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Immunological findings associated with Argentinean strains of *Mycobacterium avium* subsp. *paratuberculosis* in bovine models

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of ruminant paratuberculosis. The aim of this study was to evaluate the biological behavior of different Argentinean strains of MAP in two bovine infection models: macrophage (*in vitro*) and calf (*in vivo*) through the evaluation of early immune responses at the peripheral and local levels. Two MAP strains (A and C) were selected taking into account the different patterns of TNF-α and IL-10 secretion displayed by infected bovine macrophages *in vitro*. Two groups of calves were infected with 250 mg of total wet weight live MAP: strain A infected group (MA, n = 3), strain C infected group (MC, n = 2). Another group of animals was mock-infected (MI, n = 3). Infection was confirmed by MAP culture of feces and microscopic observation of granulomatous lesions in the gut tissue. All infected calves showed positive results in the DTH skin test. A significant increase in peripheral $CD4CD25^+$ cells in MC group on day 150 was detected. The specific cellular immune response developed allowed the identification of the infection as early as 30 days in the MA group. However, the percentage of $CD8CD25^+$ cells was significantly increased on day 120 in MC group. Significant differences between groups in proliferation and cellular responses were also detected in ileocecal lymph node samples. In summary, the strains of MAP employed herein induced differential immune responses in peripheral cells, in the proliferative responses and in cell functionality at the local level. Our findings support the hypotheses that the *in vitro* behavior displayed by macrophages could be a tool to identify differences among MAP strains infecting bovines and that the host-pathogen interactions occurring upon infection are dependent on the strain of MAP involved.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Johne's disease), a disease widely distributed in ruminants. This microorganism is responsible for substantial economic losses due to the reduction of productivity. MAP infections are typically transmitted through milk or colostrum ingestion; manure-contaminated elements or vertically (*in utero*) (Whittington and Windsor, 2009). It is assumed that animals become infected early in life, resulting in a development of resistance to infection, the generation of a lifelong subclinical infection, or the appearance of clinical disease. A balance between the pro- and anti-inflammatory immune responses generated is of paramount importance for the control of MAP infection by the host. The levels of TNF-α and IL-10 are suitable parameters to assess such balance. The long sub-clinical phase is generally considered to be due to the development of an adaptive T cell mediated cellular immune response.

There are two major groups of MAP strains known as "sheep strains" (also termed "S-type") and "cattle strains" (also termed "C-type") (Collins et al., 1990). In addition to the genotypic distinctions between C and S strains of MAP, phenotypic differences have been found both *in vitro* and *in vivo* (Abendaño et al., 2013; Fernández et al., 2014). The use of IS*900* restriction fragment length polymorphism (RFLP) and Mycobacterial Interspersed Repetitive Unit-Variable Nucleotide Tandem Repeat (MIRU-VNTR) typing have been extensively used for typing MAP isolates (Pavlik

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et al., 1999; Thibault et al., 2007); however, this methodology can detect diversity only within a small region of the genome (Bryant et al., 2016).

Different immunological and pathological findings have been detected among MAP- infected animals. Such differences can be related to mycobacterial load, location, cell types involved and the number of acid-fast bacilli present in the granulomas (Fernández et al., 2014), and to individual factors as well (Magombedze et al., 2016). The influence of bacterial genotype has also been proposed, mainly focusing in differences between strain types (Verna et al., 2007). In this work, we investigated the responses exerted by MAP strains of the same type but having a different RFLP pattern. Many authors have reviewed the suitability of animal models of paratuberculosis. In such studies, inocula with laboratory adapted strains of MAP were used. However, few studies have been conducted with clinical isolates of MAP (Hines et al., 2014).

The aim of this study was to evaluate the biological behavior of different Argentinean strains of MAP on two bovine infection models: macrophage (*in vitro*), through the evaluation of TNF-α and IL-10 secretion, and calf (*in vivo*), through the evaluation of early immune responses.

2. Materials and methods

2.1. Bacteria

Three MAP strains from the INTA Balcarce collection isolated from feces of adult bovines with paratuberculosis in Argentina were previously characterized by PCR and IS*900*-RFLP as A, B and C (Moreira et al., 1999) and by MIRU-VNTR as INMV 1 code. Bacteria were grown in Middlebrook 7H9 broth (pH 6.8) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO, USA), oleic acid–albumin–dextrose complex (Becton Dickinson Microbiology, San Jose, CA, USA) and sodium pyruvate (Sigma-Aldrich Corp., St. Louis, MO, USA). Prior to infection, 1×10^9 cells/mL of frozen bacterial stocks were thawed and clumps were dispersed by multiple passages through a 25 G needle attached to a tuberculin syringe. The number of viable MAP in the inocula preparations was monitored by performing viable counts (Mundo et al., 2013).

