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RESEARCH ARTICLE

Evaluation of the PBMC Proliferation, Apoptosis and Cytokines Profiling in Cattle Infected with *Mycoplasma bovis* Strain 07801

Hussam Askar[1, 2], Huafang Hao[1], Xinmin Yan[1], Lina Ma[1], Shimei Lan[1], Yongsheng Liu[1], Shengli Chen[1*] and Yuefeng Chu[1*]

[1]State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Xujiaping 1, Lanzhou, 730046, Gansu, People's Republic of China; [2]Al-Azhar University, Faculty of science, Assuit 71524, Egypt: Hussamaskar@azhar.edu.eg
*Corresponding author: chuyuefeng@caas.cn, chenshengli@caas.cn

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A B S T R A C T

Bovine respiratory diseases are widespread and too costly disease impacting contributes to economically essential diseases like mastitis and pneumonia worldwide. The present study aimed to explore the influence of *M. bovis* field strain 07801 on the status of PBMCs in the challenged cattle group and the immunized one. For this aim, the PBMCs proliferation, apoptosis, and cytokine profile changes were determined. In this study, *M. bovis* strain Mb 07801 and reference strain PG45 were used; ten calves (2-3 months old) were arranged into two groups (5 calve each): 1) PBS-challenged group (with *M. bovis* 07801 at about 10^{10} CFU/ml), and 2) immunized group (by inactivated *M. bovis* 07801); both groups were treated nasally and intra-tracheal. Blood samples were obtained from both groups and examined for PBMCs proliferation and apoptosis, as well as serum cytokine profile, before infection and at day zero and days 7, 14, 21, and 36 post bacterial treatment. The results revealed that *M. bovis* strain (07801and PG45) antigen increased the proliferative response of the stimulated PBMC compared with the unstimulated cells and ConA-alone stimulated. The PBMC apoptosis showed a non-significant increase in both challenged and immunized groups compared to the negative and positive control (treated with apoptosis inducer) groups. Besides, the levels of cytokines profile showed a significant up-regulation in IFN-γ, IL-4, IL-2, IL-18, TNF-α, IFN-α, IL-6, IL-10 IL-1β, and IL-13, in both groups, except IL-4 and IL-18 those recorded a down-regulation in the immunized group at days 7,14, 21, and 36 post-*M. bovis* infection. In conclusion, immunization markedly ameliorated the immune deterioration induced by *M. bovis* strain 07801.

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INTRODUCTION

In the last half-century, *Mycoplasma bovis* became a worldwide pathogen for cattle. It causes various clinical syndromes such as otitis media, conjunctivitis, arthritis, genital tract, abscesses, respiratory disease (BRD), and mastitis. Mycoplasmas are without a cell wall and have a wide variety of cytoplasmic membrane proteins that possess characteristics, such as surface antigen variety, biofilm development, and ability to interact with their environment (Maunsell and Chase, 2019). The local immune response was characterized by T and B cells' activation in the infected lung; however, the T cell reaction was more incredible. Post-infection, the high expression of antigen cells and phagocytes indicated local lung immunity activation. Despite stimulation, in separating *M. bovis* from the host and avoiding specific lung lesions, the cellular antimicrobial pathways tend to be unsuccessful, suggesting the pathogen's capacity to suppress the host immune response to bovine mycoplasma disease pneumonia (Dudek *et al.*, 2020). Recent studies have demonstrated that *M. bovis* inhibits bactericidal immune systems in bovine animals from occurring by multiple pathways, including the activation of lymphocyte immune-suppressing factor and neutrophil extracellular traps deterioration. Lymphocytes and epithelial cells are

non-phagocytic cells that can invade by *M. bovis*, in its turn, lead to *M. bovis's* potential to inhibit the host immune system and antibiotic resistance (Nishi *et al.*, 2021). Some previous studies have shown that *M. bovis*-induced infection plays a role in activating and developing specific cytokines (IFN-γ, IL-4, and TNF-α) and nitric oxide from bovine macrophages in different populations of T cells (γδT, CD4+, and CD8+ cells) (Jungi *et al*., 1996; Vanden Bush and Rosenbusch, 2003). It stated that *M. bovis* could suppress the proliferation and retard apoptosis by invading peripheral blood mononuclear cell subsets (PBMC). *Mycoplasma bovis* may attack the bovine lung and tracheal epithelial cells, live in macrophages and monocytes for broncho-alveolar lavage of fluid (Suleman *et al.*, 2018). A recent study observed that the organisms invade cells after attachment bronchiolar epithelial cells (Nunoya *et al.*, 2020). The immune responses of bovine pulmonarias to *M. bovis* are primarily anti-inflammatory with elevated IFN-γ and IgG1 antibodies and IL-4 (Vanden Bush and Rosenbusch, 2003; Hermeyer *et al.*, 2012). Previous Research proved upregulation for IL-1β, IL-6, IL-8, MMP-1, and MMP-3 mRNAs in the culture of the synovial peripheral blood mononuclear cells activated by *M. bovis*, indicating that the pathogenesis of *Mycoplasma arthritis* is charged with inflammatory cytokines and MMPs from synovial cells. In the presence of *M. bovis*, associations between synovial cells and mononuclear cells demonstrated that these cytokines and MMPs in synovial cells are released when the joints are severely inflammable (Nishi *et al.*, 2019). Previously illustrated, bovine PBMC apoptosis is delayed following *M. bovis* infection *in vitro* (Nicholas and Ayling, 2003), and did not know the causes. The current study aimed to evaluate the influence of *M. bovis* field strain 07801 on PBMCs proliferation, apoptosis, and cytokine protein profile differences in the challenged cattle group and the immunized one.

