami, FL, USA) supplemented with RPMI-1640 medium (Mediatech Inc., Herndon, VA, USA) added with 20000 UI/mL penicillin and 1 mg/mL streptomycin (Gibco/Invitrogen, Miami, FL) at 37°C in 6 well flat bottom culture plates (Falcon BD Labware, Franklin Lakes, NJ) and cultured under humidified atmosphere with 5% CO₂. Non-adherent cells were discarded by washing with cold medium. Plastic-adherent cells were recovered and evaluated for CD14 expression by flow cytometry using a PE-conjugated mouse anti-human CD14 mAb (clone 61D3) (Biosource, San Diego, CA, USA) that recognizes bovine CD14 [15].



For evaluation of the phagocytic activity, bovine MDMs (10^6 cells/mL) were incubated with PE-conjugated microbeads (CalIBRITE™ PE beads, Becton-Dickinson, CA, USA) at $37^\circ\text{C}/60\text{min}$. The beads were washed, and the phagocytic cells were evaluated by flow cytometry using a Coulter Epics XL y BD FACScanto II cytometer. As a positive control, we used PE-coupled microbeads alone.

For the infection of MDM *in vitro*, it was used the Map 19698 ATCC® reference strain (American Type Culture Collection, Rockville, MD, USA). The strain was thawed and cultured in 50 mL Middlebrook 7H10 medium (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% OADC (Becton Dickinson, Franklin Lakes, NJ, USA), 0.5% Tween 80 and 0.0002% mycobactin-J (Allied Monitor Inc., Fayette, USA). The strain was cultured at 37°C for six weeks and growth of Map was evidenced by checking of the turbidity in culture media followed by identification of the growing bacteria using Ziehl-Neelsen (Sigma-Aldrich, St. Louis, MO, USA) staining. The concentration of Map was calculated using a Spectronic® 20 Genesys Spectrophotometer (Sigma-Aldrich, St. Louis, MO, USA) which resulted in 8.3×10^6 mycobacteria/mL. Cultures of Map were centrifuged at $1,075 \text{ g}/10\text{min}$, the supernatant was discarded, and the mycobacteria-containing sediment was washed in sterile 1x Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco/Invitrogen, Miami, FL) at $1,075 \text{ g}/10\text{min}$. The mycobacteria were resuspended in glycerol and Tween 80-containing 1x DPBS (Sigma-Aldrich, St. Louis, MO, USA). Then they were subjected to four cycles of sonication at 140 Watts/10 secs/ 4°C with 2 min pause between cycles. After final washing at $67 \text{ g}/5\text{min}$, the supernatant was stored in 400 μL aliquots and was frozen at -80°C until processing.

Bovine MDMs were infected with the MAP 19698 ATCC® strain in a 5:1 Multiplicity of Infection (MOI) in the presence of 10% inactivated autologous serum. The MDM concentration was adjusted to 1×10^6 cells/mL, whereas Map was added to meet a final concentration of 5×10^6 bacteria/mL, meeting the criteria for 5:1 DOI. Map-infected MDMs were incubated in RPMI-1640 medium (Mediatech Inc., Herndon, VA, USA) at 37°C and 5% $\text{CO}_2/2\text{h}$ in a humidified atmosphere. After completing the time of infection, non-phagocytized mycobacteria were washed three times using the cold-RPMI-1640 medium at $151 \text{ g}/10\text{min}$. Then the pellet containing Map-infected MDM was reconstituted at 1×10^6 cells/mL (Figure 1).

After infection with the reference strain of Map, MDMs were pre-incubated with 10 ng/mL LPS (Sigma, St. Louis, MO, USA),

$3 \times 10^6 \text{ IU}/500\mu\text{L}$ human recombinant (hr)-IFN-g, or $1 \mu\text{g}/\text{mL}$ Dexamethasone (Merck, Bogota, Colombia). Map-infected MDMs were added with each independent stimulus diluted in 10% FBS (Gibco/Invitrogen, Miami, FL, USA) containing RPMI-1640 (Mediatech Inc., Herndon, VA, USA) at $37^\circ\text{C}/2\text{h}$. As a control, MDMs were cultured in complete RPMI-1640 media alone. After 24 h of culture, cells were washed for eliminating the stimulus. After washing, Map-infected MDM ($1 \times 10^6/\text{mL}$) were cultured in 12 well plates in a humidified atmosphere at 37°C for 6, 72, or 120h. At each time-point, supernatants were recovered and stored at -196°C until processing for cytokine measurements by Luminex®. Also, Map-infected MDM and control MDM, were lysed by sonication, and cell lysates were processed for DNA extraction and amplification of IS900 sequence of Map by real-time PCR (Figure 1).