2.2. Selection of MAP strains by macrophage in vitro infection

The Bomac cell line of macrophages (Stabel and Stabel, 1995) was infected with Argentinean bovine MAP strains (A–C), at a multiplicity of infection (MOI) of 10:1. Supernatants were collected 2 and 48 h after monolayer infection for TNF-α and IL-10 determination by ELISA as described by Karcher et al., 2008. The following reagents were used: rabbit polyclonal anti-bovine TNF-α, biotinylated anti-IL-10 mAb, alkaline phosphatase-conjugated polyclonal anti-rabbit IgG (KPL) and streptavidin followed by a solution of HRP-conjugated avidin. Results were expressed as ELISA Relative Units (ERU) calculated as the product of the sample OD and the basal OD ratio × 100. Basal values are those ODs corresponding to the supernatants obtained from uninfected macrophages.

2.3. Animals

Holstein calves (2 or 3 weeks old) born to mothers with neither clinical signs nor serological evidences of paratuberculosis were obtained from a dairy herd of the Pampa region of Argentina in which no clinical cases of paratuberculosis have been reported over the past 5 years.

2.4. Experimental design

Recommendations made by Hines et al. (2007) for the experimental challenge of bovines were taken into account in our experimental design. Six to seven week old calves were randomly assigned to the following treatment groups: MI: Mock infected group (n = 3), MA: MAP strain A infected group (n = 3) and MC: MAP strain C infected group (n = 2). On days 0 and 1 of the study, calves in infected groups were fed with milk replacer containing an average of 250 mg of total wet weight live MAP belonging to strains A or C (total average of 6.8×10^9 CFU). Blood samples were collected from the jugular vein into 10 mL tubes containing either heparin or no anticoagulant. Fecal samples were collected *per rectum* from each animal. Delayed type hypersensitivity (DTH) responses were determined on day 160. On day 180 post-infection, calves were euthanized by the intravenous injection of a sodium pentobarbital overdose (Euthanyle, Brower, Buenos Aires, Argentina) followed by exsanguination and necropsy (Fig. 1). The whole experiment was performed under the approval and supervision of the institutional committee for the use of experimental animals belonging to the School of Veterinary Medicine, Buenos Aires University (no. 2010/27).

2.5. Identification of the infection status

2.5.1. Fecal culture

Fecal cultures were carried out in HEYM with mycobactin J and pyruvate. The identity of MAP isolates was confirmed by the detection of the specific IS*900* insertion sequence by PCR (Mundo et al., 2013).

2.5.2. PPA-ELISA

Sera were evaluated by Paratuberculosis-protoplasmic antigen-ELISA (PPA-ELISA). Results were classified as positive or negative considering an OD cut-off value of 0.205 (Fernández et al., 2012).

2.5.3. Histopathology

As ileocecal lymph node (ICLN) is considered the primary site of infection in bovines (Wu et al., 2007), the presence of typical lesions in this tissue was evaluated. At necropsy, samples were fixed by immersion in 10% neutral buffered formalin and processed by standard histological techniques before being embedded in paraffin. 5 μm sections were cut from each paraffin block and stained with haematoxylin and eosin. The number of granulomas observed in each sample was recorded.

2.6. Tissue processing for flow cytometry and proliferation

For lymph node derived lymphocytes, fresh ICLN sections were minced, filtered, and washed twice with PBS. Cells were diluted to 1×10^6 cells/mL in RPMI 1640 (Life Technologies, Grand, island, NY, USA) with 10% fetal calf serum (FCS, Life Technologies).