MATERIALS AND METHODS

Mycoplasma Strains: *Mycoplasma bovis* reference strain PG45 and a Chinese field pneumonia strain 07801 were used in this study. *M. bovis* were grown in a modified PPLO medium which containing 2 g/L glucose as previously described (Chen *et al.*, 2018), then the cells were harvested; the supernatant was separated, divided into aliquots, and stored at -70°C until further usage. A part of *M. bovis* cells was heat-killed for 5 minutes in a water bath at 70°C. *Mycoplasma* cells were then suspended in RPMI 1640 medium with l-glutamine, containing 10% fetal bovine serum to a cell density of 10^8 CFU/ml. The suspension was stored at -70°C until it could use.

Animals: Ten 2-3 months of healthy calves used in the present study. After two weeks of adaption, they were housed individually and divided randomly into experimental challenge groups and immunized groups (5 calves each). For a challenge, the calves were infected with *M. bovis* 07801 (at about 10^{10} CFU/ml concentration), firstly via nose-spraying (with 2 ml/cattle) and secondly through intra-tracheal injection (20 ml/cattle). Used 2ml inactivated *M. bovis* 07801 to immunize by intramuscular injection. After the first immunization, the second immunization was carried out three weeks later with the first immunization. Animal tests complied with guidelines the ethical and technical specifications and were approved by the Committee for Animal Experimentation of the LVRI, China.

Extraction of Peripheral Blood Mononuclear Cells (PBMC): One day before infection and at indicated different time points intervals after that, peripheral blood samples were withdrawn from each animal in heparinized vacutainers and mixed gently. As previously described, peripheral blood mononuclear cells (PBMCs) were isolated (Vanden Bush and Rosenbusch, 2003). In brief, the heparin blood sample layered on Ficoll gradients (TBD, LTS1087, China), and then centrifugation (1295 ×g at 20ºC for 30 min, No brakes), and the layer containing the PBMC (buffy coat) collected by Pasteur pipette. The PBMCs were obtained from the gradient, washed with PBS three times, and counted cells by Countstar, Automated cell counter (Biotech) before re-suspended in complete media (RPMI 1640 containing 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin) based on the experimental parameters at the desired concentration.

In vitro Proliferation Assays: Determined PBMC proliferation following concanavalin A (ConA, Sigma-Aldrich, Canada, C5275) stimulation and/or *M. bovis* seeding of 96-well tissue culture plates (3×10^5 cells/well). In the presence of ConA (1μg/ml), and/or live/heat-killed *M. bovis*, the cells were incubated (37°C in 5% CO_2 at MOI of 5:1 for 72 h, in triplicate). Added a solution containing 10μl of Cell-counting-Kit (Abmole Bioscience, USA, M4839) (CCK-8)/well according to the manufacturer's instruction. The cells were incubated for 4 h; finally, used a microtiter plate ELISA reader to determine the absorbance at 450 nm.

Assays for Apoptosis: At the early and late stages of infection, the apoptotic markers' concentration was determined using commercially available apoptosis assay kits FITC Annexin V Apoptosis Detection Kit I (556547) purchased from DB Bioscience, USA. Briefly, PBMCs incubate with *M. bovis* 07801(Live and Heat-killed) for 24 h (MOI 5:1). Controls included staurosporine-treated PBMC (5 μM, 45 min) (Abmole Bioscience, USA, M2066), and untreated PBMC. Cells were gated by guavaSoft 3.1.1 is used.

Measurement of Cytokines in vivo: Blood samples were collected before infection and at indicated different time points, the sera were separated, divided into aliquots, and stored at -80°C till subsequent analysis. The concentrations of IL-1β, IL-2, IL-13, IL-18, IL-6, IFN-α, TNF-α, IFN-γ, IL-4, and IL-10 were assessed using commercially available bovine ELISA reagent kits purchased from RayBiotech, Inc., USA; At the same time, IL-6 level was measured using bovine ELISA reagent kit purchased from Abcam, China. All samples were run in duplicate.

Statistical analysis: The differences between mean values of the obtained results were statistically analyzed using non-parametric F test and One-Way ANOVA multiple comparison tests followed by post hoc test (Duncan); *p*-value less than or equal to 0.05 was considered significant. Data were analyzed using GraphPad Prism 7.00 (GraphPad App, La Jolla California USA, www.graphpad.com).

RESULTS

Effect of M. bovis strain (PG45 and 07801) on PBMCs proliferation: We examined the changes in the PBS-challenge group and immunized group when incubated with *M. bovis* and ConA to decide whether *M. bovis* PG45 or 07801 has or has not been able to induce Bovine PBMC proliferation.