Quantification of cytokines and chemokines in the supernatants of MAP-infected MDM by Luminex

The cytokines TNF- α , IL-6, IL-8, IL-10 and IL-12 (p40/p70), and the chemokines IP-10 and CCL3, were measured in culture supernatants of Map-infected MDM or control MDM collected at 0, 6, 72 and 120h after culture, following the manufacturer's instructions (Human Cytokine/Chemokine Magnetic Bead Panel, Milliplex® Map Kit, Billerica USA). The detection limit for each cytokine and chemokine were: TNF- α , 90 pg/mL; IL-6, 104 pg/mL; IL-8, 93 pg/mL; IL-10, 103 pg/mL; IL-12, 110 pg/mL; IP-10 (also known as CXCL10), 95 pg/mL; and CCL3, 148 pg/mL. Samples were evaluated by duplicates. The manufacturer provided negative and positive controls for each cytokine and chemokine. This kit because of the lack of reagents for measuring bovine cytokines and chemokines at the time we performed the study.

The amplification of the IS900 sequence of Map by real-time PCR

Real-time PCR test was carried out in a final volume of 25 μL containing 200nM dNTP each (Invitrogen, Miami, FL, USA), 12.5 μL of 1x SYBR Green I PCR Master mix (Invitrogen, Miami, FL, USA), 6.5 μL H_2O DEPC, 0.5 μL of forward (62.5 nM) and reverse (62.5 nM) primers, and 5 μL of sample DNA (5-25 $\mu\text{g}/\mu\text{L}$). The sequences of forward and reverse primers for the IS900 of Map were obtained from GenBank (National Center for Biotechnology Information, USA) as follows: Forward (FW) 5'CGACGTGTCCTTACACAGC3' and reverse (RW) 5'GTATGGTTTCATGTGGTT3'. Melting temperatures and molecular weight of the primers were 55.8°C and 5,748.8 g/mol, and 50.1°C and 5,895.9 g/mol, respectively. The thermal profile of the real-time PCR for the IS900 sequence was as follows: Denaturation at $95^\circ\text{C}/15\text{min}$, amplification phase of 50 cycles at $95^\circ\text{C}/30 \text{ sec}$, $60^\circ\text{C}/30 \text{ sec}$, $72^\circ\text{C}/30 \text{ sec}$, and a final elongation step at $72^\circ\text{C}/3\text{min}$. The range of temperature for the melting curve was 65 to 85°C . The expression of GAPDH gene was used as housekeeping gene using the following forward and reverse primers: FW 5'TGCTGGTGCTGAGTATGTGGT3' and RW 5'AGTCTTCTGGGTGGCAGTGAT3'. The melting temperature was 58.7°C and 58.4°C , respectively. The thermal profile of the real-time PCR for the GAPDH gene was as follows: Denaturation at $95^\circ\text{C}/15\text{min}$, an amplification phase of 45 cycles at $95^\circ\text{C}/8\text{sec}$, $60^\circ\text{C}/5\text{sec}$, $72^\circ\text{C}/10\text{sec}$, and a final elongation step at $72^\circ\text{C}/10\text{min}$. The range of temperature for the melting curve was 65 to 95°C . All real-time PCR reactions were performed in a Rotor-Genet 6000 real-time rotary analyzer

(Corbett Research, Mortlake, Australia) and results were analyzed using the Rotor-Gene 6000 series 1.7 software. PCR curves and fluorescence normalization were established by the Threshold value (Ct), which resulted in 0.003 for the IS900 sequence of Map and 0.001 for the GAPDH gene. The LinRegPCR® software was used for calculation of amplification efficiency. All samples were processed in triplicate, and data were analyzed by the $-(\Delta\Delta Ct)$ method [16]. The GAPDH gene was used as a control for calculating ΔCt , whereas cultures added with Dexamethasone, IFN-g and LPS were used for calculation of $\Delta\Delta Ct$.

Recovery of Map from lysates of Map-infected MDM for bacterial culture

At time 0 (after 24h incubation with each stimulus) and at 6, 72 and 120h after culture and elimination of excess of stimulus, Map-infected MDM and control MDM were collected and lysed using the CV33 sonicator (Sonics Vibra Cell, Newtown, CT, USA) and lysates were frozen until processing. After thawing, six serial dilutions of the supernatant were performed, and the final dilution was seeded in OADC-supplemented Middlebrook 7H10 medium (Becton Dickinson, Franklin Lakes, NJ, USA) without mycobactin-j [17]. Cultures were incubated at 37°C/3weeks. Finally, the number of Colony Forming Units (CFU) was measured by direct observation of the plates.

Statistical Analysis

All quantitative variables including the Relative Concentration of the Gen (RCG) for the IS900 sequence, cytokine concentration in culture supernatants, and Map CFU were evaluated for normality by the method of Levene's homogeneity of variances. The Kruskal-Wallis test evaluated differences between medians. With the Ling-PCR® software it was obtained the relative quantification units for each gene by calculating the mean amplification difference of $\Delta\Delta Ct$ between the IS900 sequence and the GAPDH gene. Differences between means from data that did not meet the assumption of normality were evaluated by the Kruskal-Wallis test and were confirmed by Dunns posthoc test. P-value was established as $P < 0.05$.