2.7. Proliferation assays

Proliferation assays were performed using tissue cells or whole blood diluted 1:10 in sterile PBS. Cells were kept in RPMI supplemented with 10% FCS and stimulated either with 20 μg/mL of Concanavalin A (ConA, Sigma-Aldrich Corp.) or with avian purified protein derivative (PPDa, National Health Service and food quality, SENASA) or with bovine purified protein derivative (PPDb, SENASA) and cultured in U-shaped 96-well plates in a 5% CO_2 atmosphere at 37 °C for 5 days. Non-stimulated control cells were incubated only with culture medium. Then, 0.5 μCi of methyl-^3H-thymidine (NEN, Perkin Elmer, MA, USA) were added to each well and incubated for

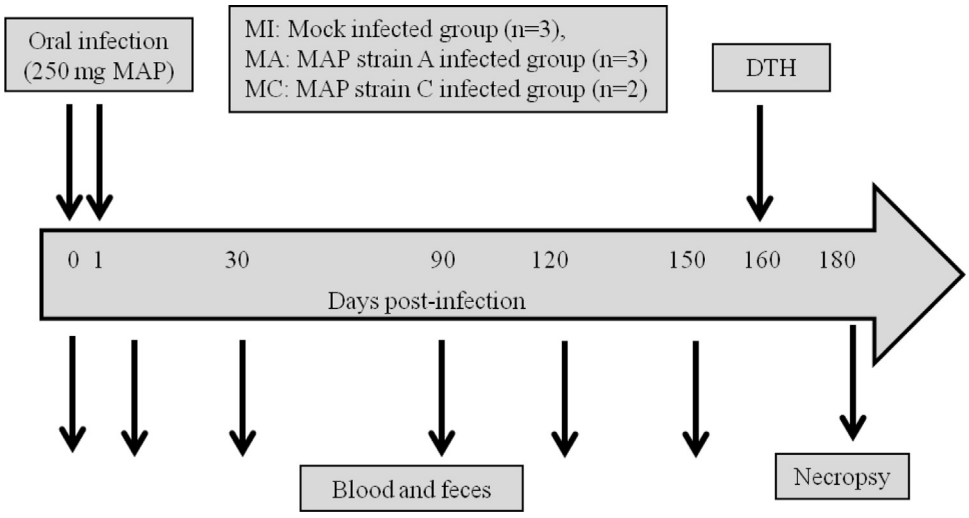

Fig. 1. Experimental design.

another 20 h. After incubation, cells were harvested and the incorporated radioactivity (counts per minute, cpm) was measured by liquid scintillation counting (Beta Counter 1214 Rackbeta, Wallac, Pharmacia, Turku, Finland).

2.8. Flow cytometry

Peripheral and tissue cells were labelled for flow cytometry using a double staining procedure. The following antibodies were used: an anti-bovine CD4 mouse IgG1 (CACT138A, VMRD, Pullman, WA, USA), an anti-bovine CD8 mouse IgG1 (BAT82A, VMRD), an anti-bovine WC1 mouse IgG1 (BAQ4A, VMRD), an anti-bovine CD25 mouse IgG2a (CACT108A, VMRD), a FITC-conjugated goat anti–mouse IgG1 (Life Technologies) and a PE-conjugated goat anti–mouse IgG2a (Life Technologies). Cells were also incubated with a mouse IgG1 (e-Bioscience Inc., San Diego, CA, USA) and a mouse IgG2a (Life Technologies) as isotype controls. Flow cytometric data were collected using a Partec PAS III (Partec Flomax, Münster, Germany) with standard optical equipment, using an argon ion laser set at 488 nm. A total of 10.000 events were collected per sample. Peripheral and tissue cells were stimulated as described above and stained 5 days after stimulation. Results were expressed as percentage of $CD25^+$ cells in relation to the number of $CD4^+$, $CD8^+$ or $WC1^+$cells.

2.9. Delayed type hypersensitivity

DTH responses were determined with 0.1 mL of PPDa (0.5 mg/mL) or PPDb (1 mg/mL) according the manufacturerís instructions (SENASA Res no. 128/2012).

2.10. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test employing the STATISTIX 8.0 software (Analytical software, Tallahassee, USA). Significance was determined as a p-value < 0.05.

3. Results and discussion

Our results showed the immune responses exerted by the two Argentinean strains of MAP in *in vitro* and *in vivo* models. Bovine macrophages infected *in vitro* with either the three evaluated MAP strains showed similar secretion levels of TNF-α (A: 113.0 ± 7.1 ERU; B: 130.5 ± 9.9 ERU; C: 114.8 ± 7.8 ERU, p = 0.22) at 2 h, with slightly higher levels than the basal production by non-infected cells. In contrast, the strains induced significant differences in IL-10 secretion levels (A: 161.1 ± 12.7 ERU; B: 133.3 ± 5.6 ERU; C: 77.8 ± 4.9 ERU, p = 0.005) at 48 h. Taking into account the different patterns of cytokine induction, the strains A and C were selected to be used in the *in vivo* infection model.