On day zero (MOI of 5:1), the obtained in vitro results showed a significant increase in the proliferative response of the stimulated PBMCs in comparison to the unstimulated cells. Also, live *M. bovis* co-cultured with ConA stimulant-induced a considerable rise in the PBMC proliferative response in contrast to ConA stimulation without *M. bovis* (Fig. 1 A, E).

Days, from day 7 to day 36, post-infection recorded a significant elevation in the proliferative responses of PBMC that stimulated with ConA and co-cultured with live *M. bovis* both in control and immunized groups (Figure 1 A-H). On the other hand, heat-killed *M. bovis* compared with unstimulated bovine PBMC was found significantly higher proliferation on day 0 and 7 in control and immunized groups, respectively (Fig. 1 A, B, E and F).

The current study results recorded a marked increase in proliferation of the stimulated PBMC of the cattle treated with either PG45 or 07801 compared with the untreated and ConA PBMCs (Fig. 2 A, B).

Effect of M. bovis strain (07801) on PBMCs apoptosis: The obtained data illustrated that M. bovis 07801 never affect the bovine PBMC apoptotic level in both challenged and immunized cattle groups as compared to that of both negative or untreated control (PBMC alone) or that of the positive control (treated with the apoptosis inducer, Staurosporine) which displayed an apoptotic event without the addition of *M. bovis* 07801 (Fig. 3 and 4).

Effect of M. bovis strain (07801) on cytokines profile during the study intervals: The effect of *M. bovis* 07801 on the measured cytokine levels of both PBS-challenge and Immunized-challenge groups during the study intervals (days 7, 14, 21, and 36) is illustrated in Figs. 5, 6.

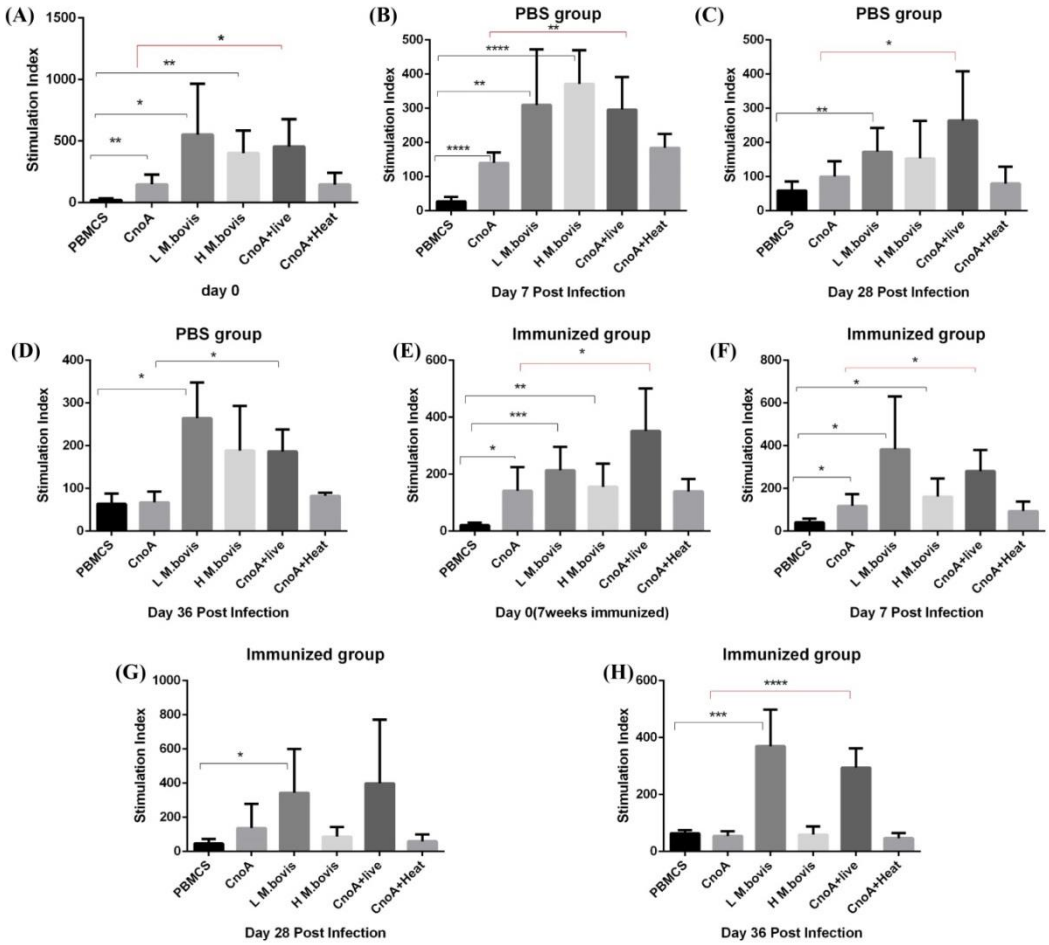

Fig. 1: *Mycoplasma bovis 07801* incubated with cattle PBMCs for all proliferation assays from animals. (A – D) reveals results from PBMC challenge cattle, while (E-H) shows data from PBMC proliferation of immunized cattle. In addition to the *M. bovis*, the treatments also included ConA stimulation. Live *M. bovis* 07801 showed high motivation compared to unstimulated cells and co-culture with ConA. Also, Heat-killed *M. bovis* compared with that of unstimulated bovine PBMC was found to have significantly higher bovine PBMCs proliferation (day 0 and 7) in control, in the challenge and immune-challenge group, respectively.