Results

The ability of primary bovine MDMs to become infected by and sustain the growth of Map

Results of real-time PCR evidenced the amplification of IS900 sequence of Map in samples of primary bovine MDMs that were previously incubated *in vitro* with a reference strain of Map (Map-infected MDM), were further incubated with Dexamethasone, IFN-g or LPS for two hours, and finally culture during 6, 72 and 120h. As shown in figure 2, the IS900 fragment of Map was amplified in all samples harvested from Map-infected MDM, with no statistically significant differences between groups ($P > 0.05$). Also, it was found a high level of variability in results, particularly in Map-infected MDM stimulated with Dexamethasone. In none of the control wells, it was amplified the IS900 fragment of Map.

Furthermore, the growth of Map present in samples harvested of lysed Map-infected MDM was evaluated in bacterial culture using Middlebrook 7H10 medium without mycobactin-j. Map growth was evidenced in all cultures of samples taken from Map-infected MDM. Only in samples of Map-infected MDM stimulated with Dexamethasone and cultured for 120h, the number of CFU of Map was significantly higher compared to control MDM (Figure 3). The amount of

CFU was higher in Map-infected MDM at time zero and after 6h of culture, then decreased at 72h and was negligible at 120h (Figure 3). In samples harvested of control MDMs, there was no growth of Map in bacterial culture.

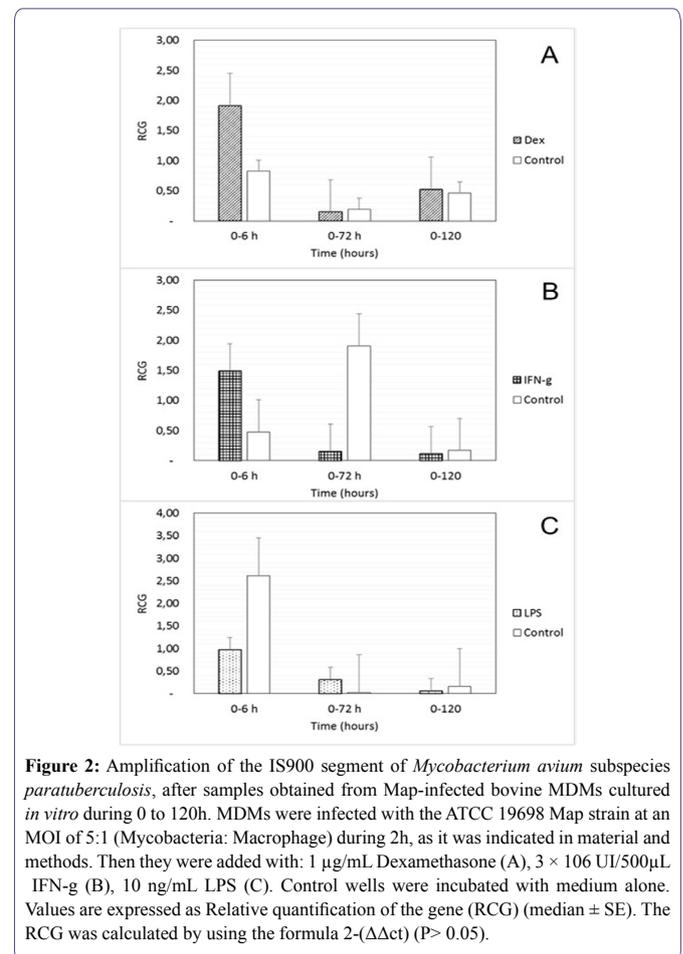


Figure 2: Amplification of the IS900 segment of *Mycobacterium avium* subspecies *paratuberculosis* after samples obtained from Map-infected bovine MDMs cultured *in vitro* during 0 to 120h. MDMs were infected with the ATCC 19698 Map strain at an MOI of 5:1 (Mycobacteria: Macrophage) during 2h, as it was indicated in material and methods. Then they were added with: 1 µg/mL Dexamethasone (A), 3 × 10⁶ UI/500µL IFN-g (B), 10 ng/mL LPS (C). Control wells were incubated with medium alone. Values are expressed as Relative quantification of the gene (RCG) (median ± SE). The RCG was calculated by using the formula 2^{-(ΔΔCt)} ($P > 0.05$).

