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Short communication

Evidence of a pro-apoptotic effect of specific antibodies in a bovine macrophage model of infection with *Mycobacterium avium* subsp. paratuberculosis

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic granulomatous enteritis in ruminants. Understanding the protective immune response following infection is crucial to improve the diagnosis and the development of vaccines against this disease. The goal of this work was to assess whether specific antibodies were able to modulate the macrophage response to MAP infection by evaluating apoptosis and TNF-α secretion in an *in vitro* model. Sera from healthy ($n = 2$), MAP-infected ($n = 3$) and lipoarabinomannan (LAM)-immunized ($n = 3$) bovines were evaluated. LAM was chosen as immunogen due to its relevant role in mycobacterial pathogenesis. We demonstrated by two different techniques (Acridine Orange/Ethidium Bromide microscopy and Annexin V/7-Amino-Actinomycin D flow cytometry) that the immune sera from both, MAP-infected and LAM-immunized bovines, significantly increased macrophage apoptosis in infected cultures. Comparable levels of apoptosis were detected when MAP was pre-incubated with purified specific antibodies instead of whole serum. Furthermore, this effect was accompanied by a significantly higher secretion of TNF-α. These results strongly suggest that specific antibodies could limit the impact of MAP on the apoptosis of bovine cells. This work would contribute to elucidate the role of the specific antibody response in bovine JD and its prevention.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease (JD), a chronic granulomatous enteritis of ruminants. The World Organisation for Animal Health classifies JD as a disease affecting multiple species with socio-economic and/or public health importance, which is likely to have a significant impact in the trade of animals and animal products (WOAH, 2012). Bovine paratuberculosis has proved to be intractable and challenging, hampered by a dearth of effective diagnostic tests (reviewed in Geraghty et al., 2014). Available vaccines (containing live attenuated or killed whole cells) reduce MAP shedding and clinical disease; however, they do not prevent infection. Besides, paratuberculosis vaccination in cattle could interfere with tuberculosis diagnosis. Mainly, interferences have been reported to occur when a single tuberculin test was performed, showing 17 times more positive reactions among vaccinated animals than the non-vaccinated. In the same study, when the comparative intradermal test was used, the percentage of cross-reactions dropped from 6.55% to 0.15% (Garrido et al., 2013). Moreover, tuberculosis control in herds in some countries such as Argentina is still based on the single intradermal test, and it has recently been informed that MAP vaccination would not be economically recommendable in regions with high frequency of tuberculosis, a disease that needs to be tested (Groenendaal et al., 2015).

Many studies about mycobacterial pathogenesis focus on the macrophage, as this cell plays a central role in both the innate and adaptive immune responses to mycobacteria (reviewed in Arsenault et al., 2014). After penetrating the intestinal epithelial barrier, MAP invades sub-epithelial macrophages by interacting with several families of receptors that recognize complement factors (CRs), immunoglobulins (FcRs), mannose (C-type lectins, TLRs and CD14), and also scavenger receptors. These different routes of entry might have important consequences for bacterial

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intracellular survival and for cytokine secretion patterns thus inducing different immune responses (reviewed in Souza et al., 2008). In particular, preferential uptake of mycobacteria through the C3–CR pathway might represent a strategy to evade critical host defenses (Schlesinger, 1998). In contrast, it has been demonstrated that the binding of Ab to FcRs could alter the trafficking of pathogenic intracellular bacteria into the lysosomal compartment (Joller et al., 2010). Other macrophage responses such as phagosome–lysosome fusion and apoptosis could also be modulated by MAP (Cheville et al., 2001; Kabara and Coussens, 2012; Abendaño et al., 2014). TNF-α is a key pro-inflammatory and pro-apoptotic cytokine associated with macrophage function and is a relevant mediator in the innate response to mycobacterial pathogens (Basler et al., 2010; Borrmann et al., 2011). In addition, apoptosis of infected macrophages might be considered a successful host response that limits intracellular growth of mycobacteria. Moreover, it has been recently suggested that regulation of macrophage apoptosis is an important immune evasion mechanism for *Mycobacterium tuberculosis* (Lee et al., 2009) and *Mycobacterium avium* subsp. *paratuberculosis* (Kabara and Coussens, 2012).