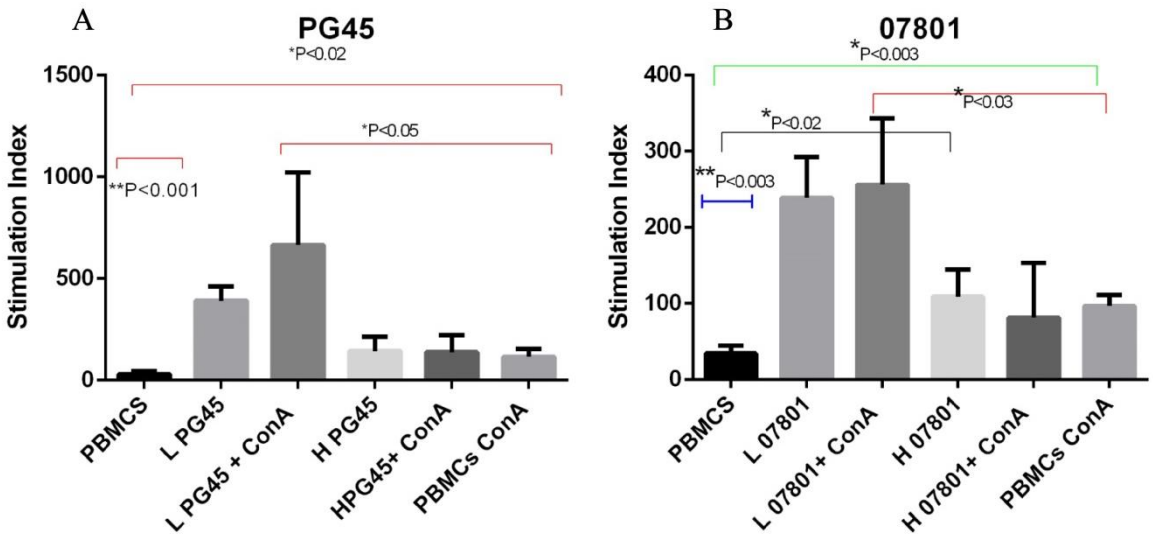

Fig. 2: PBMCs incubated with *M. bovis* PG45(A) and 07801(B) from cattle for all proliferation assays. Live *M. bovis* PG45 and 07801 showed high motivation compared to unstimulated cells (MOI of 5:1) and co-culture with ConA.

Regarding serum IFN-γ level, the present study recorded a markable increase at the day 7 post-infection in both groups (PBS-challenged and immunized-challenged) compared to its level day zero, before infection (Figs. 5A and 6A). The PBS-challenged group recorded a non-significant rise in serum IL-4 level at days 7, and 14, while the immunized-challenged group showed a significant decrease at days 14, 21, and 36 as compared to that of day 0. The IL-4 levels of the immunized-challenged group were reduced significantly compared to that of the PBS-challenged group at day 7, 14, 21, and 36 post-infection (Figure 5B and 6B). Similarly, the data monitored that the PBS-challenged group showed a non-significant increase in the level of serum IL-2 on days 7, 14, 21, and 36 post-challenge; while the immunized-challenged group recorded a significant rise in the level of serum IL-2 on days 7, 14, 21 and 36, post-challenge compared to its level on day zero before challenge (5C and 6C).

The levels of serum IL-18, TNF-α, IFN-α, IL-1β, IL-13, IL-6, and IL-10 obtained post-infection are shown in figures (5D-J and 6D-J). The value of IL-18 of the PBS-challenged group was significantly high on days 7, 14, 21, and 36 post-challenge and significantly lowered its levels in the immunized-challenged group at the same time intervals post-challenge compared to its level at day zero of each group, respectively. Besides, the serum TNF-α level was increased slightly in both groups on days 7, 14, 21, and 36 compared to its level at day zero of each group, respectively. Also, serum IFN-α level showed a significant increase in both groups at day 7, 21, and 36 compared to its level at day zero of each group, respectively.

Serum IL-1β and IL-13 levels of both groups were significantly upregulated from day 7 to day 36 compared to their grades at day 0 of each group, respectively (Fig. 5G, 5H, 6G and 6H). Higher serum IL-1β and IL-13 were found in the PBS-challenged group compared to the immunized-challenged group at the same time intervals.

In respect to the IL-10 and IL-6 serum levels at the intervals from day 7 to day 36 post-infection, both groups (PBS-challenged and immunized-challenged groups) recorded a markable increase in comparison to their grades at day zero of each group, respectively.