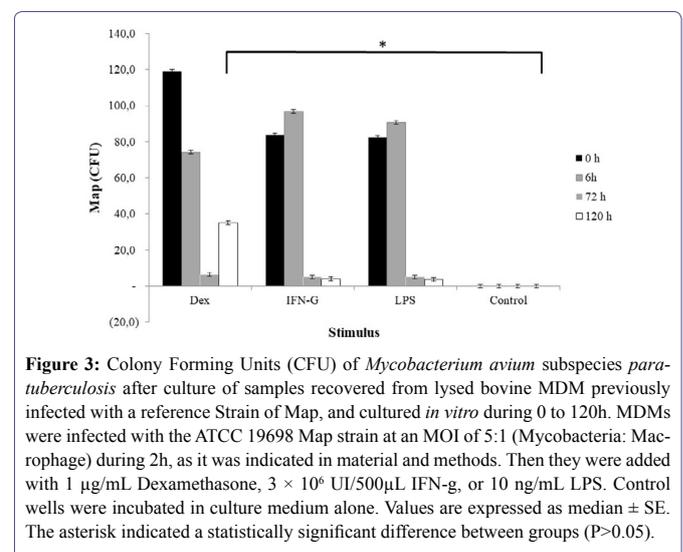


Figure 3: Colony Forming Units (CFU) of *Mycobacterium avium* subspecies *paratuberculosis* after culture of samples recovered from lysed bovine MDM previously infected with a reference Strain of Map, and cultured *in vitro* during 0 to 120h. MDMs were infected with the ATCC 19698 Map strain at an MOI of 5:1 (Mycobacteria: Macrophage) during 2h, as it was indicated in material and methods. Then they were added with 1 µg/mL Dexamethasone, 3 × 10⁶ UI/500µL IFN-g, or 10 ng/mL LPS. Control wells were incubated in culture medium alone. Values are expressed as median ± SE. The asterisk indicated a statistically significant difference between groups ($P > 0.05$).

Cytokine and chemokine production by Map-infected MDM

Map-infected MDM previously stimulated with Dexamethasone, IFN-gamma or LPS for 24h and then cultured until 120h, presented a variable profile of cytokine production at all time-points evaluated, suggesting Map-infected MDM still functional until 120h in our system. Values of cytokine production by Map-infected MDM are presented in table 1. Values of Map-infected cytokine production presented in figures 4-8 are weighted to cytokine production of control MDM at each time-point of evaluation, and represents variation over and under control values.

TNF- α production

TNF- α production was significantly reduced from 6 to 120h of incubation in Map-infected primary bovine MDMs stimulated with Dexamethasone compared to control (Table 1 and Figure 4). On the contrary, TNF- α production was significantly increased at 6, and 72h in Map-infected MDM incubated with IFN-g or LPS.

IL-6 production

Interleukin-6 production significantly decreased from 6 to 120h in Map-infected MDM incubated with Dexamethasone compared to

control. No effect of IFN-g or LPS was observed on IL-6 production by Map-infected MDMs compared to control MDMs (Table 1 and Figure 5).

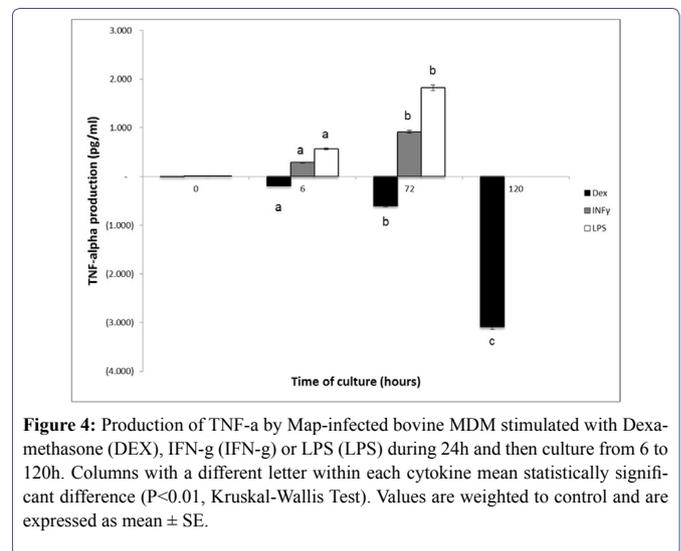


Figure 4: Production of TNF- α by Map-infected bovine MDM stimulated with Dexamethasone (DEX), IFN-g (IFN-g) or LPS (LPS) during 24h and then culture from 6 to 120h. Columns with a different letter within each cytokine mean statistically significant difference ($P < 0.01$, Kruskal-Wallis Test). Values are weighted to control and are expressed as mean \pm SE.