Table 1
Identification of infection status.

Diagnostic test	% of positive results (+/n)		
	MI	MA	MC
Fecal culture[a]	0 (0/3)	67 (2/3)	100 (2/2)
PPA-ELISA	0 (0/3)	0 (0/3)	0 (0/2)
ICLN Histopathology	0 (0/3)	100 (3/3)	50 (1/2)

MI: Mock infected. MA: MAP strain A infected group. MC: MAP strain C infected group. ICLN: ileocecal lymph node.
[a] Confirmed by IS*900* PCR, animals with positive fecal culture at any time from 30 days post-infection were considered to be infected.

IL-10 is believed to play a critical role in the regulation of host immune responses to MAP infection and potentially orchestrate the reversal of Th1/Th2 immune dominance during disease progression (Magombedze et al., 2016). Moreover, Stabel and Robbe-Austerman (2011) have detected higher levels of IL-10 secretion in calves experimentally infected with a clinical isolate compared with a laboratory strain.

Our experiment resulted in a successful infection, since the isolation of MAP from feces and/or the detection of granulomatous lesions at the ileocecal lymph node could be achieved in all experimentally infected animals. No MAP colonies were detected in the feces obtained from the uninfected control calves throughout the study (Table 1). None of the calves developed neither clinical signs of disease nor macroscopic lesions attributable to intestinal MAP infection. No humoral immune response was detected in the animals. The latter finding is in agreement with our previous studies, showing that PPA-ELISA can identify clinically infected animals but had low efficiency to detect subclinically infected cattle (Fernández et al., 2012).

When peripheral cell subpopulations were evaluated, a significant increase in the percentage of $CD4CD25^+$ lymphocytes of MC group was observed, as compared to MA and MI groups on 150 days post-infection (Fig. 2a). These results suggest that such subpopulation could be involved in the early phases of paratuberculosis pathogenesis. A significant decrease in the number of $WC1CD25^+$

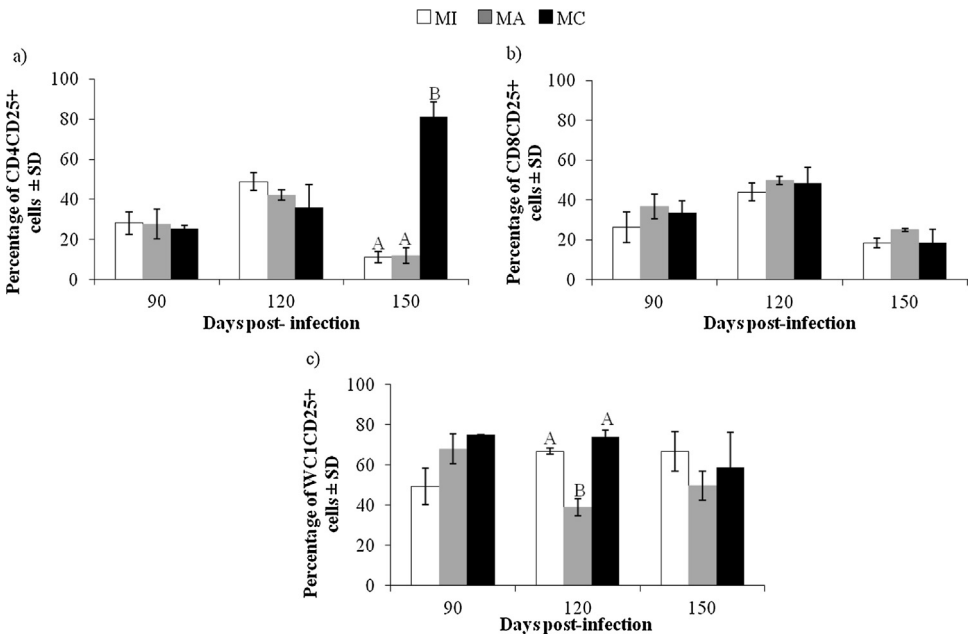

Fig. 2. Kinetics of the percentages of peripheral: (a) $CD4CD25^+$, (b) $CD8CD25^+$ and (c) $WC1CD25^+$ cells in calves from the experimental groups. MI = mock infected, MA = MAP strain A infected group and MC = MAP strain C infected group. Error bars indicate standard deviation (SD) and the letters indicate significant differences among groups ($p < 0.05$). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed.