DISCUSSION

Mycoplasma bovis causes various chronic inflammatory diseases, including mastitis and bronchopneumonia in dairy and feed cattle, and it suppresses the immune response during infection is found, leading to chronic development. Our study aimed to explore the influence of *M. bovis* field strain 07801 on the status of PBMCs in the challenged cattle group and the immunized one. This aim evaluated the PBMCs proliferation, apoptosis, and cytokine profile changes. Recent studies have shown that *M. bovis* suppresses lymphocyte function such as Th1 cytokine and induces in vitro lymphocyte apoptosis (Sajiki *et al.*, 2020). Our results revealed a massive increase in stimulated bovine PBMCs with ConA and *M. bovis* than bovine PBMCs stimulated with ConA or *M. bovis* alone in this study. This finding agreed with Gondaira, Higuchi *et al*. (2015), who reported that bovine PBMCs were have triggered proliferative responses with ConA and live *M. bovis*, which were considerably higher than phytohemagglutinin-P (PHA) and *M. bovis* (Gondaira *et al*., 2015) and discordant with earlier studies indicated that PBMCs proliferation was inhibited directly by mitogenic infection with *M. bovis* (Thomas *et al*., 1990; Vanden Bush and Rosenbusch, 2003; van der Merwe *et al*., 2010; Suleman *et al*., 2018). The interaction mechanisms of *M. bovis* and bovine PBMCs require more clarification, while PBMCs can stimulate with *M. bovis* and mitogens related to intracellular signal transduction characteristics. *M. bovis* isolates have different antigenic variations (Sachse *et al*., 2000), and the ability of other *M. bovis* field isolates to suppress cell metabolism and of bovine leukocyte cell production of radical oxygen species varies (Wiggins *et al*., 2011). Despite these advancements, significant information gaps persist in understanding mycoplasma bovine pathogenesis.