Cytokine (Units)	Time (Hours)	Control	Dexamethasone	IFN-gamma	LPS
TNF-alpha (pg/mL)	0	14,7 \pm 0,4	9,7 \pm 0,2	18,0 \pm 0,5	16,0 \pm 0,5
	6	382,4 \pm 11,3	193,7 \pm 1,1	668,6 \pm 19,8	951,2 \pm 28,1
	72	1224 \pm 36,2	619,8 \pm 1,9	2.139,6 \pm 63,3	3.043,9 \pm 90,1
	120	5000 \pm 0	1.913,4 \pm 3,3	5.000,0 \pm 0	5.000,0 \pm 0
IL-6 (pg/mL)	0	52,7 \pm 5,5	58,4 \pm 6,1	56,8 \pm 6,0	52,7 \pm 5,5
	6	366,8 \pm 38,4	123,9 \pm 13,0	365,4 \pm 38,3	366,8 \pm 38,4
	72	1.137,1 \pm 119,2	384,0 \pm 40,3	1.132,9 \pm 118,8	1.137,1 \pm 119,2
	120	3.638,6 \pm 381,4	1.228,9 \pm 128,8	3.625,3 \pm 380,0	3.638,6 \pm 381,4
IL-10 (pg/mL)	0	14,2 \pm 2,0	16 \pm 1,7	2,7 \pm 1,6	14,2 \pm 2,0
	6	274,1 \pm 38,3	150 \pm 15,7	44,3 \pm 25,6	274,1 \pm 38,3
	72	724,4 \pm 210,0	464 \pm 48,7	137,4 \pm 79,3	724,4 \pm 210,0
	120	724,4 \pm 210,0	1.485 \pm 155,7	439,7 \pm 253,9	724,4 \pm 210,0
IP-10 (pg/mL)	0	37,7 \pm 2,1	22,2 \pm 1,2	41,5 \pm 1,1	39,2 \pm 2,2
	6	112,9 \pm 6,2	258,7 \pm 14,2	920,7 \pm 5,4	444,9 \pm 24,5
	72	327,3 \pm 18,0	750,4 \pm 41,2	2.670,2 \pm 9,2	1.290,1 \pm 70,9
	120	1.014,7 \pm 55,8	2.326,0 \pm 127,8	8.277,4 \pm 16,2	3.999,3 \pm 219,8
CCL3 (pg/mL)	0	10,6 \pm 0,3	11,7 \pm 0,3	13,9 \pm 0,4	12,6 \pm 0,4
	6	180,0 \pm 5,3	107,0 \pm 3,2	191,5 \pm 5,7	286,5 \pm 8,5
	72	576,0 \pm 17,1	342,3 \pm 10,1	612,8 \pm 18,2	916,8 \pm 27,1
	120	1.670,3 \pm 49,5	992,7 \pm 29,4	1777,1 \pm 52,6	2.658,6 \pm 78,7
IL-8 (pg/mL)	0	334,7 \pm 18,4	340,8 \pm 18,7	338,5 \pm 18,6	337,1 \pm 18,5
	6	1.141,3 \pm 62,7	1.126,7 \pm 61,9	1215,1 \pm 66,8	1.344,2 \pm 73,9
	72	3.309,7 \pm 181,9	3.267,5 \pm 179,6	3523,8 \pm 193,7	3.898,2 \pm 214,2
	120	5.000,0 \pm 0	5.000,0 \pm 0	5000 \pm 0	5.000,0 \pm 0
IL-12 (pg/mL)	0	15 \pm 0,8	11,2 \pm 0,6	12 \pm 0,7	14 \pm 0,8
	6	16 \pm 0,9	12,4 \pm 0,7	16 \pm 0,9	14 \pm 0,8
	72	14 \pm 0,8	20,2 \pm 1,9	23 \pm 1,3	20 \pm 1,1
	120	39 \pm 2,2	58,6 \pm 3, 1,12	68 \pm 3,7	59 \pm 3,2

Table 1: Cytokine production in supernatants of Map-infected MDM incubated with Dexamethasone, Interferon gamma or Lipopolysaccharide for 24h and then cultured until 120h.

*Values are expressed as the mean \pm standard error.

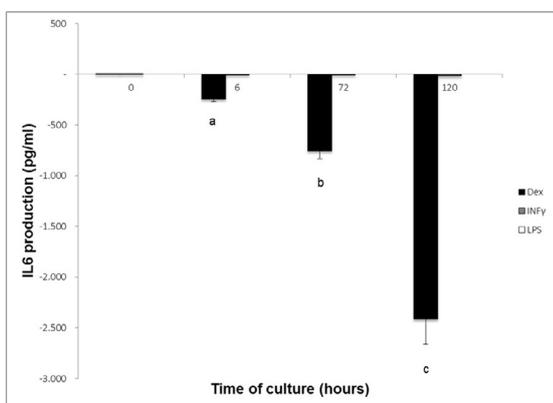


Figure 5: Production of IL-6 at 0, 6, 72 and 120h post-infection, by Map-infected bovine MDM stimulated with Dexamethasone (DEX), IFN-g (IFN-g) or LPS (LPS) during 24h. CN, Control MDM. Columns with a different letter within each cytokine mean statistically significant difference ($P < 0.01$, Kruskal-Wallis Test). Values are weighted to control and are expressed as mean \pm SE.

