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IMMUNE RESPONSE OF CALVES VACCINATED WITH *Brucella abortus* S19 OR RB51 AND REVACCINATED WITH RB51

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Área de concentração: Medicina Veterinária Preventiva

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SUMMARY

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RESUMO

As vacinas de *Brucella abortus* B19 e RB51 têm sido utilizados com sucesso para controlar a brucelose bovina em todo o mundo, no entanto, no presente, a maior parte do nosso entendimento da resposta imunológica protetora induzida pela vacinação vem de estudos realizados em camundongos. Assim, o objetivo deste estudo foi caracterizar e comparar as respostas imunes induzidas em bovinos primo imunizados com *B. abortus* B19 ou RB51 e revacinados com RB51. Bezerras com idades entre 4 a 8 meses foram imunizados com as vacinas B19 ou RB51, no dia 0, e revacinados com RB51 no dia 365 do experimento. A caracterização da resposta imunológica foi realizada utilizando soro e as células mononucleares do sangue periférico. As amostras de sangue foram coletadas nos dias 0, 28, 210, 365, 393 e 575 após-imunização. Os resultados mostraram que a vacinação com B19 e RB51 induziu uma resposta imune caracterizada pela proliferação de células T $CD4^+$ e células T $CD8^+$; produção de IFN-γ e IL-17A por células T $CD4^+$; indução de células T citotóxicas $CD8^+$; secreção de IL-6; indução de células de memória T $CD4^+$ e $CD8^+$; indução de imunoglobulinas da classe IgG1; e expressão dos fenótipos de ativação nas células T. As diferenças na resposta imune estimulada por B19 em comparação com RB51 foram a maior persistência de IFN-γ e células T $CD4^+$ de memória, a indução de células $CD21^+$ de memória e maior secreção de IL-6. Após a revacinação com RB51, a resposta imunológica foi caracterizada principalmente por aumento da expressão de IFN-γ, proliferação de células T $CD4^+$ e células T $CD8^+$ antígeno específicas, células T $CD8^+$ citotóxicas e diminuição de IL-6 em ambos os grupos. No entanto, uma polarização diferente da resposta imune, CD4 ou CD8-dominante, foi observado após o reforço com RB51, em animais primo vacinados com RB51 e B19, respectivamente. Os nossos resultados indicam que após a primeira vacinação, ambas as estirpes de vacina (B19 e RB51) induziram uma resposta imune forte e complexa dominada por um perfil Th1, embora após a revacinação RB51 as diferenças entre os perfis imunológicos induzidos pela primo vacinação tornaram-se mais acentuadas.

Palavras-Chave: vacinas de brucelose, resposta imune, *B. abortus*, bovinos.

ABSTRACT

Brucella abortus *S19 and RB51 have been successfully used to control bovine brucellosis worldwide, however, at the present, most of our understanding of the protective immune response induced by vaccination comes from studies in mice. Therefore, the aim of this study was to characterize and compare the immune responses induced in the cattle prime immunized with* B. abortus *S19 or RB51 and RB51 revaccination. Calves aged 4 to 8 months were immunized with either vaccine S19 or RB51 on day 0, and revaccinated with RB51 on day 365 of the experiment. The characterization of the immune response was performed using serum and peripheral blood mononuclear cells. The blood samples were collected on days 0, 28, 210, 365, 393 and 575 post-immunization. Results showed that S19 and RB51 vaccination induced an immune response characterized by proliferation of $CD4^+$ and $CD8^+$ T-cells; IFN-γ and IL-17A production by $CD4^+$ T-cells; cytotoxic $CD8^+$ T-cells; IL-6 secretion; $CD4^+$ and $CD8^+$ memory cells; immunoglobulins of IgG1 class; and expression of the phenotypes of activation in T-cells. The differences in the immune response stimulated by S19 compared to RB51 were the higher persistency of IFN-γ and $CD4^+$ memory cells, induction of $CD21^+$ memory cells and higher secretion of IL-6. After RB51 revaccination, the immune response was chiefly characterized by increase in IFN-γ expression, proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells, cytotoxic $CD8^+$ T-cells and decrease in IL-6 production in both groups. However, a different polarization of the immune response, CD4- or CD8-dominant, was observed after the booster with RB51, for S19 and RB51 prime vaccinated animals, respectively. Our results indicate that after first vaccination both vaccine strains (S19 and RB51) induce a strong and complex immune response dominated by Th1 profile, though after RB51 revaccination the differences between immune profiles induced by prime vaccination become more accentuated.*

Keywords: brucellosis vaccines, immune response, B. abortus, cattle

1. INTRODUCTION

The microorganisms of the genus *Brucella* are causing brucellosis, an infectious disease that affects numerous livestock and wildlife animal species besides man (Corbel, 2006; OIE, 2009). Brucellosis is a major zoonosis in public and animal health, and found disseminated worldwide (Corbel, 2006). *Brucella* infection is a highly contagious disease that affects man and numerous livestock and wildlife animal species (Corbel, 2006). Approximately 500,000 cases of human brucellosis are reported annually to the World Health Organization (WHO) (Pappas et al., 2005). The infection in man is almost invariably transmitted by direct or indirect contact with infected animals or their products and a human *Brucella* vaccine does not exit. Moreover, *Brucella* microrganims are classified as a biosafety level 3 pathogen and considered to be a potential bioterrorist agent (Pappas et al., 2005).

Cattle are the preferred hosts of *Brucella abortus* (Corbel, 2006; OIE, 2009) and the economic importance attributed to bovine brucellosis is based on direct losses caused by abortions, stillbirths, weight loss, decreased milk production and the establishment of sanitary barriers to international trade of animals and their products (Bernues et al., 1997). Moreover, transmission of the disease to humans occurs almost invariably through the direct or indirect contact with infected animals or their products (Corbel, 2006).

The measures employed in the control of bovine brucellosis are justified by their importance to public health and the economic losses it causes, and can be classified into two main general categories: hygiene and vaccination (Lage et al., 2005). The hygiene whose purpose is to limit the exposure of susceptible animals, includes all processes, as the agent isolation, diagnosis, restraint of trade and slaughter of positive animals (Lage et al., 2005). Vaccination is the most effective measure in reducing the prevalence and incidence of brucellosis. This procedure has contributed enormously to the success of many programs, especially at the control stage of the disease (Olsen and Stoffregen, 2005).

The best results in the brucellosis prevention are observed for vaccines prepared with live attenuated strains of *Brucella* (Olsen and Stoffregen, 2005), despite countless recent advances in the development of new vaccines, mainly recombinant. Currently, S19 and RB51 are the *B. abortus* vaccine strains more widely used in the prevention of the brucellosis in cattle. Both vaccines are effective in the prevention of abortion and infection, besides offer long lasting protection (McDiarmid, 1957; Manthei, 1959; McDiarmid, 1960; Cheville et al., 1993; Cheville et al., 1996; Elzer et al., 1998; Poester et al., 2006). *B. abortus* S19 is a stable smooth attenuated organism with relatively high immunogenicity and antigenicity that has been used for the prevention of brucellosis for more than seven decades. RB51 vaccine is a lipopolysaccharide O-antigen deficient mutant derived from the virulent smooth strain *B. abortus* 2308, which does not induce an antibody response detectable by routine serological tests (Schurig et al., 1991). This feature allows RB51 vaccination to be performed at any age, while vaccination with S19 is normally restricted to calves between 3 and 8 months of age to avoid the vaccinal interference in the routine serological tests (Brasil, 2006).

At the present, almost all the knowledge available about the protective response induced by both vaccines strains comes from research papers using mouse model. Studies in mice have shown us that RB51 vaccine induces a strong Th1 cell response with production of INF-γ but not IL-4 in immunized animals, besides $CD8^+$ specific cytotoxic cells (Vemulapalli et al., 2000a; Vemulapalli et al., 2000b; He et al., 2001; Pasquali et al., 2001; Andrews et al., 2006; Luo et al., 2006; Cabrera et al., 2009). Furthermore, recent results suggest that Th17 cells may act synergistically with Th1 cells in protection conferred by RB51 in mice, mainly in mucosal immunity, by secretion of IL-17 and IL-22 (Clapp et al., 2011). As expected S19 vaccination also induces Th1 type response, with production of IL-2, TNF-α and IFN-γ but not IL-4 or IL-10 by murine cells, besides higher levels of antigen-specific CD4+ and Granzyme B-secreting CD8+ T-cell responses (Zhan et al., 1995; Rosinha et al., 2002; Yu et al., 2007a; Yu et al., 2007b; Fu et al., 2012). In addition, in mouse, both major T lymphocytes subpopulations, $CD4^+$ and $CD8^+$, have been proved important in the protection against *Brucella* infection, and IFN-γ, TNF-α, and IL-12 are the main cytokines associated with the desired Th1 (type 1 T helper cells) immunity response induced by *B. abortus* infection (Araya et al., 1989; Jiang and Baldwin, 1993; Zhan et al., 1993a; Zhan et al., 1993b; Caron et al., 1994; Oliveira and Splitter, 1995; Zhan and Cheers, 1995; Zhan et al., 1996; Zhan and Cheers, 1998; Agranovich et al., 1999; Pasquali et al., 2001).