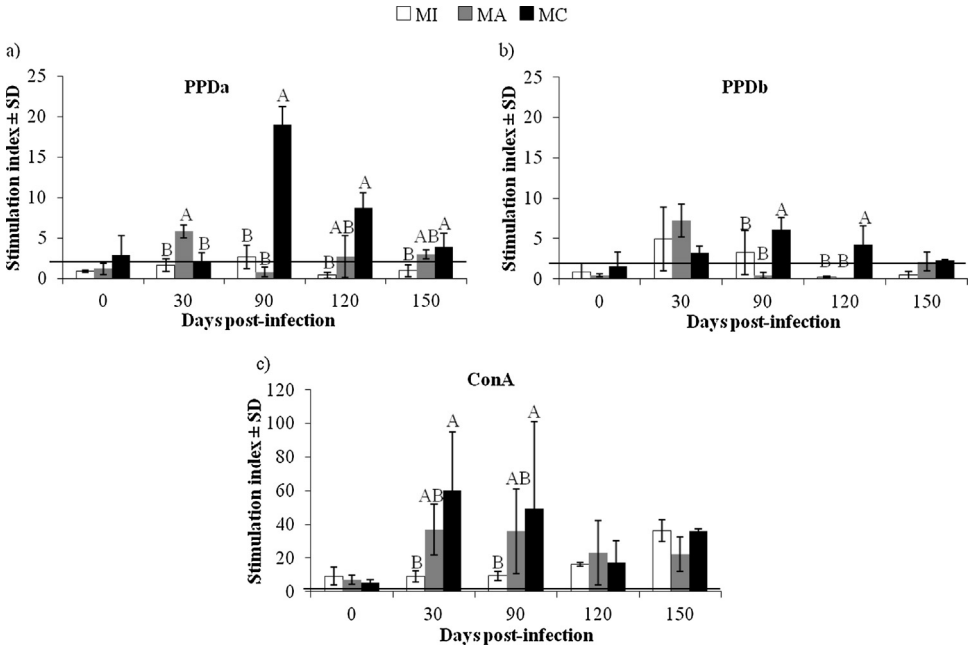

Fig. 3. Kinetics of the specific proliferation responses by peripheral cells stimulated with (a) PPDa, (b) PPDb or (c) ConA in the experimental groups. Results are expressed as stimulation index (SI = mean cpm stimulated wells/mean cpm non-stimulated wells) from cells of MI = mock infected, MA = MAP strain A infected group and MC = MAP strain C infected group. Error bars indicate standard deviation (SD) and the letters indicate significant differences among groups ($p < 0.05$). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed.

cells ($p < 0.05$) was also detected in MA on day 120 post-infection (Fig. 2c). When antigen-specific proliferation responses to PPDa and PPDb were assessed, the MA group showed an early response on day 30, while MC group displayed a higher specific response on day 90, which remained positive throughout the experimental period (Fig. 3a and b). Other authors have reported a detectable response between 80–270 days post-infection in a cattle model (Stabel et al., 2011).

When peripheral cells were stimulated with PPDa, the MC group showed an increase in the percentage of $CD8CD25^+$ cells (MI: 48.9 ± 3.8; MA: 42.5 ± 4.6; MC: 56.3 ± 1.6, $p < 0.05$) on day 120 post-infection, suggesting that this subpopulation could be at least partially responsible for the higher proliferative response observed in the MC group. No differences in $WC1CD25^+$ cells (MI: 53.6 ± 3.2; MA: 54.1 ± 5.2; MC: 61.4 ± 3.5, $p = 0.24$) could be detected on day 120 post-infection.