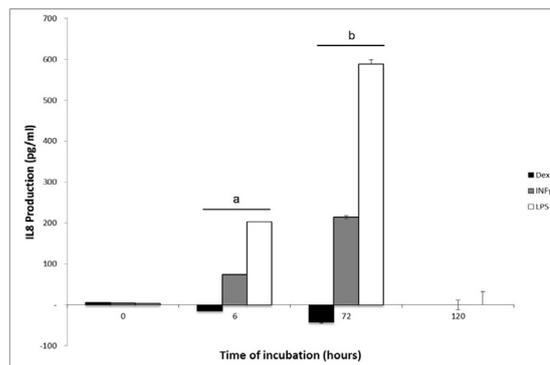


Figure 8: Production of IL-8 by Map-infected bovine MDM stimulated with Dexamethasone (DEX), IFN-g (IFN-g) or LPS (LPS) during 24h and then cultured for 0, 6, 72 and 120h post-infection. Groups of columns with different letter mean statistically significant difference within each cytokine compared to control MDM ($P < 0.01$, Kruskal-Wallis Test). Values are weighted to control and are expressed as mean \pm SE.

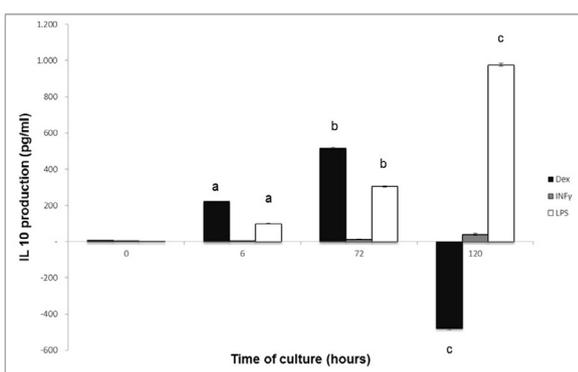


Figure 6: Production of IL-10 by Map-infected bovine MDM stimulated with Dexamethasone (DEX), IFN-g (IFN-g) or LPS (LPS) during 24h and then cultured for 0, 6, 72 and 120h post-infection. Columns with a different letter within each cytokine mean statistically significant difference ($P < 0.01$, Kruskal-Wallis Test). Values are weighted to control and are expressed as mean \pm SE.

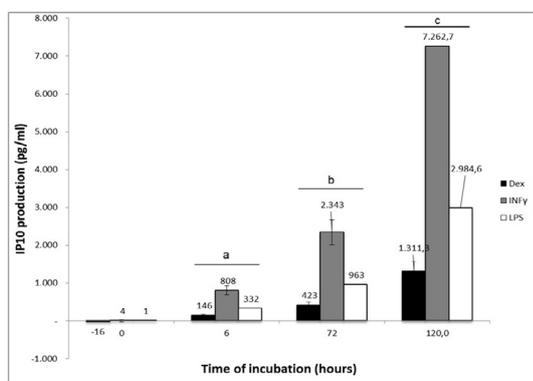


Figure 7: Production of IP-10 by Map-infected bovine MDM stimulated with Dexamethasone (DEX), IFN-g (IFN-g) or LPS (LPS) during 24h and then cultured for 0, 6, 72 and 120h post-infection. Groups of columns with different letter mean statistically significant difference within each cytokine compared to control MDM ($P < 0.01$, Kruskal-Wallis Test). Values are weighted to control and are expressed as mean \pm SE.

IL-10 production

Interleukin-10 production by Map-infected bovine MDM significantly increased from 6 to 72h and then significantly decreased from 72 to 120h in Map-infected MDMs added with Dexamethasone, compared with control MDM ($P > 0.05$). On the contrary, its production significantly increased from 6 to 120h in Map-infected MDM added with LPS ($P < 0.01$), with no statistically significant effect ($P > 0.05$) of IFN-g (Table 1 and Figure 6) compared to control MDM.

IP-10 production

Production of the IP-10 factor by Map-infected MDMs significantly increased in cultures added with Dexamethasone, IFN-g or LPS, compared to control. The highest IP-10 production was observed in Map-infected MDM incubated with IFN-g, in comparison to LPS, Dexamethasone or control, respectively. Regarding the time of culture, the highest level of IP-10 production was observed at 120h of culture (Table 1 and Figure 7).

CCL3 production

CCL3 production significantly increased from 6 to 120h in Map-infected MDMs incubated with LPS compared to control MDM. On the contrary, its production was significantly reduced from 6 to 120h in Map-infected MDMs stimulated with Dexamethasone (Table 1).

IL-8 production

IL-8 production by Map-infected MDMs did not significantly increase from 6 to 72h in Map-infected MDMs added with IFN-g or LPS, and then its values did not significantly decrease from 72 to 120h compared with control MDM (Table 1 and Figure 8).