In contrast, for cattle there is limited information concerning the immune mechanism by which the *B. abortus* vaccines confer protection. Some studies show that IFN-γ is induced after RB51 vaccination in cattle (Polci et al., 2006; Singh et al., 2012) and that immunization with S19 has been shown to elicit both $CD4^+$ and $CD8^+$ T cell–responses after vaccination (Hu et al., 2009). However, T lymphocyte response specifically induced by *B. abortus* vaccination in cattle has been extensively evaluated only through the blastogenic response (Confer et al., 1985; Smith et al., 1990; Wyckoff et al., 1993; Stevens et al., 1994, 1995; Palmer et al., 1997), whose results indicate the development of cell mediated immune response following vaccination. Even though not able to differentiate between the various biological functions of B and T-cells subpopulations, proliferation assays promotes experimental evidence of the stimulation of cell-mediated immune response components (Banks et al., 2011).

Characterize protective immunity conferred by *B. abortus* vaccines in cattle is critical for the development of new vaccines, more effective and safer, besides new methods to assess these potential vaccines. The incomplete characterization of *B. abortus*-specific T and B lymphocytes subsets preclude a definitive conclusion about the exact responsibility of the immune cells subpopulations in protective response. Furthermore, it remains to be determined whether animals vaccinated with RB51 or S19 have equal profile and persistence of the immune response and, still if revaccination of adult animals previously vaccinated with S19 or RB51 promotes some improve in immune response, which would strengthen the argument in favor of using RB51 in regions of focus. Also, understanding of the immunological mechanisms involved in *Brucella* spp. immunization / infection promotes the opportunity to know more details of the host / pathogen relationship. Additionally, some studies have shown promising results using RB51 and S19 as vaccine vector for heterologous antigens (Vemulapalli et al., 2000a; Sanakkayala et al., 2005; Ramamoorthy et al., 2007a; Ramamoorthy et al., 2007b; Vemulapalli et al., 2007; Sabio y Garcia et al., 2008; Sabio y Garcia et al., 2010), in this sense, is essential to understand in details the immune response generated by this strain so that its use as a vector may be appropriate and maximized.

2. OBJECTIVES

The objectives of this study are to evaluate and compare:

i - The subset of lymphocytes ($CD4^+$, $CD8^+$, $CD21^+$, Treg) induced by calfhood vaccination with S19 or RB51 and by adult revaccination with RB51;

ii - The in vitro production of IL-4, IL-6, IL-10, IL-17A, TGF-β and IFN-γ by peripheral blood mononuclear cells of vaccinated animals;

iii - The in vitro lymphocyte ($CD4^+$ and $CD8^+$) proliferation of vaccinated animals;

iv - The cellular activation phenotypes ($MHCII^{high}$ and $CD25^{high}$) of peripheral mononuclear leukocytes ($CD4^+$, $CD8^+$, $CD21^+$) vaccinated animals;

v - The induction of T and B memory cells in vaccinated animals;

vi - The cytotoxic activity (Granzyme B and Perforin) of $CD8^+$ T lymphocytes in vaccinated animals.

3. REFERENCES

Agranovich, I., Scott, D.E., Terle, D., Lee, K., Golding, B., 1999. Down-regulation of Th2 responses by *Brucella abortus*, a strong Th1 stimulus, correlates with alterations in the B7.2-CD28 pathway. Infection and Immunity 67, 4418-4426.

Andrews, E., Salgado, P., Folch, H., Onate, A., 2006. Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide-dismutase: II. Induction of specific CD8+ cytotoxic lymphocytes and sensitized CD4+ IFN-gamma-producing cell. Microbiology and Immunology 50, 389-393.

Araya, L.N., Elzer, P.H., Rowe, G.E., Enright, F.M., Winter, A.J., 1989. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. Journal of Immunology 143, 3330-3337.

Banks, H.T., Sutton, K.L., Thompson, W.C., Bocharov, G., Roose, D., Schenkel, T., Meyerhans, A., 2011. Estimation of cell proliferation dynamics using CFSE data. Bulletin of mathematical biology 73, 116-150.

Bernues, A., Manrique, E., Maza, M.T., 1997. Economic evaluation of bovine brucellosis and tuberculosis eradication programmes in a mountain area of Spain. Preventive Veterinary Medicine 30, 137-149.

Brasil, 2006. Manual Técnico do Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose – PNCEBT. Ministerio da Agricultura Pecuária e Abastecimento, Brasília.

Cabrera, A., Saez, D., Cespedes, S., Andrews, E., Onate, A., 2009. Vaccination with recombinant Semliki Forest virus particles expressing translation initiation factor 3 of *Brucella abortus* induces protective immunity in BALB/c mice. Immunobiology 214, 467-474.

Caron, E., Peyrard, T., Kohler, S., Cabane, S., Liautard, J.P., Dornand, J., 1994. Live *Brucella* spp. fail to induce tumor necrosis factor alpha excretion upon infection of U937-derived phagocytes. Infection and Immunity 62, 5267-5274.

Cheville, N.F., Olsen, S.C., Jensen, A.E., Stevens, M.G., Palmer, M.V., Florance, A.M., 1996. Effects of age at vaccination on efficacy of *Brucella abortus* strain RB51 to protect cattle against brucellosis. American Journal of Veterinary Research 57, 1153-1156.

Cheville, N.F., Stevens, M.G., Jensen, A.E., Tatum, F.M., Halling, S.M., 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. American Journal of Veterinary Research 54, 1591-1597.

Clapp, B., Skyberg, J.A., Yang, X., Thornburg, T., Walters, N., Pascual, D.W., 2011. Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infection and Immunity 79, 4165-4174.

Confer, A.W., Hall, S.M., Faulkner, C.B., Espe, B.H., Deyoe, B.L., Morton, R.J., Smith, R.A., 1985. Effects of challenge dose on the clinical and immune responses of cattle vaccinated with reduced doses of *Brucella abortus* strain 19. Vet Microbiol 10, 561-575.

Corbel, M.J.E., S.S.; Cosivi O., 2006. Brucellosis in humans and animals. World Health Organization Geneva.

Elzer, P.H., Enright, F.M., Colby, L., Hagius, S.D., Walker, J.V., Fatemi, M.B., Kopec, J.D., Beal, V.C., Jr., Schurig, G.G., 1998. Protection against infection and abortion induced by virulent challenge exposure after oral vaccination of cattle with *Brucella abortus* strain RB51. American Journal of Veterinary Research 59, 1575-1578.

Fu, S., Xu, J., Li, X., Xie, Y., Qiu, Y., Du, X., Yu, S., Bai, Y., Chen, Y., Wang, T., Wang, Z., Yu, Y., Peng, G., Huang, K., Huang, L., Wang, Y., Chen, Z., 2012. Immunization of mice with recombinant protein CobB or AsnC confers protection against *Brucella abortus* infection. PloS One 7, e29552.

He, Y., Vemulapalli, R., Zeytun, A., Schurig, G.G., 2001. Induction of Specific Cytotoxic Lymphocytes in Mice Vaccinated with *Brucella abortus* RB51. Infection and Immunity 69, 5502-5508.

Hu, X.D., Yu, D.H., Chen, S.T., Li, S.X., Cai, H., 2009. A combined DNA vaccine provides protective immunity against Mycobacterium bovis and *Brucella abortus* in cattle. DNA and Cell Biology 28, 191-199.

Jiang, X., Baldwin, C.L., 1993. Effects of cytokines on intracellular growth of *Brucella abortus*. Infection and Immunity 61, 124-134.

Lage, A.P., Poester, F.P., Gonçalves, V.S.P., 2005. Controle da brucelose bovina. Cadernos Técnicos de Veterinária e Zootecnia 47, 30-41.

Luo, D., Ni, B., Li, P., Shi, W., Zhang, S., Han, Y., Mao, L., He, Y., Wu, Y., Wang, X., 2006. Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infection and Immunity 74, 2734-2741.

Manthei, C.A., 1959. Summary of controlled research with strain 19. In, Proceedings 63rd Ann Meet US Livestock Sanitary Association, 91-97.

McDiarmid, A., 1957. The Degree and Duration of Immunity in Cattle Resulting from Vaccination with S. 19 *Br. abortus* Vaccine and its Implication in the Future Control and Eventual Eradication of Brucellosis. Veternary Record 69, 877-879.

McDiarmid, A., 1960. The prophylaxis of brucellosis in cattle. Veternary Record 72, 917-920.

OIE, 2009. Bovine Brucellosis. Manual of diagnostic tests and vaccines for terrestrial animals. World Organisation for Animal Health.

Oliveira, S.C., Splitter, G.A., 1995. CD8+ type 1 CD44hi CD45 RBlo T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice. European Journal of Immunology 25, 2551-2557.