The lipoarabinomannan (LAM) is the main glycolipid antigen of the mycobacteria envelope (reviewed in Briken et al., 2004). Its role in mycobacterial pathogenesis has been well–recognized (Thirunavukkarasu et al., 2014). *M. bovis* LAM has been shown to inhibit the production of pro-inflammatory cytokines and to be involved in the suppression of apoptosis induced by mycobacteria (Wojtas et al., 2011). Besides, Souza et al. (2013) have recently reported the results of *in vitro* experiments supporting LAM as a virulence factor that facilitates the survival of MAP within macrophages.

Understanding the protective immune response elicited by the mycobacterial infection is crucial to improve the diagnosis and the development of vaccines against JD. A substantial body of recent studies suggests that protection against intracellular bacteria is not strictly limited to Th1 responses and provides evidence for a protective role of humoral immunity (Glatman-Freedman, 2006; Joller et al., 2010; Robinson et al., 2011; Allen et al., 2012). In fact, an active role of Ab on the MAP-macrophage interaction has been suggested (Hostetter et al., 2005; Mundo et al., 2008; Jolly et al., 2011). Besides, the beneficial effect of experimental vaccination in bovines with a MAP recombinant protein has been shown to be related to the humoral arm of the immune response rather than to cellular mechanisms (Koets et al., 2006; Santema et al., 2011, 2013). Moreover, a study recently conducted by Begg et al. (2015) strongly suggests that MAP specific mucosal Ab may play a role in the protection against JD. In this context, the classical paradigm of an early Th1 response skewing towards a Th2 response as JD progresses is now under reconsideration (Begg et al., 2011; Magombedze et al., 2014). The goal of this work was to assess whether specific antibodies opsonizing MAP are able to modulate the macrophage response to infection by potentiating its pro-inflammatory response and/or by affecting its apoptotic ability.

2. Materials and methods

2.1. Bovine macrophages

2.1.1. BoMac cells

The sv-40-transformed bovine peritoneal macrophage cell line (Stabel and Stabel, 1995) was cultured in RPMI-1640 medium (GIBCO™, Invitrogen Corp., Carlsband, CA, USA) supplemented with 5% decomplemented FCS (Invitrogen) at 37 °C and 5% CO_2 in a humidified atmosphere.

2.1.2. Blood monocyte-derived macrophages (BMDM)

Anticoagulated-blood (9 parts of blood with 1 part of 2.2% sodium citrate, 2.45% anhydrous dextrose, and 0.73% citric acid) was collected from 2 healthy adult Holstein cows testing negative for JD by fecal culture and serum PPA-ELISA (Fernández et al., 2012).

After blood centrifugation, the buffy coats were diluted in PBS, seeded onto an equal volume of Hystopaque® 1077 (Sigma-Aldrich Corp., St. Louis, MO, USA) and centrifuged (2700 rpm, 30 min at room temperature). Mononuclear cells were washed twice in PBS, resuspended to a final concentration of 1×10^7 viable cells/mL in RPMI-1640 supplemented with 20% FCS and seeded onto 24-well tissue culture plates. After 2.5 h of incubation at 37 °C and 5% CO_2, non-adherent cells were removed by washing with warm PBS. Fresh RPMI–FCS medium was added and adherent cells were allowed to mature during 1 week.

2.2. Bacteria

The ATCC 19698 MAP reference strain was grown at 37 °C in Middlebrook 7H9 broth (Difco, BD biosciences, FranklinLakes, NJ, USA) containing 10% albumin–dextrose–catalase, 0.05% Tween 80 (Sigma-Aldrich Corp.), and 2 μg/mL mycobactin J (Allied Monitor Inc., Fayette, MO, USA). Titration was performed by serial dilution and seeding onto Middlebrook 7H9 agar plates. The bacterial stock was centrifuged and frozen at −70 °C in 15% glycerol medium. For each assay, MAP was thawed and cultured overnight at 37 °C, then centrifuged, disaggregated by passages through a 25-gauge needle, and resuspended in RPMI medium to a final concentration of 10^9 CFU/mL

2.3. Serum samples

Sera from healthy ($n = 2$), MAP-naturally infected ($n = 3$), and LAM-immunized ($n = 3$) bovines were obtained as previously described (Jolly et al., 2011). Evaluation of MAP-specific IgG isotypes were assessed as described elsewhere (Fernández et al., 2012). Before apoptosis assays, serum samples were filtered through 0.22 μm membrane, decomplemented at 56 °C for 30 min and diluted in RPMI. For the assays with purified Ab, purification by protein G affinity chromatography was carried out as previously described (Jolly et al., 2011). MAP-recognizing ability was assessed by ELISA after purification.