IL-12 production

Although with negligible levels, IL-12 production by Map-infected MDM significantly increased from 72 to 120h in MDM treated with Dexamethasone, IFN-g or LPS (Table 1).

Discussion

In this study, we provide evidence on the effect of Dexamethasone, IFN-g, and LPS on the ability of MDM for allowing survival of Map and modify their profile of cytokine production when cultured *in vitro*. This system showed that bovine MDM infected with Map at 5:1 MOI, added with Dexamethasone, IFN-gamma or LPS for 24h and further culture for 2 to 120h, remains functional during all time-points of culture evaluated. We propose this model could be used for studying the interaction of Map with primary bovine MDM, mimicking stressful conditions of the macrophages characterized by high circulating levels of cortisol and LPS, such as those found when bovines suffering JD are under stress caused by endogenous glucocorticoids or LPS-related conditions. With the working hypothesis that Dexamethasone, IFN-g or LPS, could affect the ability of primary bovine MDM for supporting the survival of Map *in vitro*, we selected a cow free of JD as a donor of primary MDM to be infected with a reference strain of Map from a group of 17 potential donors.

Interestingly, Map growth in bacterial culture after recovering Map-infected MDM decreased from 6 to 72h regardless of the stimulus, although the statistically significant growth of Map was observed in samples recovered from Map-infected MDM added with Dexamethasone from 72 to 120h of culture, compared to control MDM.

Glucocorticoids exert their effects on macrophages in a dose-dependent manner: At nanomolar doses, glucocorticoids increases adhesion, chemotaxis, phagocytosis and cytokine production, whereas at micromolar doses glucocorticoids can exert immunosuppression [18] and induce monocytes to differentiate toward macrophages [19,20]. In our study, we used 1 µg/mL Dexamethasone a value considered in the micromolar range. Although we do not evaluate the precise mechanism of Dexamethasone in our study, the dose used resulted in the ability of MDM to support the growth of Map from 72 to 120h of culture. In that context, Nozawa et al. [21], found that Dexamethasone at the above-indicated concentration impaired the ability of mouse macrophages to destroy *Candida parapsilosis* when compared with untreated macrophages [21]. This finding was further corroborated in a rabbit model using BCG [22] and in mouse macrophages and human monocytes [23,24] and Microglia cells [25] infected with *Mycobacterium tuberculosis*. We found no reports in the literature on the effect of Dexamethasone on the response of bovine macrophages against Map.

Regarding cytokine production by Map-infected MDM, the addition of Dexamethasone resulted in a statistically significant reduction of TNF-α and IL-6 production, compared to control MDM in agreement with previous reports on cytokine production by Th1 and Th2 lymphocytes [26,27]. TNF-α is one of the most critical pro-inflammatory cytokines produced by macrophages infected with pathogenic strains of Map [28]. TNF-α production is also induced during protective immune responses elicited against mycobacterial infections in mouse [5,28] and humans [29,30]. For example, the addition of TNF-α to macrophages *in vitro* potentiates the death of *M. tuberculosis* (Mtb) by increasing Nitric Oxide (NO) production [28,31]. Similarly, high production of TNF-α was related with the elimination of mycobacteria by Map-infected murine macrophages, whereas a low production was related to Map survival into the macrophages [5]. In our study, Map-infected MDM incubated with Dexamethasone produced lower TNF-α compared with control MDM (Table 1 and Figure 4).

On the contrary, its production significantly increased in MDM incubated with LPS or IFN-g at 72h of culture, a finding reflecting the physiological response of macrophages to these cytokines. In that context it was reported that IFN-g promotes phagosome acidification and its maturation, resulting in lysis of the intracellular pathogen [32].

Interleukin-6 was significantly reduced in Map-infected MDM stimulated with Dexamethasone, whereas IFN-gamma and LPS did not affect its production (Figure 5). IL-6 is a pro-inflammatory cytokine produced by macrophages stimulated including mycobacteria cell wall extracts [24]. Macrophages collected from cows infected by Map significantly produced more IL-6 *in vitro*, compared to healthy cows [33] as it was reported for human and bovine macrophages [34]. In our study, control MDM did produce IL-6. Although no reports were found in the scientific literature related to cytokine production by non-stimulated bovine macrophages, in a study published by Lee et al. [33], reported higher levels of IL-6 gene expression (evaluated by *in situ* hybridization) and protein expression (evaluated by immunohistochemistry) in cows infected with Map compared to healthy cows, in samples of ileal tissue [33]. No other reports were found, suggesting that to our knowledge, this is the first report on IL-6 production by non-stimulated bovine MDM.

The immunomodulatory cytokine IL-10 acts suppressing the antimicrobial activity of macrophages; e.g., it promotes Th2 responses and impairs Th1 responses [35] as it was corroborated in studies performed with Map-infected macrophages [36]. Also, IL-10 inhibits cytokine production and antigen presentation by macrophages and dendritic cells [37]. In our study, IL-10 production significantly increased at 120h of culture in Map-infected MDM incubated with LPS, compared to Map-infected MDM incubated with Dexamethasone, IFN-g or control MDM. These finding would probably reflect the impairment of macrophage function induced by LPS in agreement with the report by Langelaar et al. [38].