Olsen, S.C., Stoffregen, W.S., 2005. Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert review of vaccines 4, 915-928.

Palmer, M.V., Olsen, S.C., Cheville, N.F., 1997. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. American journal of veterinary research 58, 472-477.

Pappas, G., Akritidis, N., Bosilkovski, M., Tsianos, E., 2005. Brucellosis. The New England Journal of Medicine 352, 2325-2336.

Pasquali, P., Adone, R., Gasbarre, L.C., Pistoia, C., Ciuchini, F., 2001. Mouse cytokine profiles associated with *Brucella abortus* RB51 vaccination or B. abortus 2308 infection. Infection and Immunity 69, 6541-6544.

Poester, F.P., Goncalves, V.S., Paixao, T.A., Santos, R.L., Olsen, S.C., Schurig, G.G., Lage, A.P., 2006. Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. Vaccine 24, 5327-5334.

Polci, A., Leone, A., Di Provvido, A., 2006. Study of the gene expression of Th1 and Th2 cytokines in the immune response of cows vaccinated with *Brucella abortus* RB51. Veterinaria Italiana 42, 147-155.

Ramamoorthy, S., Sanakkayala, N., Vemulapalli, R., Duncan, R.B., Lindsay, D.S., Schurig, G.S., Boyle, S.M., Kasimanickam, R., Sriranganathan, N., 2007a. Prevention of lethal experimental infection of C57BL/6 mice by vaccination with *Brucella abortus* strain RB51 expressing *Neospora caninum* antigens. International Journal for Parasitology 37, 1521-1529.

Ramamoorthy, S., Sanakkayala, N., Vemulapalli, R., Jain, N., Lindsay, D.S., Schurig, G.S., Boyle, S.M., Sriranganathan, N., 2007b. Prevention of vertical transmission of *Neospora caninum* in C57BL/6 mice vaccinated with *Brucella abortus* strain RB51 expressing *N. caninum* protective antigens. International Journal for Parasitology 37, 1531-1538.

Rosinha, G.M., Myioshi, A., Azevedo, V., Splitter, G.A., Oliveira, S.C., 2002. Molecular and immunological characterisation of recombinant Brucella abortus glyceraldehyde-3-phosphate-dehydrogenase, a T- and B-cell reactive protein that induces partial protection when co-administered with an interleukin-12-expressing plasmid in a DNA vaccine formulation. J Med Microbiol 51, 661-671.

Sabio y Garcia, J.V., Bigi, F., Rossetti, O., Campos, E., 2010. Expression of MPB83 from *Mycobacterium bovis* in *Brucella abortus* S19 induces specific cellular immune response against the recombinant antigen in BALB/c mice. Microbes and Infection 12, 1236-1243.

Sabio y Garcia, J.V., Farber, M., Carrica, M., Cravero, S., Macedo, G.C., Bigi, F., Oliveira, S.C., Rossetti, O., Campos, E., 2008. Expression of *Babesia bovis* rhoptry-associated protein 1 (RAP1) in *Brucella abortus* S19. Microbes and Infection 10, 635-641.

Sanakkayala, N., Sokolovska, A., Gulani, J., Hogenesch, H., Sriranganathan, N., Boyle, S.M., Schurig, G.G., Vemulapalli, R., 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clinical and Diagnostic Laboratory Immunology 12, 1429-1436.

Schurig, G.G., Roop, R.M., 2nd, Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

Singh, R., Basera, S.S., Tewari, K., Yadav, S., Joshi, S., Singh, B., Mukherjee, F., 2012. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in cross bred cattle calves in India. Indian Journal of Experimental Biology 50, 239-242.

Smith, R., 3rd, Adams, L.G., Sowa, B.A., Ficht, T.A., 1990. Induction of lymphocyte responsiveness by the outer membrane-peptidoglycan complex of rough strains of Brucella abortus. Veterinary Immunology and Immunopathology 26, 31-48.

Stevens, M.G., Olsen, S.C., Cheville, N.F., 1994. Lymphocyte proliferation in response to immunodominant antigens of *Brucella abortus* 2308 and RB51 in strain 2308-infected cattle. Infection and Immunity 62, 4646-4649.

Stevens, M.G., Olsen, S.C., Cheville, N.F., 1995. Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. Veterinary Immunology and Immunopathology 44, 223-235.

Vemulapalli, R., He, Y., Boyle, S.M., Sriranganathan, N., Schurig, G.G., 2000a. *Brucella abortus* strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infection and Immunity 68, 3290-3296.

Vemulapalli, R., He, Y., Cravero, S., Sriranganathan, N., Boyle, S.M., Schurig, G.G., 2000b. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infection and Immunity 68, 3286-3289.

Vemulapalli, R., Sanakkayala, N., Gulani, J., Schurig, G.G., Boyle, S.M., Lindsay, D.S., Sriranganathan, N., 2007. Reduced cerebral infection of *Neospora caninum* in BALB/c mice vaccinated with recombinant *Brucella abortus* RB51 strains expressing *N. caninum* SRS2 and GRA7 proteins. Veterinary Parasitology 148, 219-230.

Wyckoff, J.H., 3rd, Howland, J.L., Confer, A.W., 1993. Comparison of *Brucella abortus* antigen preparations for in vitro stimulation of immune bovine T-lymphocyte cell lines. Veterinary Immunology and Immunopathology 36, 45-64.

Yu, D.H., Hu, X.D., Cai, H., 2007a. A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against *Brucella abortus* 2308 by inducing specific CTL responses. DNA and Cell Biology 26, 435-443.

Yu, D.H., Li, M., Hu, X.D., Cai, H., 2007b. A combined DNA vaccine enhances protective immunity against *Mycobacterium tuberculosis* and *Brucella abortus* in the presence of an IL-12 expression vector. Vaccine 25, 6744-6754.

Zhan, Y., Cheers, C., 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. Infection and Immunity 63, 1387-1390.

Zhan, Y., Cheers, C., 1998. Control of IL-12 and IFN-gamma production in response to live or dead bacteria by TNF and other factors. Journal of Immunology 161, 1447-1453.

Zhan, Y., Kelso, A., Cheers, C., 1993a. Cytokine production in the murine response to *Brucella* infection or immunization with antigenic extracts. Immunology 80, 458-464.

Zhan, Y., Kelso, A., Cheers, C., 1995. Differential activation of *Brucella*-reactive CD4+ T cells by *Brucella* infection or immunization with antigenic extracts. Infection and Immunity 63, 969-975.

Zhan, Y., Liu, Z., Cheers, C., 1996. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. Infection and Immunity 64, 2782-2786.

Zhan, Y., Yang, J., Cheers, C., 1993b. Cytokine response of T-cell subsets from Brucella abortus-infected mice to soluble *Brucella* proteins. Infection and Immunity 61, 2841-2847.

CHAPTER 1 - RECENT ADVANCES IN *Brucella abortus* VACCINES

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ABSTRACT

Brucella abortus vaccines play a central role in bovine brucellosis control/eradication programs and have been successfully used worldwide for decades. Strain 19 and RB51 are the approved *B. abortus* vaccines strains most commonly used to protect cattle against infection and abortion. However, due to some drawbacks shown by these vaccines much effort has been undertaken for the development of new vaccines, safer and more effective, that could also be used in other susceptible species of animals. In this paper, we present a review of the main aspects of the vaccines that have been used in the brucellosis control over the years and the current research advances in the development of new *B. abortus* vaccines.

Keywords: *B. abortus*; RB51; S19; DNA; subunit; vector; mutants; and outer membrane vesicles (OMVs) vaccines.

1. INTRODUCTION

B. abortus is the main causative agent of brucellosis in cattle, causing abortion and infertility in adult animals [1]. Bovine brucellosis is a worldwide zoonotic disease, endemic in some regions of the world such as Latin America, Middle East, Africa and Asia [1] and responsible for large economic losses due to animal and human health problems.

Due to public health importance of brucellosis and the damage that it causes to the livestock industry, much effort has been expended to control and eradicate the disease in cattle. The development of an efficacious vaccine for brucellosis control/eradication has been a challenge for scientists for many years. Despite enormous advances and the development of *B. abortus* S19 and RB51 vaccines, the search for improved vaccines has never ends. Although the available vaccines are effective in controlling brucellosis, they have numerous drawbacks, such as interference with diagnostic tests, pathogenicity for humans, potential to cause abortion in pregnant animals, among others. In this paper, we present a review of the main aspects of the vaccines that have been used in the bovine brucellosis control and eradication over the years and some of the current advances in the research for a new *B. abortus* vaccine.