No differences among the groups were observed for specific $CD4CD25^+$ cells on any of the days evaluated. However, it has previously been demonstrated that CD4+ T cells expressing CD25 are the first cell population that responds to antigen stimulation in calves

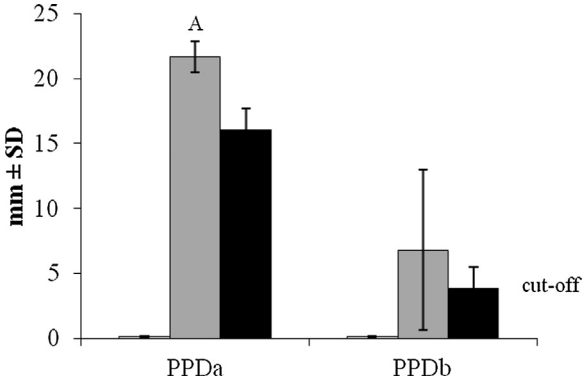

Fig. 4. Delayed-type hypersensitivity immune response in calves. Data are presented as mean increase in skin thickness (mm) ± standard deviation (SD) on day 160. MI = mock infected, MA = MAP strain A infected, MC = MAP strain C infected, PPDa = avian purified protein derivative and PPDb = bovine purified protein derivative. Error bars indicate SD and the letters indicate significant differences among groups ($p < 0.05$). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed. A thickness > 3 mm was considered a positive reaction for *M. avium* sp.

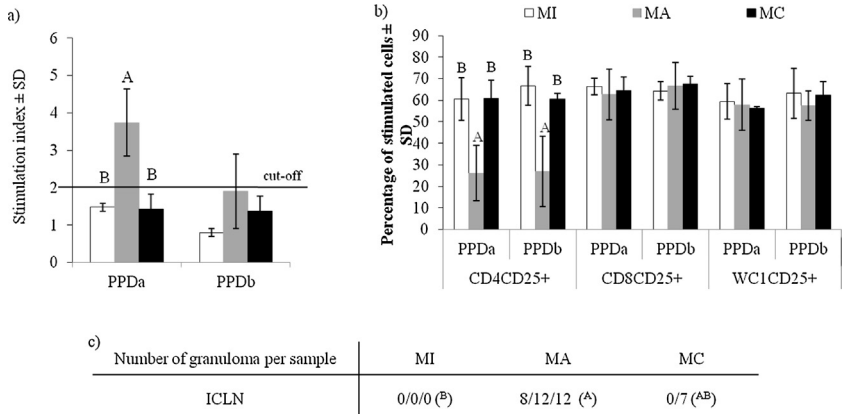

Fig. 5. (a) Proliferation responses, (b) percentages of $CD4CD25^+$, $CD8CD25^+$ and $WC1CD25^+$ cells induced by PPDa and PPDb in ileocecal lymph node (ICLN) and (c) number of granulomas per sample in ICLN on day 180 (necropsy). MI = mock infected, MA = MAP strain A infected group and MC = MAP strain C infected group. Results are expressed as stimulation index (SI = mean cpm stimulated wells/mean cpm non-stimulated wells) or percentage of stimulated cells. Error bars indicate standard deviation (SD), A, B and AB indicates significant differences among groups ($p < 0.05$). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed.

within 6 months of oral administration of MAP (Koo et al., 2004). Probably, these differences could be related to the strain of MAP employed or the antigen used as *in vitro* stimulus.

The DTH test was able to differentiate all experimentally infected from uninfected calves. All infected calves showed positive results in the DTH skin test using PPDa (Fig. 4). The cross-reaction induced by PPDb probably reflects the interference caused by MAP in tuberculosis diagnosis (Brito et al., 2014).

At necropsy (day 180 post-infection), the specific proliferation of ICLN cells was evaluated against PPDa and PPDb. The MA group showed a significantly higher stimulation index with PPDa (Fig. 5a). At the same time, a significant specific decrease in $CD4CD25^+$ cells was observed in the MA group. A reduced $CD4^+$ effector T cell capacity and the development of regulatory T cell (Tregs) subpopulations at the local level have been reported to correlate with disease progression in cattle (Coussens et al., 2012; Bull et al., 2014).

All infected calves developed incipient focal granulomatous lesions in the lymphoid tissue that were mainly made up of macrophages. No lesions were observed in the MI group (Fig. 5c). In summary, differences in immune responses induced by strains in peripheral cells, in proliferative responses and in local cell functions were detected. These findings support the hypothesis that differences in the *in vitro* behavior found in the macrophage model could be a tool to identify biological differences among MAP strains infecting cattle. This work is a contribution to the understanding of the early anti-MAP immunity induced by strains isolated in Argentina. Further studies should be carried out in order to obtain an in-depth understanding of the influence of MAP strains in host-pathogen interactions and the outcome of disease.

Conflict of interest

The authors declare having no conflict of interest.

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