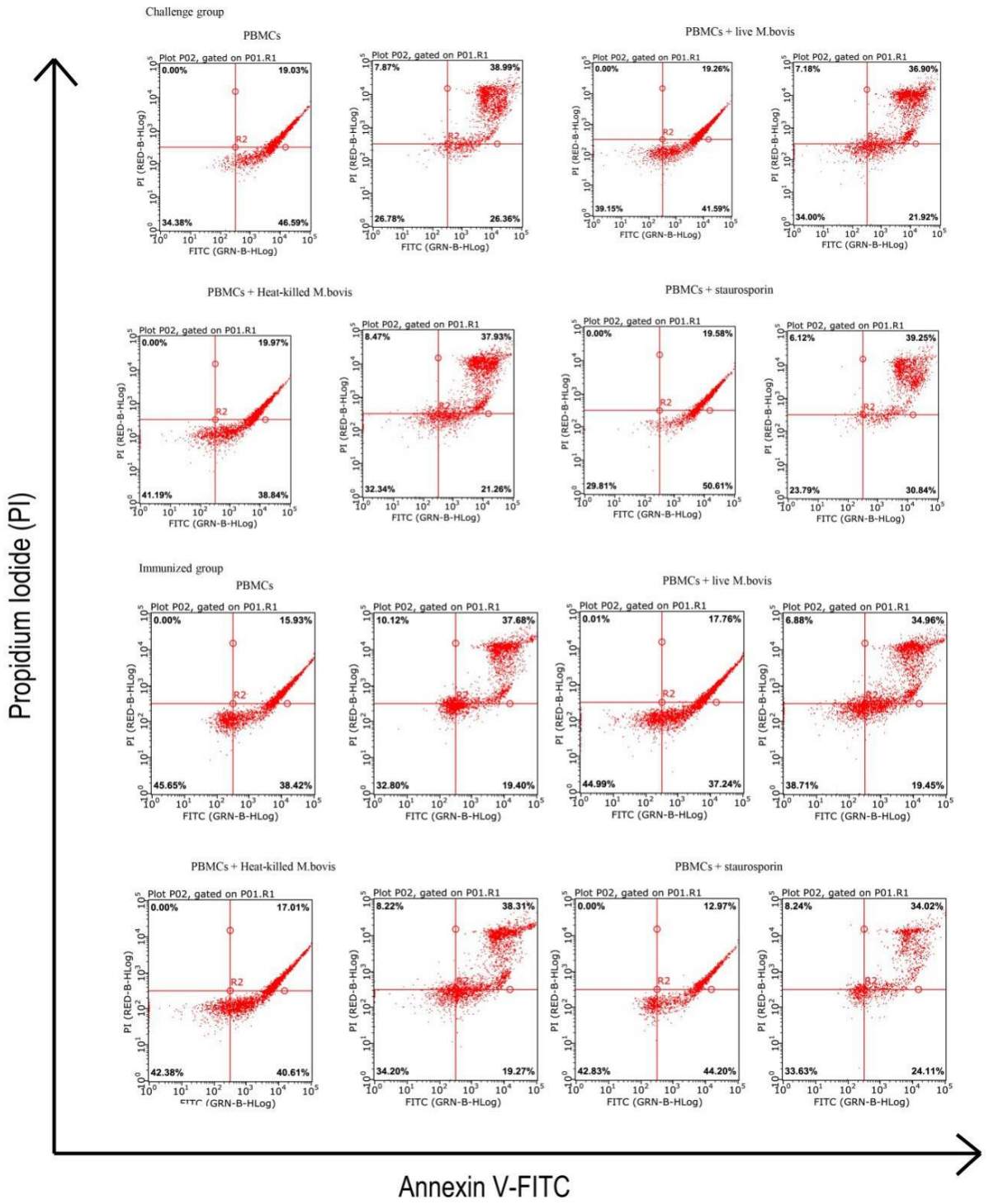

Fig. 3: Bovine PBMCs were incubated either with or without *M. bovis* 07801 during the challenge and immunized group. The flow cytometry monitored both early and late apoptotic events. There is no significant difference for Apoptosis of PBMCs in the challenge and immunized group, respectively.

The previous analysis found that *M. bovis* PG45 can efficiently inhibit cattle PBMC proliferation in the presence of ConA (Suleman *et al.*, 2016). In this report, we saw *M. bovis* PG45 and 07801 for their ability to stimulate cattle PBMC proliferation and found inverse results. We have therefore chosen PG45 as a reference strain for our proliferation experiment.

Our data obtained showed that *M. bovis* 07801 did not significantly delay or induced the bovine PBMCs apoptosis post infection. This finding disagrees with previous studies stated that *M. bovis* triggers apoptosis in bovine lymphocytes, monocytes, and neutrophils in vitro (Chao *et al.*, 2019; Sajiki *et al.*, 2020), Studies previously indicated that *M. bovis* Mb1 suppressed apoptosis in bovine PBMC and monocytes (Maina *et al.*, 2019), proposed that this should be a survival strategy for long-term infections (Suleman *et al.*, 2016). Pathogens such as *Mycobacterium tuberculosis* (Behar *et al.*, 2010) and *Salmonella typhimurium* (Salamone *et al.*, 2010) have recorded delays in apoptosis. This inconsistent may be due to the difference in *M. bovis* strains used (Wiggins *et al.*, 2011) and need further study.