2. VACCINES, VACCINATION AND THEIR USE IN BRUCELLOSIS CONTROL AND ERADICATION PROGRAMS

According to Schurig et al.[2] and Ko and Splitter [3], an ideal vaccine against brucellosis should possess the following characteristics: (i) be live and able to provide a strong type 1 T helper immune response (Th1); (ii) do not induce antibodies that interfere with the serological tests employed in the diagnosis of infected cattle, regardless of route, dose of administration, age or sex of the animals; (iii) be attenuated and do not cause disease or persistent infection in immunized animals nor be pathogenic for humans; (iv) be able to induce a strong and long-lasting protection against systemic and uterine infection, besides preventing

abortion, even in pregnant animals inoculated with a single dose; (v) do not lead to seroconversion on revaccination; (vi) be stable and do not revert virulence in vivo nor in vitro; and (vii) be inexpensive, easy to produce and to administer.

Even though we still do not have an ideal vaccine, vaccination with available vaccine strains remains the most successful method for the prevention and control of brucellosis in cattle, being a critical component of most brucellosis control and eradication programs throughout the world [4]. Numerous countries have adopted control measures against bovine brucellosis in order to reduce the prevalence or eradicate the disease from domestic livestock in an effort to prevent transmission to humans and mitigate economic losses. Vaccination of female calves is the central point of any brucellosis control program, since it has performed well in the reduction of disease prevalence, therefore useful at the disease control stage. Considering that vaccination alone is not enough to control and eradicate the disease, it should be associated with continuous elimination of infected animals [5], as they are the source of new infections. Thus, besides vaccination, most bovine brucellosis eradicate programs also include test–and–slaughter policies, surveillance and hygiene measures [4].

The aim of vaccination is the reduction of susceptibly individuals in the population and the success of any vaccination program depends mainly on the effectiveness of the vaccine used and its coverage in the target population. Vaccines against brucellosis have been evaluated with respect to their potency by three different approaches: (i) testing in laboratory animals or (ii) testing in natural hosts experimentally challenged and (iii) testing under natural conditions [6]. Of these, test in natural hosts shows more significant response and is the only one able to measure the efficacy of *B. abortus* vaccines [7, 8]. In experimental studies of vaccine efficacy, vaccinated and non-vaccinated controls will receive a known infectious dose of a virulent *B. abortus* strain at the most susceptible period (mid-gestation), and the protection is measure by the ability of the vaccine in preventing abortion [4]. However, it is important to emphasize that the experimentally obtained effectiveness may differ from field efficacy, that can be influenced by other factors, such as nutrition, environmental stress, age at vaccination, vaccination management or immunological status [4]. Besides, the above three classical methodologies, *B. abortus* vaccines could also be assessed by measuring the immune responses and determination of correlates of protection by mathematical modeling. The identification of protection markers can be a useful approach to screen vaccine candidates whether validated by vaccine potency tests [9]. Since experiments involving challenge of pregnant cattle are very expensive, time-consuming and requires large animal biosafety level 3 facilities, the rational way for the future of *B. abortus* vaccines testing and development could be the characterization and identification of the correlates of protection.

Another important aspect related to the success of brucellosis control programs is the quality of the vaccine used. Despite the cost of the vaccine being just one fraction of the total cost of a control program, its quality will affect directly and dramatically the outcome of the program. Assessing the quality of live *Brucella* vaccines is usually based on in vitro criteria, including physico-chemical and microbiological in vitro tests as to purity, dissociation, and determination of pH, humidity and count of viable bacteria [10]. Recently, genetic stability has also been proposed as an additional criterion in assessing of the quality of *Brucella* spp. vaccines [11-13]. Although less frequently, immunogenicity in mouse can also be included in *Brucella* vaccines tests, however not having cutoff points (protection zone) defined for vaccine [10, 12] and as mouse immune system does not accurately represent bovine immune system, it is very difficult to use such data.

Attenuated *B. abortus* strains have demonstrated the best results in the prevention of bovine brucellosis. *B. abortus* live modified vaccines are highly effective in decreasing transmission and production losses caused by brucellosis, but are less effective at preventing infection by field strains [4]. Since, abortion is the key for the brucellosis transmission in cattle, a vaccine that can effectively prevent abortion is able to reduce disease transmission and largely reduce economic losses caused by the disease. Immunization with live modified *B. abortus* vaccines is generally performed in young female calves in a single dose by intramuscular or subcutaneous injection [6]. However, in zones of high prevalence of brucellosis, massive vaccination, including adult cows, is performed [5].

It is also important take into account that although cattle are the main target of the vaccination against *B. abortus* within bovine brucellosis control and eradication programs, they are not the unique species infected by this agent. Goats, feral swines, elks, bisons and other hosts can also be infected by *B. abortus* and some of them are even able to sustain the disease, being considered important source of the re-emergency of the disease in cattle [1].

Only a few vaccines have been used massively in cattle immunization against *B. abortus*, S19, RB51, 45/20 and SR82, being S19 and RB51 the most widely used vaccines [4]. However, many *B. abortus* vaccine candidates have been developed, including DNA, subunit, recombinant *B. abortus* and recombinant vector vaccines. All of them are evaluated principally in mouse model [14-47], and with a few exceptions the majority of these new vaccines, have not been tested in cattle or were not protective in cattle, the target species.

3. S19 VACCINE

Strain 19 is a live attenuated vaccine and the first *B. abortus* vaccine to be used extensively for bovine brucellosis control [48]. In USA, this vaccine was used for more than five decades from 1941 and is still being used in several other countries [4].

Brucella abortus S19 was isolated in 1923 from milk of a Jersey cow by Dr. John Buck [49]. This virulent culture was accidentally left out at room temperature for one year and when tested in guinea pigs showed lower virulence compared with previous tests [50]. Subsequently, S19 showed to be highly successful in immunization of calves [49, 51]. The efficacy of *B. abortus* S19 has been proven by extensive efficacy tests in cattle [48, 52] and has been evaluated under field conditions [6, 53] and its main characteristics are stable low pathogenicity, relatively high immunogenicity, and moderate antigenicity [54]. Strain 19 is a smooth attenuated *B. abortus* biovar 1 that induces antibody response that cannot be distinguished from the antibody response induced by infection with field strains [4, 51]. The lipopolysaccharide (LPS) O-side chain is an immunodominant antigen to which the majority of antibodies resulting from S19 immunization or natural infection are directed [55]. Antibody titers resulting from vaccination may persist for a prolonged period in a small proportion of vaccinated calves: approximately two animals per 100,000 calfhood vaccinated ones [55]. Residual antibody titers increase with the age at which the animal was vaccinated [6], and to address this issue, vaccination is usually performed on young female calves between three and eight months of age [48]. However, vaccination of this age group does not appear to significantly differ in immunity induced [48]. Restriction on age of vaccination, due to the interference in the brucellosis diagnosis, is the main disadvantage of vaccination with S19. This has contributed greatly to their replacement by RB51 vaccine strain, which does not have this problem.

In calves, S19 vaccination can be performed with full dose [$2.5 – 12 \times 10^{10}$ colony forming units (CFU)], orginal dosage used in S19 classical experiments, or with reduced dosage ($3\text{-}10 \times 10^9$ CFU) to minimize residual antibody titers and to prevent occasional persistent vaccinal infection [4]. After calfhood-vaccination, S19 is usually cleared from superficial cervical lymph node within 10 to 12 weeks [56]. Vaccination of adult cattle with S19, low dosage ($0.3\text{-}3 \times 10^9$ CFU), was also successfully employed in infected herds [6, 57, 58]. S19-adult vaccination was tested as a strategy to be used in infected herds in order to reduce abortions and subsequently brucellosis transmission; however, it was discontinued because vaccination of pregnant animals can cause abortion and mainly because of the persistence of vaccinal antibodies [58, 59].

In general, after calfhood vaccination, S19 do not persist in the reproductive tracts of mature heifers and do not cause abortion in these animals [55]. Nonetheless, even with markedly infrequent occurrence, some cattle remain chronically infected and may abort and excrete the vaccine strain in the milk via the mammary gland. Another disadvantage of S19 vaccination includes the fact that in some circumstances S19 can cause abortion in pregnant animals [59]. After vaccination of cattle with one, two or three doses prior to breeding age, McDiarmid [52] recovered S19 from 10% of milk samples and 1.5% of samples from cases of abortion. In males, calfhood S19 vaccination usually results in persistent antibody titers, testicular infection, and

hence infertility [60]. Furthermore, the vaccination of infected animals with S19 does not cure nor alter the normal course of the disease [48, 51].

On the other hand, duration of immunity induced by S19 in cattle vaccinated as calves has proven to be quite long, reaching almost the entire productive lifespan of the animal [48, 52]. The immunity in cattle vaccinated between 6 and 8 months of age does not decrease from the first through the fifth pregnancy [48, 52]. Moreover, revaccination experiments with S19 and killed *B. abortus* vaccines demonstrated no apparent benefit in cattle-challenge experiments compared with just S19-calfhood immunization [48], despite McDiarmid [52] having observed a small gain from S19 revaccination. Under field conditions, 82 to 95% of vaccinated cattle have been shown to have complete protection against infection with virulent strains [51]. However, it has also been demonstrated that the effectiveness of the vaccine decreases proportionally with an increasing dose of bacterial exposure [48, 51].