2.4. In vitro *infection of macrophages*

BoMac cells (1×10^6 viable cells/mL) were seeded onto 24-well tissue culture plates and incubated at 37 °C overnight in 5% CO_2. Bacteria were opsonized with 5% decomplemented sera in RPMI medium at 37 °C for 1 h in a shaker. Immediately prior to inoculation of monolayers, the bacterial suspension was disaggregated by passages through a 25-gauge needle. The suspension was then inoculated into BoMac or BMDM cultures, in duplicate at a multiplicity of infection (MOI) of 10:1 (bacteria:cell). Cells in duplicate wells were kept uninfected to determine basal apoptosis levels. After 2 h, monolayers were washed with PBS and incubated with RPMI–FCS medium during 24 h, until apoptosis was evaluated.

For the assays with labeled bacteria, MAP was stained with fluorescein isothiocyanate (FITC, Sigma-Aldrich Corp.) as described previously (Mundo et al., 2008) and then opsonized as described above.

2.4.1. Acridine orange/Ethidium bromide staining and fluorescence microscopy

For Acridine orange/Ethidium bromide (AO/EB) staining, 20 μl of dye mix containing equal amounts of AO and EB (1 mg/ml, final concentration each) were added to the centrifuged cells for

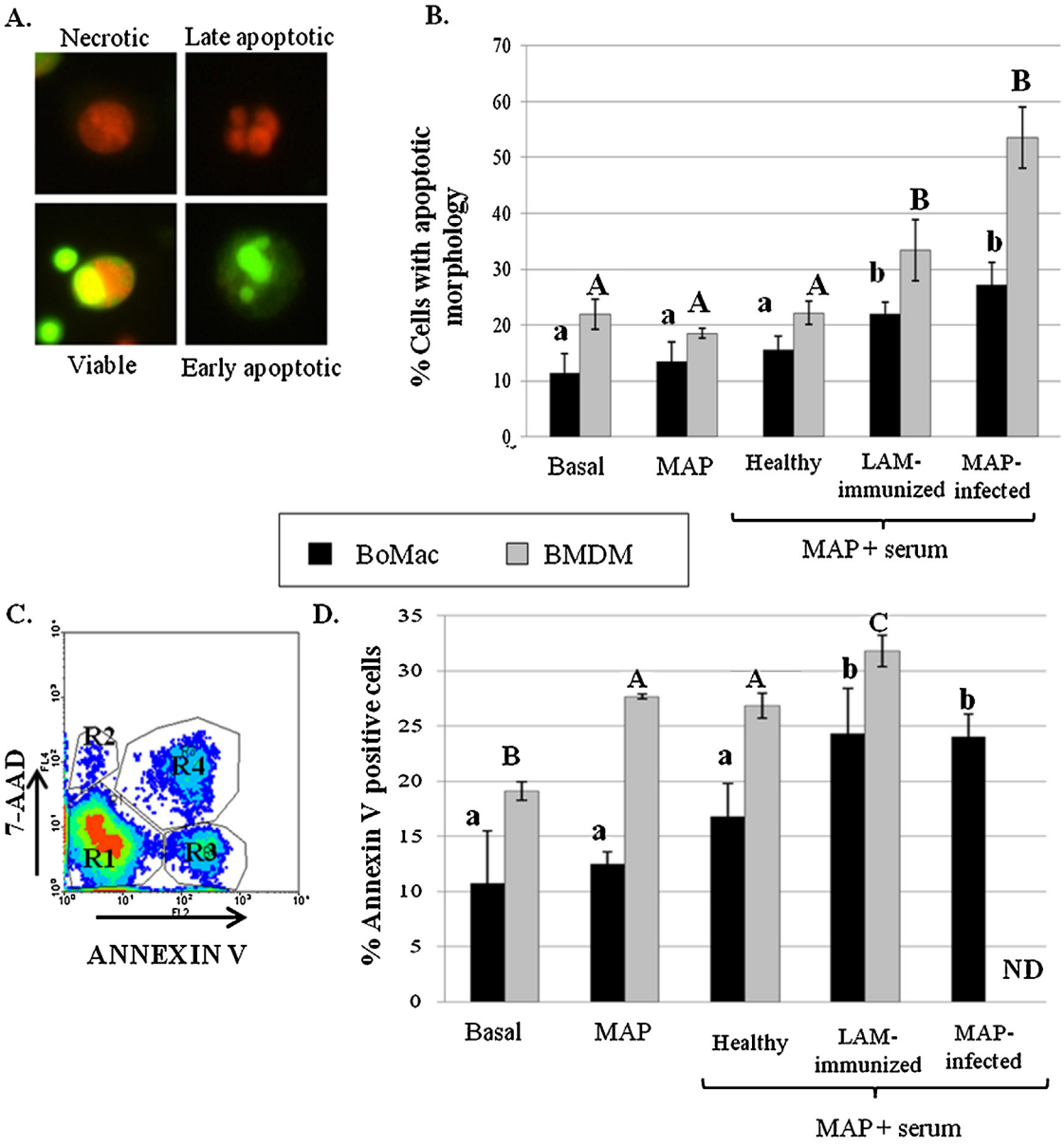

Fig. 1. Immune sera increased apoptosis percentage in both BoMac lineage cells and Blood Monocyte-Derived Macrophages (BMDM), infected with: MAP alone or pre-incubated with serum from healthy ($n = 2$), LAM-immunized ($n = 3$) or MAP-infected ($n = 3$) cows. (A) Fluorescence patterns in Acridine Orange/Ethidium Bromide (AO/EB) stain. (B) Percentage of cells with apoptotic morphology assessed by AO/EB stain. (C) Set regions used for flow cytometry analysis shown in D.: viables (R1), apoptotic (R2 + R3) and necrotic (R4) cells. (D) Percentage of Annexin V positive cells obtained by flow cytometry with Annexin V/7AAD. ND: not determined. ANOVA and Dunnett's Test with MAP as control level were ran for BoMac and BMDM ($P < 0.05$, lower case and capital letters, respectively).