Regarding our findings on chemokine production by Map-infected MDM, few studies were available in the literature on the effect of chemokines on MDM infection *in vitro* [39,40]. IP-10 was shown to induce inflammatory responses at the site of infection in Mycobacteria-induced colitis. Our finding on the effect of IFN-gamma on IP-10 production by Map-infected MDM, suggests that bovine MDM reacts against intracellular bacteria by producing its provision of cytokines and chemokines and probably reflects the *in vivo* situation when infected macrophages are preparing for encountering CD4⁺ T cells. Conversely, IP-10 production was significantly lower in Map-infected MDM incubated with Dexamethasone, in comparison with MDM incubated with IFN-g or LPS (P<0.01) (Figure 7 and Table 1). It suggests that the immunoregulatory role of glucocorticoids in MDM function should be exerted through inhibition of chemokine production.

IL-12 is an effect or cytokine protecting the cells [41]. Its production by macrophage and dendritic cells is required for inducing the Th1 responses necessary for controlling mycobacterial infection [9]. Our data on the low production of IL-12 (Both IL-12 p40 and IL-12 p70) by Map-infected bovine MDM are in agreement with previous reports [9,41]. Altogether, cytokine and chemokine production in our system indicates that MDM still viable and functional at all time-points of evaluation, with variations in cytokine production related to the effect of the stimulus added.

Taking into consideration that Map-infected MDMs and control MDMs did support growth and survival of Map and did produce several cytokines during the time points evaluated, we propose this model could serve for testing several hypotheses on the effect of physiological conditions of cows on their immune response against Map. For example, it could be tested the capability of macrophages recovered under stress conditions such as parturition and lactation peak, or the effect of infection (postpartum uterine disease, mastitis, laminitis) for eliminating Map, comparing the response between cows suffering Johne's disease and healthy cows. This model would be particularly useful because of the difficult to achieve growth of Map under conventional microbiological culture, and the difficult for isolating Map of cowshaving stages I and II of the disease. The finding that Map-infected MDM added with Dexamethasone allowed the highest growth of Map in bacterial culture suggest this compound was able to render macrophages more susceptible to maintain Map infection *in vitro*.

Finally, the model must be tested under field conditions to evaluate if circulating macrophages isolated from cows suffering the phase I or phase II of JD, could be used for detecting Map-infected circulating macrophages. In that sense, in a case report, it was found that circulating and resident Map-infected macrophages were isolated from milk, peripheral blood and lymph nodes of a cow suffering the terminal phase IV of Johne's disease [7].

Study limitations

One of the key limiting factors of this study is the use of a single donor of primary MDM. We wonder whether use primary macrophages or a macrophage cell line because of the functional differences and most intense response against *Mycobacterium* challenge exerted by primary macrophages compared to macrophage cell lines, so finally we choose the primary source [42]. Then because primary macrophages could present a high level of variability under the culture conditions [43] and strain of intracellular pathogens [44] compared to cell lines of macrophages, we decide to use a single donor of cells for avoiding variability due to donor source.

The second limiting factor was the off-label use of a kit for measurement of human cytokines. At the time we performed this study there was not a commercial kit available for measurement of bovine cytokines using the Luminex principle. Based on the high conservation of sequence homology between human and bovine genes [45] particularly cytokines and chemokines [46], this option was chosen. Nowadays there are kits available for measurement of bovine cytokines for further experiments on this subject.

Finally, Map growth in bacterial culture of samples obtained from Map-infected primary bovine MDMs was cultured without mycobactin-j. In a report by Aduriz et al. [17], in an ovine model of infection with Map, the authors reported the capability of Middlebrook 7H10 medium to support the growth of Map without mycobactin [17]. Authors argued that primary cultures of Map cultured with mycobactin-j exhibits the capability of growing in the absence of mycobactin-j in further bacterial culture, by a carry-over effect of mycobactin taken from the primary culture. Although this fact was not tested in our experiment, it was the basis for culture Map without mycobactin.

In summary, our results showed that primary bovine MDM that were infected *in vitro* with a reference strain of Map, still functional up to 120h of culture as evidenced by its cytokine and chemokine

production. The addition of Dexamethasone to Map-infected MDM resulted in the higher growth of Map in bacterial cultures of samples harvested at 120h, suggesting this glucocorticoid could be used as for favoring Map survival and proliferation into MDM for research purposes and early diagnosis of cows with Johne's disease.

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Conflict of Interest

The authors state there were no conflicts of interest all through the study. There was no dependency on the results between the financial support from Universidad de Antioquia and the group of researchers.

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