Regarding the immune response triggered by S19 vaccination, most of our knowledge come from mice studies, which have been showed a strong Th1 immune response with production of IFN-γ and high levels of antigen-specific $CD4^+$ and granzyme B-secreting $CD8^+$ T-cell responses [33].

Being pathogenic to man, the utilization of S19 requires safety training of the personal involved and the use of personal protection equipaments as gloves, long sleeve coats, protection glasses, and N95 masks.

4. RB51 VACCINE STRAIN

B. abortus strain RB51 is a rough rifampicin-resistant strain, which exhibited a lack of expression of the LPS O-side chains (OPS) [61]. RB51 vaccine strain was developed in 1982 by Prof. Gerhardt Schurig's group and is derived from a virulent smooth *B. abortus* biovar 1 strain 2308 [61]. This is a natural mutant derived by serial passages on media containing subinibitory concentrations of rifampicin or penicillin and by selecting single colonies with rough morphology [61]. The rough characteristic is stable during in vitro and in vivo passages and does not revert to virulent phenotype [61].

The protection against abortion and infection induced by RB51 vaccination in cattle has been sufficiently demonstrated under experimental conditions [7, 8, 56, 62, 63]. Also, the use of RB51 is highly effective under field conditions, in herds with high and low brucellosis prevalence [5, 64, 65].

The literature shows that calves vaccinated with RB51 at three, five and seven months of age are protected against infection and abortion [56], as well as heifers vaccinated at age of 10 or 24 months, after challenge with the virulent *B. abortus* 2308 [7, 62]. Nevertheless, it has been suggested that under experimental circumstances the vaccination with S19 is slightly (not significant) more efficacious than RB51 [4, 56]. After vaccination, RB51 is usually cleared from calf superficial cervical lymph node within 6 to 10 weeks [56]. RB51 is considered more attenuated than S19, based on results of clearance and histologic examination of infected tissues of vaccinated animals [62, 66].

In general, the recommended dosage for RB51 calfhood vaccination is $1.0 – 3.4 \times 10^{10}$ CFU [4]. Protection against *B. abortus* infection is similar through the suggested dosage, although higher antibody titers and longer persistence of bacteria had been associated with the full dose (3.4×10^{10} CFU) [8]. Reduced dosage (1×10^9 CFU), generally recommended for adult animals, also protects against infection and abortion caused by virulent 2308 [63]. Despite RB51 having highly reduced abortifacient characteristics [61, 67], it is not completely safe for pregnant cow, mainly when full dose is administrated [66]. However, some results indicate that non-vaccinated cattle and cattle vaccinated with S19 as calves can be safely vaccinated with RB51 (full dose) during the pregnancy [5, 7, 68]. Furthermore, data indicates that RB51 vaccination is a safe procedure also for males [69].

In addition, as S19 vaccine, RB51 can cause infection in humans especially immunosuppressed individuals [70]. RB51 is resistant to rifampin [61], one of the antibiotics of choice in the treatment of human brucellosis, and failure to be detected by routine serological tests are the two most important points one has

to be aware during diagnosis and treatment of humans. Therefore, the same protective measures recommended for S19 also applies to RB51 use.

Because of the rough phenotype, RB51 does not induce the production of anti-OPS antibodies in immunized animals, overcoming the serologic problems observed after S19 vaccination [8, 61, 63]. Consequently, RB51 vaccinated cattle can be easily and accurately differentiated from naturally infected animals, allowing the effective use of the test-and-slaughter and vaccination policies simultaneously. Vaccination with RB51 does not induce antibodies detectable by routine serologic brucellosis diagnostic tests, even after S19 calfhood vaccination and multiple RB51 boosters or use of full dose of RB51 (3.4×10^{10} CFU) [8, 63, 68]. However, RB51-specific antibodies can be detected by dot enzyme-linked immunosorbent assay or ELISA using killed RB51 antigens [71, 72], until approximately 12 weeks after vaccination, with the peak occurring four weeks after vaccination or revaccination with decreasing titers after ten weeks [63, 73]. Interestingly, S19-vaccinated cattle exhibit higher titers against RB51 antigens in ELISA than animals vaccinated with RB51, probably due to persistence of S19, which may result in high levels of cross-reacting antibodies against RB51 antigens [73].

So far, there are no experiments that evaluated the duration of immunity, but Olsen and Stoffregen [4] suggest that a booster vaccination is required between 4 and 5 years of age to maintain high levels of protection after RB51 calfhood vaccination. Also, RB51 revaccination has been recommended six months and one year after calfhood vaccination in northern Mexico [74]. Nonetheless, findings from blastogenic response of $CD4^+$ and $CD8^+$ T-cells and the production of IFN-ɣ and IL-4 by the lymphocyte subsets six months after RB51 revaccination indicate that there was no increase or improvement in the immunological response resulting from RB51-revaccination of adult cattle [75]. Even though, RB51 revaccination may still be considered as a tool for increasing herd immunity, since not all animals are completely protected after primary immunization [51]. Furthermore, it has been demonstrated that RB51 induces a strong Th1 cellular immune response with production of IFN-γ and $CD8^+$ specific cytotoxic cells, but not IL-4 after vaccination of mice [76].

5. 45/20 VACCINE

This vaccine is prepared with heat-killed *B. abortus* biovar 1 strain 45/20 combined with oil adjuvant [77]. The 45/20 is a rough *B. abortus*, derived of smooth strain 45/0 after 20 passages through guinea pigs [78]. This bacterin was used in some European Union countries for *B. abortus* control replacing the S19, in order to eliminate the problems related to the induction of antibodies interfering in the routine diagnosis of infection [6]. However, data of experimental efficacy and immunologic response are contradictory and mostly show the superiority of vaccination with S19 [79, 80]. Furthermore, its use has some drawbacks such as the use of oil adjuvant, needing of repeat vaccination and reversion to smooth strain when used as a live vaccine [6, 78]. Furthermore, some studies have also indicated that 45/20 is not completely free of the O-side chain [81], hence this vaccine can induce antibodies detectable by rotine serologic tests employed in the diagnosis of bovine brucellosis. The variability in reported protection, along with unpredictable serological effects and the occurrence of reactions at the site of vaccine injection in some animals led to the interruption of the use of 45/20 vaccine.

6. SR82 VACCINE STRAIN

The SR82 strain is a *B. abortus* biovar 6 live attenuated vaccine used since 1974 by the former Union of Soviet Socialist Republics (USSR) for bovine brucellosis control [82]. This vaccine agglutinates in both rough and smooth anti-sera, but does not induce positive response in brucellosis agglutination tests [82, 83]. Moreover, SR82 induced protection level similar to S19, after challenge with virulent *B. abortus*, and it has been shown to be effective under field conditions [82, 83]. Currently the SR82 strain is still massively used in the Russian Federation, Azerbaijan, Tejikistan and other countries in the region [82].

7. VACCINATION WITH RECOMBINANT GENES, PROTEINS, VECTORS AND *B. abortus* RECOMBINANT MUTANTS

Classically and historically, the vaccines used in the bovine brucellosis control are live attenuated vaccines produced from spontaneously attenuated or ramdomly selected strains. Nonetheless, the numerous advances in genomics, proteomics, recombinant DNA technology and even in vaccinology, allowed the exploration of other tools for the development of safer vaccines, without drawbacks observed in classical vaccines. In this context, several studies aimed to develop, test the efficacy or assess the immulogical responses of the *B. abortus* genetically engineered vaccines (recombinant genes, proteins, vectors and modified *B. abortus* strains) have been performed essentially in mice. However, with a few exceptions the majority of these recombinant vaccines, have not been tested or did not protect cattle, their target species. Moreover, it important take into account that recombinant vaccines, especially non-living ones, have important limitations regarding economic viability, need for multiple doses and the need for combination of antigens.

7.1. DNA VACCINES

DNA vaccines offer the possibility of inducing both cellular and humoral responses, expression of antigens is prolonged, they have better stability and do not require refrigeration under storage. Therefore, several antigens have been explored for their value as DNA vaccines against *B. abortus* challenge, providing various levels of protection. DNA vaccines ecoding ribosomal L7/L12, lumazine synthase (BLS), P39 (a putative periplasmic binding protein), Omp16 (outer membrane protein) and BAB1_0278 genes have demonstrated to confer protection against *B. abortus* challenge in mice [23, 24, 28, 43]. Moreover, Cu/Zn superoxide dismutase (Cu-Zn SOD) DNA vaccine induced a protection level similar to the one induced by RB51 [26]. All these genes also proved capable of eliciting a desirable cellular immune response in mice [23, 24, 33, 34, 43]. In contrast, plasmid DNA carrying the BAB1_0263 and bacterioferritin (BFR) genes did not induce significant level of protection against challenge with virulent *B. abortus* [23, 43].