10 min, washed twice and examined at 400× using a Olympus BX-51 fluorescence microscope (OLYMPUS Corp., 2-3-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 163-0914, Japan). The nuclear morphology and fluorescence pattern were observed and cells were categorized as: (i) viable cells (VC), uniformly green with normal nuclei; (ii) early apoptotic cells (EAC), with bright green dots in the nuclei as a consequence of chromatin condensation or nuclear fragmentation, (iii) late apoptotic cells (LAC), orange-stained and displaying condensed or fragmented nuclei; and (iv) necrotic cells (NC), orange with uncondensed nuclei (Fig. 1A). The percentage of cells with apoptotic morphology was estimated by counting 200 cells in different fields randomly focused and using the formula (EAC + LAC)/(EAC + LAC + VC + NC)× 100.

2.4.2. Annexin V/7-amino-actinomycin D (7-AAD) labeling and flow cytometry

The *PE Annexin V Apoptosis Detection Kit I* (BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used following the manufacturer's instructions. Stained cells were then analysed using a PAS-III flow cytometer (PARTEC, Münster, Germany), acquiring at least 20,000 events per sample. Cells were electronically gated on the basis of size and granularity to remove debris from final analyses. Cells that showed low staining for both Annexin V and 7-amino-actinomycin D (7-AAD) were considered viable; cells that showed low Annexin V staining, but stained with 7-AAD were considered necrotic; and cells with high Annexin V staining regardless of 7-AAD status were considered apoptotic (Fig. 1C).