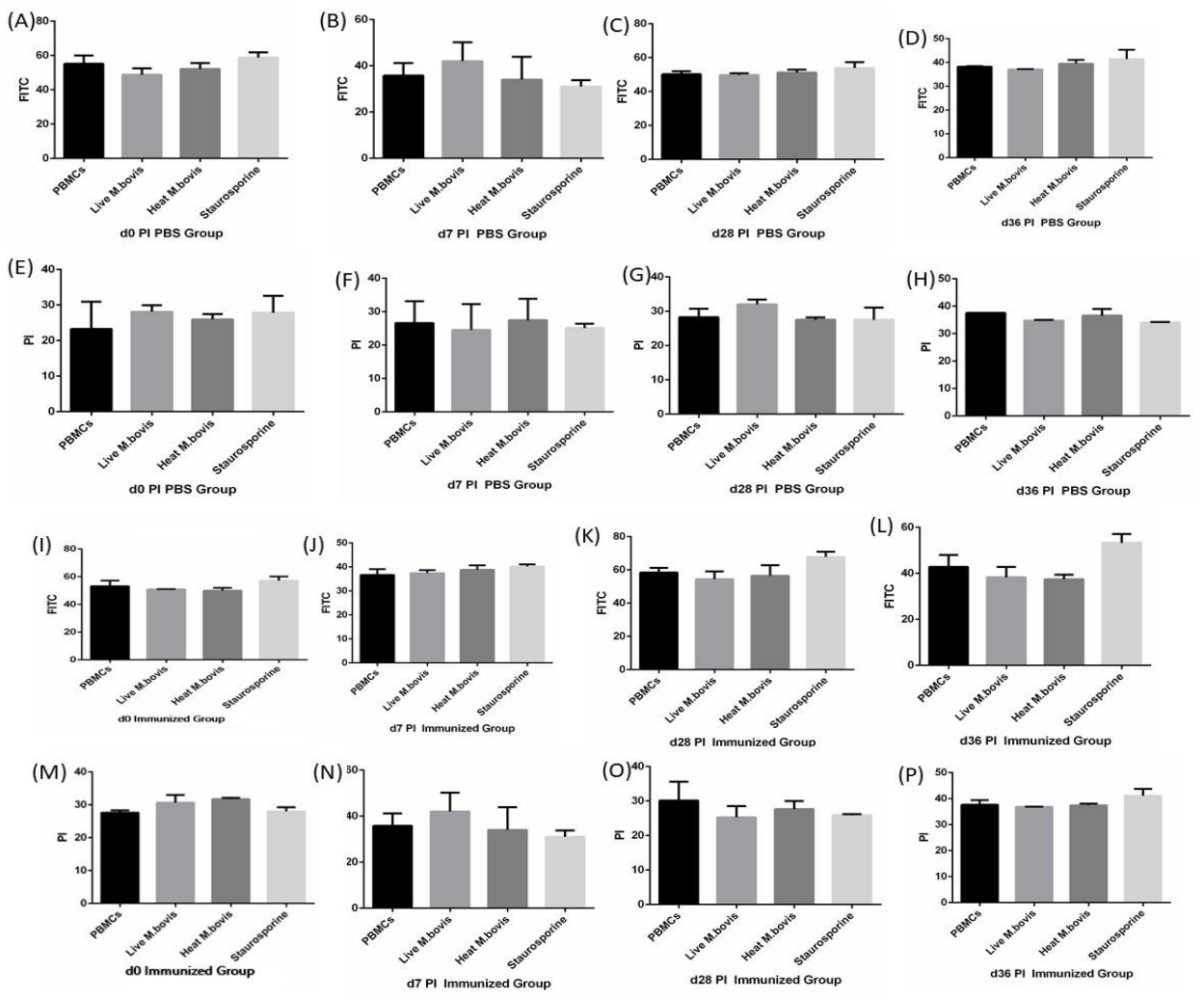

Fig. 4: Bovine PBMCs were incubated either with or without *M. bovis* 07801 during the challenge (A-H) and immunized (I-P) group for 24 h on days 0,7, 28, and 36, respectively. Early and late apoptotic events monitored by flow cytometry. Our results showed that No Significant difference for Apoptosis of PBMCs in the challenge and immunized group, respectively.

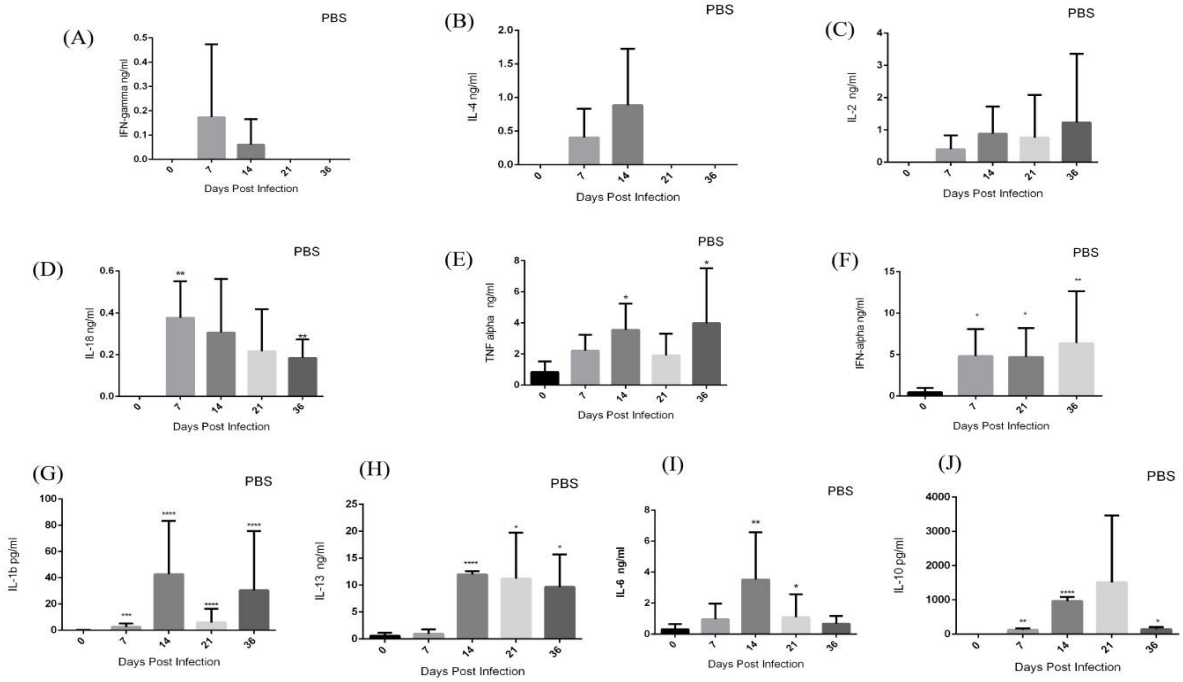

Fig. 5: Cytokines concentrations of calves' challenge with the *M. bovis* 07801. Treated each animal in duplicates, and the bar graph shows representative median data with a range of five cattle. Significant results are shown as NS (non-significant), * p(≤0.01, 0.02, 0.04, 0.03), ** p≤(0.006, 0.001, 0.002, 0.005, 0.007), *** p (≤0.0005, 0.0006, 0.0002) and **** P≤0.0001, for higher stimulation index.