Combined DNA vaccines have also demonstrated their ability to protect better against a challenge. DNA vaccines of genes coding for an immundominant *Brucella*-antigen (BCSP31) and promising *Brucella*-antigens (SOD and L7/L12) provided significantly better protection than S19 in mice [33]. This combined DNA vaccine also elicited significantly higher cytotoxic response (granzyme B–producing CD8+ T cells) compared to S19-vaccinated mice [33]. Likewise, divalent fusion DNA vaccine encoding L7/L12 and Omp16 genes also proved to be effective and able to elicit a strong T-cell proliferative response and induce a large amount of gamma interferon producing T cells [28]. Additionally, data show that combination of these *B. abortus* genes (BCSP31, SOD and L7/L12) with *Mycobacterium tuberculosis* (Ag85B, MPT64, and MPT83) or *Mycobacterium bovis* (Ag85B, MPT64, and MPT83) genes are very promising for both agents [34, 35]. DNA vaccine containing six genes encoding immunodominant antigens from *M. bovis* and *B. abortus* induced protection comparable to S19 and better than Bacillus Calmette-Guerin (BCG) vaccine in cattle, suggesting that this is a highly promising vaccine for both diseases [35]. Combined DNA vaccine containing *M. tuberculosis* and *B. abortus* genes with added IL-12 adjuvant system and showed that besides the high level of protection, IL-12 acts as an adjuvant to enhance protective immunity against *M. tuberculosis* and *B. abortus* in challenge mice [34]. Conversely, results suggest that a SOD DNA vaccine fused to IL-2 did not improve protection efficacy [30].

However, despite some of *B. abortus* DNA vaccines candidate have showed very promising results in mice, the need of several booster vaccinations (at least four) to be effective as well as the high cost for be use in large animals, make this type of vaccine impractical for cattle, the main target of brucellosis vaccination. Moreover, excluding mice studies practically no DNA vaccine has been explored in natural hosts. The potential targets for protective immunity observed using the DNA vaccine should be tested using other approaches as recombinant *B. abortus* mutants.

7.2. SUBUNIT VACCINES

Many of the same antigens tested as DNA vaccines have also been evaluated as potential antigens for subunit vaccines (L7/L12 ribossomal protein; P39; BLS; Omp16; Cu/Zn SOD) [15, 22, 25, 38, 41]. The

outer membrane proteins (OMPs) of *B. abortus*, potential immunogenic antigens, have been widely explored as subunit vaccines [38, 40, 44, 46]. Unlipidated recombinant Omp16 and Omp19, and recombinant Omp25 liposome encapsulated gave protection comparable to S19 in vaccinated mice following challenge [38, 40, 46]. Also, Omp28 subunit vaccine increased resistance against challenge with virulent *B. abortus* but at lower level than live attenuated vaccines [44].

Similarly, flagellar proteins have been screened in search for a subunit vaccine antigen candidate. Five flagellar genes, although *Brucella* spp. are non-motile, (BAB1_0260 (FlgJ); BAB2_0122 (FliN); BAB2_0150; BAB2_1086; BAB2_1093) were evaluated for their ability to induce humoral and cell-mediated responses and protect mice against *B. abortus* challenge [84]. Of these, FlgJ and FliN were found to be protective antigens that produced humoral and cell-mediated responses in mice [84].

Moreover, recombinant proteins of other proven or putative pathogenesis-associated genes such as L7/12, BLS, rSurA and rDnaK induced different levels of protective immunity and cellular immune response in mice against brucellosis [15, 25, 32]. Whereas, dihydrolipoamide succinyltransferase (rE2o) and cysteine synthase A (rCysK) provided partial protection against *B. abortus* challenge and induced primarily Th2 type of immune response [42, 47]. Furthermore, CobB, AsnC and P39 elicit protective immunity similarly to Cu/Zn SOD and S19, which is marked by both humoral and cellular immune responses [22, 85]. Also, Cu/Zn SOD recombinant protein (liposomes encapsulated) confers resistance in mice, further increased upon co-immunization with recombinant IL-18 [41]. In contrast, BAB1_0560, BAB1_1108, BAB2_0059 (VirB10), BAB2_0191, BAB2_0423 (GntR) and BRF protein vaccines did not induce protective immune response [22, 85].

The potential use of *B. abortus* subunit vaccines under field conditions is very limited, although some encouraging results showed. The requirement of multiple boosters, adjuvants and combination of several antigens, as well as observed for DNA vaccine, make it economically unviable for cattle. Moreover, it is important considered that the response observed in mice may not reflect the protection achieved in natural hosts after vaccination. Furthermore, generate a strong and protective immune response that can mimic the natural infection from a combination of few proteins of the pathogen is a hard and complex challenge.

7.3. VECTOR VACCINES

Alternatively, genes encoding immunodominant *B. abortus* antigens can be introduced into attenuated viruses or bacteria that serve as vector vaccines. *B. abortus* genes have been sucessfully expressed in viruses (Semliki Forest virus and Vaccinia virus) and bacteria (*Escherichia coli, Ochrobactrum anthropi, Lactococcus lactis, Salmonella enterica* subsp *enterica* serovar *Typhimurium* and *B. abortus*) [17, 21, 27, 29, 36, 39, 45, 86]. Escherichia coli, O. anthropi (plus unmethylated CpG motifs) and L. lactis expressing Cu/Zn SOD antigen of *B. abortus* were able to elicit a Th1 immune response and to protect mice following challenge with virulent *B. abortus* [21, 29, 45, 86]. Likewise, Semliki Forest virus-based vector carrying RNA encoding *Brucella* translation initiation factor 3 (IF3) showed a significant level of protection against a challenge with *B. abortus* 2308 in mice [36]. L7/L12 protein carried by *S. enterica* serovar Typhimurium but not by Vaccinia virus conferred protective efficacy and immunogenicity [27, 39]. Also, vaccinia virus carrying 18-kDa OMP of *B. abortus* were not able to protect mice against a challenge with the virulent strain *B. abortus* 2308 [87].

The expression of *B. abortus* antigens in viral or bacterial vectors is a superior alternative to DNA and subunit vaccines, as closely mimic a natural infection, allowing the modulation of the host immune response and the multiplication of the initial number of antigen copies within the host. However, despite not have some of the inconveniences observed in non-living vaccines, as multiple doses, need for adjuvant and high cost, other organisms expressing *B. abortus* proteins still need the perfect grouping of antigens, expressed in high amount to be effective. The amount of foreign protein expressed by the carrier organism need to be able to promote a specific protection. Moreover, the use of viral platform implies the small chance that the vector DNA is integrated into the genome of the host cell. In addition, although promising most of these above vaccines have failed or have not been tested in cattle, the target species, so no conclusion could be drawn at this time.

7.4. *B. abortus* RECOMBINANT MUTANTS

Another focus of research for new vaccines to protect against *B. abortus* infection has been the construction of RB51 recombinant mutants, which retain the rough phenotype and attenuation but have improved characteristics such as immunogenicity and protection against a challenge [18-20]. Hence, some studies have shown that the complementation of RB51 with a functional wboA gene (RB51WboA), which lead to the expression of O-side chain in its cytoplasm, or the overexpression of Cu/Zn SOD protein (RB51SOD) results in significant enhancement of the vaccine efficacy against challenge with virulent *B. abortus* in mice [18, 19]. Furthermore, the combination of these two genes in a single RB51 strain (RB51SOD/wboA) also significantly increased the protective ability of this RB51 recombinat vaccine in mice and did not alter its desirable characteristics [20]. Nonetheless, this RB51-recombinant strain was not as effectives as the parental RB51 strain in calfhood vaccination of bison after challenge with 2308 [37].

Besides RB51, *B. abortus* strain 2308 has also been tested as recombinant mutant vaccine; the deletion of the gene znuA, important protein for survival and normal growth under low Zn2+ concentrations, generated a mutant capable of conferring protection similar to S19 or RB51 against challenge with parental 2308 in mice [31]. Experiments in natural hosts, cattle, was showed that the double gene deletion (htrA cycL) PHE1 was attenuated in the bovine host when compared to the virulent parental 2308 [88]. However, due the absence of a standard challenge study using this potential vaccine, the meaning of such data is unclear. Recombinant mutants based on deletion of ABC transporter ATPase (BAB1_0542) or phosphoglycerate kinase (pgk) gene of *B. abortus* 2308 also showed protection against challenge with virulent strain in mice and the critical of role of these genes to full bacterial virulence [89, 90]. In addition to the virulence attenuation, is desired that these *B. abortus* mutants also show no interference with the diagnostics tests, hence genes associated to the smooth phenotype have been explored in the generation of deleted vaccines. Rough mutant generated by wboA gene deletion of S19 protected mice against challenge with 2308 and did not induced abortion in pregnant sheep, showing promising results to be explored in the future development of rough vaccines [91].