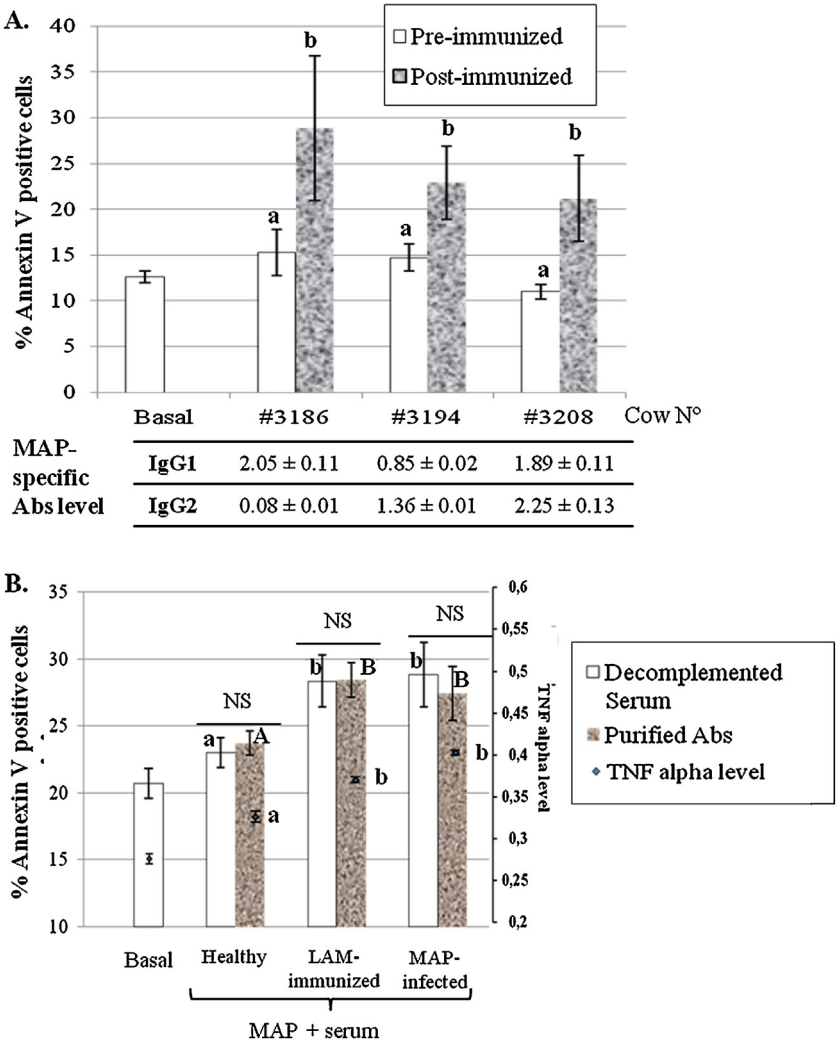

Fig. 2. Antibodies are involved in the pro-apoptotic effect of immune sera. (A) Simultaneous assessment by flow cytometry of apoptosis by pre-incubating MAP with sera from LAM-immunized cows, obtained before and after immunization. The chart below the graphic shows the reactivity against MAP detected by IgG1 and IgG2 isotype-ELISA in post-immunization serum samples (expressed in mean OD ± SD for each serum). (B) Comparative effect on the apoptosis of pre-incubating MAP with serum or purified Ab. NS: non-significant differences by paired Student's *t* test between purified Ab or its corresponding serum. Lower case and capital letters above bars show statistical comparison between groups for decomplemented sera and purified Ab, respectively). The points inside spotted-bars represent TNF-α level (mean OD ± SD) in culture supernatants measured by ELISA, and the letters show significant differences by ANOVA and Dunnett's Test with "MAP + Healthy serum" as control level, $P < 0.05$.

In MAP–FITC assays, cells were separated into infected and bystander populations, based on fluorescence level in the FITC-channel (Fig. 3A).

