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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

Ana Jolly[a], Silvina Lompardía[b], Silvia E. Hajos[b], Silvia L. Mundo[a,*]

[a] Cátedra de Inmunología, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Chorroarín 280 C1427CWO, Buenos Aires, Argentina
[b] Cátedra de Inmunología—Instituto de Estudios de la Inmunidad Humoral (IDEHU), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires—CONICET, Junín 956 C1113AAD, Buenos Aires, Argentina

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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

* Corresponding author. Tel.: +54 1145248450.
E-mail address: smundo@fvet.uba.ar (S.L. Mundo).

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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