The improvement of the existing *B. abortus* vaccines or the creation of new attenuated vaccines by deletion or complementation of some genes, it seem to be the most promising direction to find a safer and more efficient substitute for the known *B. abortus* vaccines. Modified live vaccines are highly effective in comparison to killed vaccines. This is most likely due to strong and protective cellular immune response induced by live vaccines [2, 3]. The use of this platform avoid the main disadvantages related to the non-living vaccines, as multiple delivery, low immunogenicity, need for adjuvants and costly. Furthermore, *B. abortus* strains, even if genetically modified, can colonize, be immunogenic, and therefore perfectly simulate the natural infection. They are able to multiply within animals for a short period of time expressing in vivo protective antigens. The major advantage of this approach over the use of vectors is that recombinant mutants share most of proteins with *B. abortus* field strains, whereas carrier organisms are able of expressing only few *Brucella* antigens. However, a real concern on *B. abortus* mutant strains is the presence of antibiotic selection marker. The antibiotic marker is used in the screening of transformed clones, but it is not desirable in the final vaccine due to the potential of spread an antibiotic resistance marker. Options, as an RB51 leucine auxotroph, have been explored to avoid this issue [92]. Additionally, so far, there is no data available to exclude the possibility of these live mutants will not have similar safety and diagnostic issues as live strains, especially if made from smooth strains. Also, to move towards in the control of bovine brucellosis, these recombinant mutants must be evaluated in cattle and other target species of animals. The worldwide need is a vaccine for natural hosts, since the transmission of disease occurs from cattle to people. The results obtained in mice, although favorable for some vaccines, have to be interpreted according to its limitations, as they cannot be directly transported to cattle.

On another point of view about the usefulness of *B. abortus* mutant as vaccines, S19 and RB51, the widely used *B. abortus* vaccines, has been investigated as potential vectors for heterologous protein expression, mainly using protective antigens important for other diseases of veterinary interest [93-96]. In this context, multivalent recombinant RB51 vaccines expressing *Neospora caninum* or *M. bovis* proteins have been shown to induce antigen specific immune response to heterologous antigens and, in the case of *N. caninum*, was also achieved significant level of protection in mice [93, 94]. Likewise, S19 carrying the genes

encoding for the heterologous antigens of *Babebia bovis* or *M. bovis* demonstrated successful specific cellular immune response to recombinant proteins in mouse model [95, 96]. These above bivalent live modified *B. abortus* candidate vaccines need further evaluation as to their ability to induce protective immune response as well lack of interference in the diagnostic tests.

8. OTHER *B. abortus* POTENTIAL VACCINES

Besides *B. abortus* recombinants vaccines, also vaccines based on outer membrane vesicles (OMVs) has been exploited as an acellular alternative to live vaccines [97]. OMVs are bilayer membrane vesicles release by Gram-negative and Gram-positive bacterias, which have been associated to many processes such as release of virulent factors, DNA transfer, regulation of host immune response and survival in the host cell [98]. *B. abortus* OMVs are mainly composed for outer membrane proteins (Omps) and have been associated with modulation of host immune response by inhibition of TNF-α and IL-8 response, inhibition of IFN-γ induced expression of MHC class II molecules on human monocytes and increase in expression of adhesion molecules [97-99]. A *Brucella melitensis* vaccine based on OMVs has been tested and showed promising results in BALB/c mice [100]. Furthermore, it is already available a vaccine based on OMVs, against meningococcus serogroup B (*Neisseria meningitides*) in some countries as Cuba, Norway and New Zealand [97]. Therefore, it is possible to speculate that an OMV vaccine against *B. abortus* has a great potential to be considered as part of the continuous efforts to reach *B. abortus* vaccine safer and more effective. Nonetheless, due high cost and labor intensive related to OMV production, this is a more suitable approach to human vaccine, being impracticable for cattle.

9. FINAL CONSIDERATIONS

Vaccination is a determinant strategy for brucellosis control and eradication programs, therefore it has been the target of innumerous studies over decades. Nowadays, some effective vaccines are available to control the disease in cattle. S19 and RB51 are the officially approved *B. abortus* vaccine strains more widely and successfully used to prevent bovine brucellosis worldwide. However, due to some side effects shown by these current vaccines, plus the advances in recombinant DNA technology and the lack of a vaccine for humans, there is an on going extensive efforts focused on the development of new and better vaccines. Engineered vaccines have the potential to be the future of the bovine and human brucellosis control, but many studies are still needed to develop a better vaccine than the current vaccines in terms of safety, efficacy and other desirable characteristics. Moreover, it is important consider that, mainly non-living recombinant vaccines, such as subunit and DNA, also present important issues, as necessity for multiple booster, adjuvant, optimal combination of antigens, besides induction of poor cellular immune response. In addition, although the excellent results observed for some recombinant vaccines in mice, very few of these candidate vaccines have been evaluated in cattle. The recent studies showed that drive the mutant construction to exclude the drawbacks presented by RB51 and S19 and to enhance immunogenicity offered to these vaccines is the future of a new *B. abortus* vaccine. Furthermore, concerning immune response induced after S19 and RB51 vaccination, as well as after RB51 revaccination, in cattle very little is understood. Efforts to find out the principal characteristics of the immune response triggered in cattle by the two most used and successful *B. abortus* vaccine strains are essential to try to establish an ideal vaccine. The definition of immune markers correlated with protection, by mathematical modeling or evaluation of the immune response in vaccine – challenge studies – would be very helpful in the screening *B. abortus* candidate vaccines. The search for an ideal vaccine passes through the better understanding of how existing vaccines confer protection in the target species.

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14. REFERENCES

1. Corbel MJE, S.S.; Cosivi O.: Brucellosis in humans and animals. Geneva: World Health Organization; 2006.

2. Schurig G, Boyle S, Sriranganathan N: *Brucella abortus* vaccine strain RB51: A brief review. Archivos De Medicina Veterinaria 1995, 27:19-22.

3. Ko J, Splitter GA: Molecular Host-Pathogen Interaction in Brucellosis: Current Understanding and Future Approaches to Vaccine Development for Mice and Humans. Clinical Microbiology Reviews 2003, 16:65-78.

4. Olsen SC, Stoffregen WS: Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert review of vaccines 2005, 4:915-928.

5. Martins H, Garin-Bastuji B, Lima F, Flor L, Pina Fonseca A, Boinas F: Eradication of bovine brucellosis in the Azores, Portugal-Outcome of a 5-year programme (2002-2007) based on test-and-slaughter and RB51 vaccination. Preventive veterinary medicine 2009, 90:80-89.

6. Nicoletti P: Vaccination. In Animal Brucellosis. Edited by Nielsen K, Duncan JR. Boca Raton: CRC Press; 1990: 283-299

7. Poester FP, Goncalves VS, Paixao TA, Santos RL, Olsen SC, Schurig GG, Lage AP: Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. Vaccine 2006, 24:5327-5334.

8. Olsen SC, Bricker B, Palmer MV, Jensen AE, Cheville NF: Responses of cattle to two dosages of Brucella abortus strain RB51: serology, clearance and efficacy. Research in veterinary science 1999, 66:101-105.

9. Thakur A, Pedersen LE, Jungersen G: Immune markers and correlates of protection for vaccine induced immune responses. Vaccine 2012, 30:4907-4920.

10. Miranda KL, Dorneles EM, Pauletti RB, Poester FP, Lage AP: *Brucella abortus* S19 and RB51 vaccine immunogenicity test: evaluation of three mice (BALB/c, Swiss and CD-1®) and two challenge strains (544 and 2308). Vaccine 2015, In Press.

11. Garcia-Yoldi D, Le Fleche P, Marin CM, De Miguel MJ, Munoz PM, Vergnaud G, Lopez-Goni I: Assessment of genetic stability of *Brucella melitensis* Rev 1 vaccine strain by multiple-locus variable-number tandem repeat analysis. Vaccine 2007, 25:2858-2862.

12. Miranda KL, Poester FP, Minharro S, Dorneles EM, Stynen AP, Lage AP: Evaluation of *Brucella abortus* S19 vaccines commercialized in Brazil: immunogenicity, residual virulence and MLVA15 genotyping. Vaccine 2013, 31:3014-3018.

13. Dorneles EM, de Faria AP, Pauletti RB, Santana JA, Caldeira GA, Heinemann MB, Titze-de-Almeida R, Lage AP: Genetic stability of *Brucella abortus* S19 and RB51 vaccine strains by multiple locus variable number tandem repeat analysis (MLVA16). Vaccine 2013, 31:4856-4859.

14. Oliveira SC, Harms JS, Banai M, Splitter GA: Recombinant *Brucella abortus* proteins that induce proliferation and gamma-interferon secretion by CD4+ T cells from *Brucella*-vaccinated mice and delayed-type hypersensitivity in sensitized guinea pigs. Cellular immunology 1996, 172:262-268.

15. Oliveira SC, Splitter GA: Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. Vaccine 1996, 14:959-962.

16. Vemulapalli R, Duncan AJ, Boyle SM, Sriranganathan N, Toth TE, Schurig GG: Cloning and sequencing of yajC and secD homologs of *Brucella abortus* and demonstration of immune responses to YajC in mice vaccinated with *B. abortus* RB51. Infection and immunity 1998, 66:5684-5691.