2.5. TNF-α measurement by ELISA

A sandwich ELISA was used to detect TNF-α in macrophages culture supernatants. Briefly, ELISA plates (NUNC, Rochester, NY, USA) were coated with a anti-bovine TNF-α mAb (MCA2334, Abd Serotec, Washington, DC, USA), diluted 1:100 in 0.05 M carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C. Plates were then washed with PBS–Tween 20 (0.05%) and blocked with PBS–BSA (0.2%) for 1 h at 37 °C. Undiluted culture supernatants were added to the plates. After overnight incubation at 4 °C and 3 washes, a rabbit polyclonal anti-bovine TNF-α (AHP852Z, Abd Serotec, Washington, DC, USA) was added in a 1:250 dilution and incubated at 37 °C for 3 h. After washes, plates were incubated with an alkaline phosphatase-labeled goat polyclonal anti-rabbit IgG (H + L) Ab (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:200 for 2 h a 37 °C. A color developing solution (Bluphose®, KPL, Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) was added and incubated for 1 h at room temperature. The reaction was stopped by the addition of 0.1 M EDTA, pH 8, and plates were read at 630 nm.

2.6. Statistical analysis

Three independent experiments were conducted and results are expressed as mean value for each group ± SD. Data were processed with STATISTIX 8.0 (Analytical Software, Tallahassee, FL, USA) and comparisons between groups were made by using ANOVA followed by Dunnett's Test, setting infection with MAP as the control level. Apoptosis levels were also compared by a multi-way ANOVA, considering model (BoMac and BMDM), technique (AO/EB microscopy and AnnexinV/7-AAD flow cytometry) and treatment (Basal, MAP, and MAP + Healthy, LAM-immunized, or MAP-infected sera) as main effects. Effect of pre- and post- immunization sera was compared by Student's paired *t* test. In MAP–FITC assays, differences between infected and non-infected macrophages were evaluated by Student's *t* test (for independent samples). In all cases, a *P*-value < 0.05 was considered to be statistically significant.

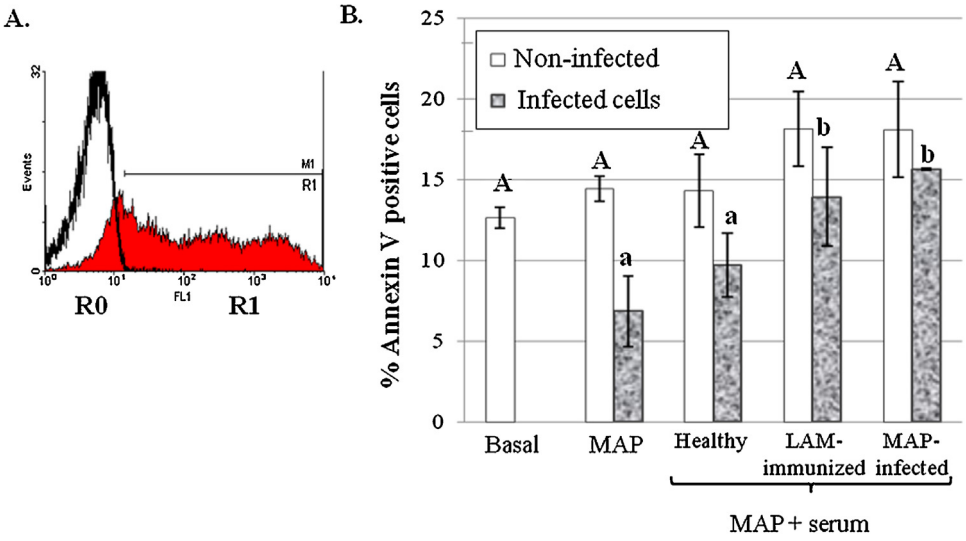

Fig. 3. Immune sera from infected cows interfered with the apoptotic inhibition effect induced by MAP in BoMac infected cells. (A) For differential apoptosis analysis in non-infected *vs*. infected cells, a marker was determined in a FL1 histogram (fluorescence due to FITC-stained MAP) and results of Annexin V *vs.* 7-AAD evaluated by gating the two resulting regions (R0 y R1). (B) Percentage of Annexin V positive cells. Mean results and SD of two experiments are shown. Different letters represent statistically significant differences by ANOVA and Dunnett's Test with MAP as control level, $P < 0.05$.

2.7. Ethics

All animals involved in this work were managed following the protocols approved by the Committee on the Ethics of Animal Experiments of the Veterinary School of the Buenos Aires University.