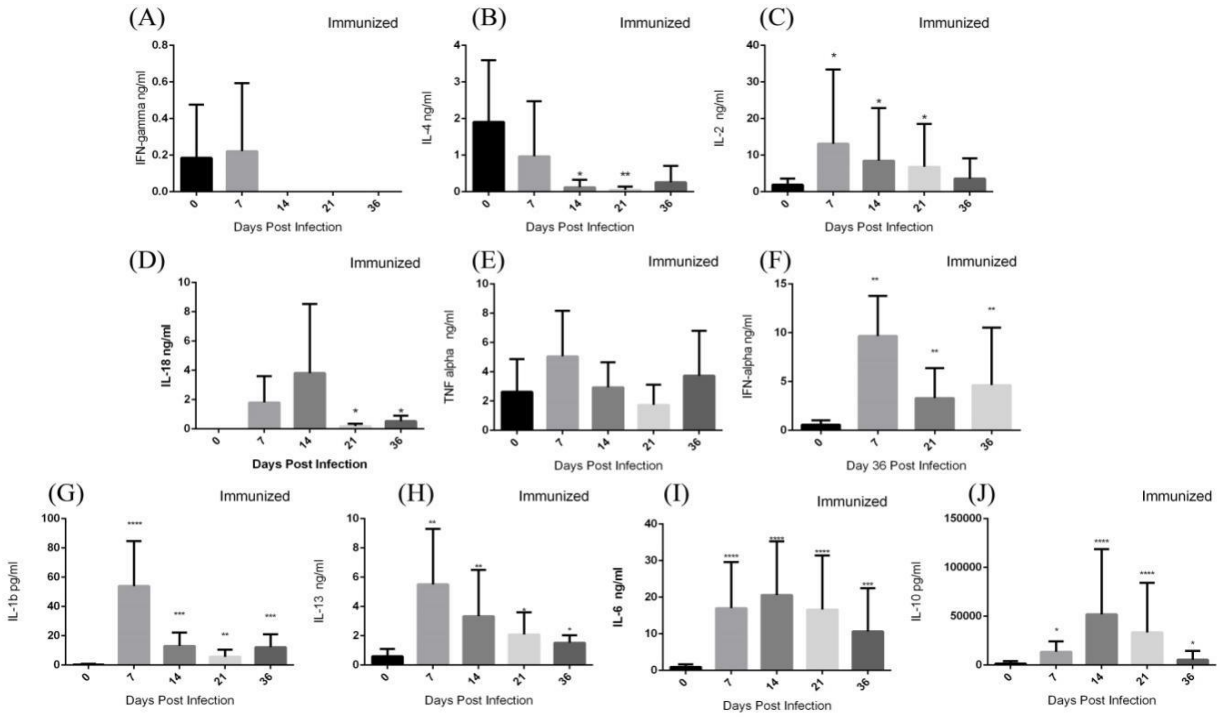

Fig. 6: Cytokines concentrations of calves' challenge after immunized with the *M. bovis* 07801. Each animal is treated in duplicates, and the bar graph shows representative median data with a range of five cattle. Significant results are shown as NS (non-significant), * p (≤0.01, 0.02, 0.04, 0.03), ** p≤(0.006, 0.001, 0.002, 0.005, 0.007), *** p(≤0.0005, 0.0006, 0.0002) and **** p≤0.0001, for higher stimulation index

Our analysis investigated the effect of live *M. bovis* 07801on the in vivo cytokine profile. The results showed that infection with *M. bovis* significantly increased IFN-γ, IL-4, IL-2, IL-18, TNF-α, IL-1β, IL-13, IL-6, IL-10, and IFN- α secretion in bovine blood in both groups, except for IL-4 and IL-18 downregulation in the Immunized group. The findings indicate that such cytokines are essential pathophysiology mediators during *M. bovis* infection. Similar to our results, previous studies have documented the enhanced expression of TNF-α, IL-4, IL-10, and IFN- γ in lung tissue from *M. bovis*-infected calves (Rodríguez *et al.*, 2015; Zbinden *et al.*, 2015; Maunsell and Chase, 2019). The detection of an interaction between enhanced cytokine production by PBMCs and *M. bovis* has been demonstrated infection (Gondaira *et al.*, 2015; Nishi *et al.*, 2019; Nishi *et al.*, 2020).

Cattle infected with *M. bovis* secrete both interferon IFN-γ and interleukin (IL-4) from peripheral blood mononuclear cells (PBMCs), indicating a mixture of Th1-Th2 systemic cytokine response. In vivo expression of TNF-α and IFN-γ proinflammatory cytokines in alveolar macrophage in the lung and local lymphoid tissue in cattle *M. bovis* in experimentally induced pneumonia. In vitro, *M. bovis* incubation with alveolar macrophages induced stimulated TNF-a production, and anti-inflammatory cytokine IL-10 is reported (Maunsell and Chase, 2019). The immunomodulatory events in cattle infected by *M. bovis* have influenced a broad array of physiological processes, including directly affecting the immune cells and indirectly affecting through induction of cytokine secretion from immune cells (Ohtsuka *et al*., 2020).

Conclusions: Our results suggest *M. bovis* induces a proliferative response in bovine PBMCs with cytokine production and has no effect on bovine PBMCs apoptosis. Nonetheless, large numbers of live *M. bovis* are necessary for bovine PBMCs to induce an immune response. These findings would help enhance *M. bovis* infection and immune response in future perspectives.

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Authors contribution: HA performed the practical studies and analyzed the experimental results. YC and HA planned the lessons; YC supported the experimental funding. SC designed experiments on statistical optimization and helped gather blood samples. SC, HH, XY, NM, YL, and YC provided reagents/materials/ analysis tools. HA wrote the manuscript.SC, SL, and YC revised the manuscript. The final document was read and approved by all authors.

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