3. Results and discussion

It is thought that apoptosis of MAP-infected macrophages is important for the effective bacterial clearance from the host (Kabara and Coussens, 2012). In this work, we demonstrated by two different techniques that immune sera could affect the ability of MAP to modulate apoptosis in both bovine BMDM and BoMac linage cells, whereas MAP pre-incubated with sera from healthy cows failed to do so. By the multi-way ANOVA analysis, we found that the model but not the technique used had an effect in apoptosis levels. In fact, as seen in Fig. 1B, some significant differences were detected between the results obtained by the two techniques for BMDM cells. When analyzed by the AO/EB microscopy technique, similar levels of apoptosis were found in basal, MAP-infected and MAP + Healthy sera infected BMDM cultures. On the other hand, significantly higher levels of apoptosis were detected by AnnexinV/7-AAD flow cytometry for these two infected treatments as compared with the basal. In an attempt to explain this difference, we hypothesize that in the absence of specific antibody, the MAP–macrophage interaction at the receptor–ligand level in BMDM could be different from that of BoMac cells, thus affecting the apoptosis levels observed. In this sense, differences in cell surface markers expression between BoMac and BMDM cells have been already described by Sager et al. (1999). Basal apoptosis rates were higher in BMDM ($21.9 \pm 2.7\%$) than in BoMac cells ($11.4 \pm 3.6\%$). Similar levels of basal apoptosis for bovine BMDM have been published elsewhere (Kabara and Coussens, 2012). The apparent relative resistance of BoMac cells to apoptosis could be related to the insertion of sv-40 virus in its genome for immortalization (Stabel and Stabel, 1995). Nevertheless, taking into account the advantages of working with a established cell line over BMDM cells and considering that BoMac cells have been demonstrated to conserve the main characteristics of macrophages (Stabel and Stabel, 1995; Tooker et al., 2002; Jolly et al., 2011) and to be a reliable and reproducible model of MAP infection *in vitro* (Abendaño et al., 2013), we decided to continue our experiments using this cell line.

As regards modulation by specific Ab, the percentage of apoptosis detected when MAP was pre-incubated with immune sera (from LAM-immunized or MAP-infected bovines) was significantly higher than the level obtained for cultures infected with MAP alone, or pre-incubated with sera from healthy bovines. This effect of immune sera was evident in BoMac and BMDM cells when analysed by AO/EB stain or by flow cytometry (Fig. 1B and D). To further investigate the relevance of specific Ab present in immune sera in the interference with MAP effect, we compared apoptosis levels induced by MAP when pre-incubated with paired samples of serum from LAM-immunized cows, obtained pre- and post-immunization. As shown in Fig. 2, only post-immunization samples resulted in a significant increased apoptosis level in macrophage cultures infected with MAP (Fig. 2A). In addition, comparable levels of apoptosis were detected when MAP was pre-incubated with serum-purified Ab or whole serum (Fig. 2B). Taking together, these results strongly suggest that specific Ab could be responsible for the pro-apoptotic effect detected after pre-treating of MAP with immune sera.

It has been described that MAP infection of macrophages could interfere with the expression of the pro-apoptotic cytokine TNF-α, at least in part through an over expression of IL-10 (Weiss et al., 2002; Abendaño et al., 2014). In an attempt to determine whether the production of such cytokine was involved in the increase of apoptosis mediated by Ab, we examined its levels in the supernatant of infected macrophage cultures. As a result, we found significantly higher levels of TNF-α when MAP was pre-treated with specific Ab, from LAM-immunized or MAP-naturally infected cows (Fig. 2B). This finding suggests that specific Ab could limit the impact of MAP infection on TNF-α secretion.

Little is known about the function of the different Ab isotypes in bovine species (Estes and Brown, 2002; Corbeil, 2002). We have previously reported the opsonizing capacity of bovine IgG1 and its role in reducing MAP intracellular viability within macrophages (Mundo et al., 2008; Jolly et al., 2011). In an attempt to evaluate whether IgG1, IgG2, or both could be involved in the pro-apoptotic effect detected herein, serum samples of the three LAM-immunized cows were iso typified by MAP–ELISA (Fig. 2A, chart). We could not find the same trend in isotypes composition in the three sera.

According to these results, it still remains unclear whether IgG1 or IgG2 were involved in the effect observed.

Taking into account that the importance of analyzing apoptosis in bystander and infected macrophages separately in MAP-infected cultures has been recently demonstrated (Kabara and Coussens, 2012), we included a final assay, using FITC pre-labeled MAP. When apoptosis was evaluated in the whole infected culture as a single biological unit, we could not detect a consistent effect of MAP infection. Nevertheless, differential analysis within each culture in MAP–FITC assays allowed us to detect a significantly diminished level of apoptosis in infected *versus* bystander macrophages ($P < 0.05$, Student's t test for independent samples) in cultures infected with MAP in the absence of Ab (Fig. 3B). The apparent inhibition of apoptosis induced by MAP observed herein supports the hypothesis of apoptosis as a beneficial process in host response against mycobacterial infection. This observation is in agreement with previous reports where reduced apoptosis in MAP-infected macrophage cultures were described (Kabara and Coussens, 2012; Abendaño et al., 2014) or where lower rates of apoptosis were found when infecting cells with pathogenic *vs.* non-pathogenic mycobacterial strains (Weiss et al., 2004; Wojtas et al., 2011). We also found that the pre-treatment of MAP with immune sera only induced a significant increase in apoptosis in the infected macrophage subpopulation, but not in bystander cells within the same culture (Fig. 3B). This result suggests that immune sera could modulate the MAP ability to interfere with apoptosis on infected cells.

To date, apoptosis modulation by Ab recognizing pathogens during infections has not been extensively studied. Kim et al. (2014) have demonstrated that antitoxin serum prevents apoptosis and other toxic effect induced by toxin A on an *in vitro* colorectal cellular model of *Clostridium difficile* infection, which is an extracellular toxigenic pathogen. To our knowledge, no literature data on this phenomenon in intracellular bacterial infections is available. Nevertheless, since it has been described that Ab–FcR interactions may lead to alternative intracellular trafficking in experiments with Ab-opsonized *Legionella pneumophila*, another intracellular pathogen (Joller et al., 2010), it is possible to speculate that internalization using this route could lead to differences in the ability of macrophages to use apoptosis as a protective mechanism. Another hypothesis is that the preincubation of MAP with specific Ab could block exposed active components such as LAM, preventing their interaction with host's molecules and the resulting biological effects. Accordingly, Santema et al. (2011) have pointed that antibodies directed against surface exposed MAP antigens contribute to a protective immune response. It is feasible to postulate that the redirection of MAP in the intracellular compartment might limit its ability to interfere with the apoptotic pathways and thus enable the cell to use this protective mechanism.

Potential correlations between *in vitro* findings in apoptosis assays and the biological role of apoptosis during *in vivo* infections must be made cautiously. Nevertheless, it is possible to speculate that the presence of specific Ab at the time of MAP infection could locally promote apoptosis in the macrophages of the intestinal mucosa. Taking into account that intracellular viability of mycobacteria has been demonstrated to decrease in apoptotic cells (Keane et al., 2000) and that apoptosis favors cross-presentation of mycobacterial antigens potentiating T cells response (Winau et al., 2006), the increase in apoptosis induced by Ab might be beneficial in the *in vivo* response to infection. Indeed, the idea of developing a pro-apoptotic vaccine has been proposed as an alternative for improving tuberculosis control (Behar et al., 2011). In this sense, evidences consistent with a beneficial role of apoptosis in JD infection have been described in deers. BMDM from JD-resistant red deers showed significantly higher apoptosis rates than cells from susceptible animals, when infected *ex vivo* with MAP (Dobson et al., 2013).

In summary, in this work we provide evidence of a pro-apoptotic effect of antibodies in an *in vitro* infection model of bovine macrophages with MAP. Collectively, the findings reported herein and in our previous works (Mundo et al., 2008; Jolly et al., 2011) suggest an active role of specific antibodies that might be proposed as beneficial for the host in the MAP–macrophage interaction. Whether the induction of an early potent specific antibody response could serve for JD protection needs to be further elucidated.

Conflict of interest statement

The authors declare having no conflict of interest.

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