17. Vemulapalli R, Cravero S, Calvert CL, Toth TE, Sriranganathan N, Boyle SM, Rossetti OL, Schurig GG: Characterization of specific immune responses of mice inoculated with recombinant vaccinia virus expressing an 18-kilodalton outer membrane protein of *Brucella abortus*. Clinical and diagnostic laboratory immunology 2000, 7:114-118.

18. Vemulapalli R, He Y, Buccolo LS, Boyle SM, Sriranganathan N, Schurig GG: Complementation of Brucella abortus RB51 with a functional wboA gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infection and immunity 2000, 68:3927-3932.

19. Vemulapalli R, He Y, Cravero S, Sriranganathan N, Boyle SM, Schurig GG: Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infection and immunity 2000, 68:3286-3289.

20. Vemulapalli R, Contreras A, Sanakkayala N, Sriranganathan N, Boyle SM, Schurig GG: Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Vet Microbiol 2004, 102:237-245.

21. He Y, Vemulapalli R, Schurig GG: Recombinant *Ochrobactrum anthropi* expressing *Brucella abortus* Cu,Zn superoxide dismutase protects mice against *B. abortus* infection only after switching of immune responses to Th1 type. Infection and Immunity 2002, 70:2535-2543.

22. Al-Mariri A, Tibor A, Mertens P, De Bolle X, Michel P, Godefroid J, Walravens K, Letesson JJ: Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvant. Infection and Immunity 2001, 69:4816-4822.

23. Al-Mariri A, Tibor A, Mertens P, De Bolle X, Michel P, Godfroid J, Walravens K, Letesson JJ: Induction of immune response in BALB/c mice with a DNA vaccine encoding bacterioferritin or P39 of *Brucella* spp. Infection and Immunity 2001, 69:6264-6270.

24. Velikovsky CA: A DNA Vaccine Encoding Lumazine Synthase from *Brucella abortus* Induces Protective Immunity in BALB/c Mice. Infection and Immunity 2002, 70:2507-2511.

25. Velikovsky CA, Goldbaum FA, Cassataro J, Estein S, Bowden RA, Bruno L, Fossati CA, Giambartolomei GH: *Brucella* Lumazine Synthase Elicits a Mixed Th1-Th2 Immune Response and Reduces Infection in Mice Challenged with *Brucella abortus* 544 Independently of the Adjuvant Formulation Used. Infection and Immunity 2003, 71:5750-5755.

26. Onate AA, Cespedes S, Cabrera A, Rivers R, Gonzalez A, Munoz C, Folch H, Andrews E: A DNA Vaccine Encoding Cu,Zn Superoxide Dismutase of *Brucella abortus* Induces Protective Immunity in BALB/c Mice. Infection and Immunity 2003, 71:4857-4861.

27. Baloglu S, Boyle SM, Vemulapalli R, Sriranganathan N, Schurig GG, Toth TE: Immune responses of mice to vaccinia virus recombinants expressing either *Listeria monocytogenes* partial listeriolysin or *Brucella abortus* ribosomal L7/L12 protein. Vet Microbiol 2005, 109:11-17.

28. Luo D, Ni B, Li P, Shi W, Zhang S, Han Y, Mao L, He Y, Wu Y, Wang X: Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infection and Immunity 2006, 74:2734-2741.

29. Andrews E, Salgado P, Folch H, Onate A: Vaccination with live Escherichia coli expressing *Brucella abortus* Cu/Zn superoxide-dismutase: II. Induction of specific CD8+ cytotoxic lymphocytes and sensitized CD4+ IFN-gamma-producing cell. Microbiology and Immunology 2006, 50:389-393.

30. Gonzalez-Smith A, Vemulapalli R, Andrews E, Onate A: Evaluation of *Brucella abortus* DNA vaccine by expression of Cu-Zn superoxide dismutase antigen fused to IL-2. Immunobiology 2006, 211:65-74.

31. Yang X, Becker T, Walters N, Pascual DW: Deletion of znuA virulence factor attenuates *Brucella abortus* and confers protection against wild-type challenge. Infection and Immunity 2006, 74:3874-3879.

32. Delpino MV, Estein SM, Fossati CA, Baldi PC, Cassataro J: Vaccination with *Brucella* recombinant DnaK and SurA proteins induces protection against *Brucella abortus* infection in BALB/c mice. Vaccine 2007, 25:6721-6729.

33. Yu DH, Hu XD, Cai H: A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against *Brucella abortus* 2308 by inducing specific CTL responses. DNA and Cell Biology 2007, 26:435-443.

34. Yu DH, Li M, Hu XD, Cai H: A combined DNA vaccine enhances protective immunity against Mycobacterium tuberculosis and *Brucella abortus* in the presence of an IL-12 expression vector. Vaccine 2007, 25:6744-6754.

35. Hu XD, Yu DH, Chen ST, Li SX, Cai H: A combined DNA vaccine provides protective immunity against *Mycobacterium bovis* and *Brucella abortus* in cattle. DNA and Cell Biology 2009, 28:191-199.

36. Cabrera A, Saez D, Cespedes S, Andrews E, Onate A: Vaccination with recombinant Semliki Forest virus particles expressing translation initiation factor 3 of *Brucella abortus* induces protective immunity in BALB/c mice. Immunobiology 2009, 214:467-474.

37. Olsen SC, Boyle SM, Schurig GG, Sriranganathan NN: Immune responses and protection against experimental challenge after vaccination of bison with *Brucella abortus* strain RB51 or RB51 overexpressing superoxide dismutase and glycosyltransferase genes. Clinical and Vaccine Immunology : CVI 2009, 16:535-540.

38. Pasquevich KA, Estein SM, Garcia Samartino C, Zwerdling A, Coria LM, Barrionuevo P, Fossati CA, Giambartolomei GH, Cassataro J: Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific $CD4^+$ and $CD8^+$ T cells as well as systemic and oral protection against *Brucella abortus* infection. Infection and Immunity 2009, 77:436-445.

39. Zhao Z, Li M, Luo D, Xing L, Wu S, Duan Y, Yang P, Wang X: Protection of mice from *Brucella* infection by immunization with attenuated *Salmonella enterica* serovar Typhimurium expressing A L7/L12 and BLS fusion antigen of *Brucella*. Vaccine 2009, 27:5214-5219.

40. Pasquevich KA, Ibanez AE, Coria LM, Garcia Samartino C, Estein SM, Zwerdling A, Barrionuevo P, Oliveira FS, Seither C, Warzecha H, et al: An oral vaccine based on U-Omp19 induces protection against *B. abortus* mucosal challenge by inducing an adaptive IL-17 immune response in mice. PloS One 2011, 6:e16203.

41. Singha H, Mallick AI, Jana C, Fatima N, Owais M, Chaudhuri P: Co-immunization with interlukin-18 enhances the protective efficacy of liposomes encapsulated recombinant Cu-Zn superoxide dismutase protein against *Brucella abortus*. Vaccine 2011, 29:4720-4727.

42. Verma SK, Jain S, Kumar S: Immunogenicity and protective potential of a bacterially expressed recombinant dihydrolipoamide succinyltransferase (rE2o) of *Brucella abortus* in BALB/c mice. World Journal of Microbiology & Biotechnology 2012, 28:2487-2495.

43. Sislema-Egas F, Cespedes S, Fernandez P, Retamal-Diaz A, Saez D, Onate A: Evaluation of protective effect of DNA vaccines encoding the BAB1_0263 and BAB1_0278 open reading frames of *Brucella abortus* in BALB/c mice. Vaccine 2012, 30:7286-7291.

44. Lim JJ, Kim DH, Lee JJ, Kim DG, Min W, Lee HJ, Rhee MH, Kim S: Protective effects of recombinant *Brucella abortus* Omp28 against infection with a virulent strain of *Brucella abortus* 544 in mice. Journal of Veterinary Science 2012, 13:287.

45. Saez D, Fernandez P, Rivera A, Andrews E, Onate A: Oral immunization of mice with recombinant *Lactococcus lactis* expressing Cu,Zn superoxide dismutase of *Brucella abortus* triggers protective immunity. Vaccine 2012, 30:1283-1290.

46. Goel D, Rajendran V, Ghosh PC, Bhatnagar R: Cell mediated immune response after challenge in Omp25 liposome immunized mice contributes to protection against virulent *Brucella abortus* 544. Vaccine 2013, 31:1231-1237.

47. Jain S, Afley P, Kumar S: Immunological responses to recombinant cysteine synthase A of Brucella abortus in BALB/c mice. World Journal of Microbiology & Biotechnology 2013, 29:907-913.

48. Manthei CA: Summary of controlled research with strain 19. In Proceedings 63rd Ann Meet US Livestock Sanitary Association. 1959: 91-97.

49. Buck JM: Studies on vaccination during calfhood to prevent bovine infectious abortion. The Journal of Agricultural Research 1930, 41:667-689.

50. Graves RR: The Story of John M. Buck's and Matilda's Contribution to the Cattle Industry. Journal of American Veterinary Medical Association 1943, 102:193-195.

51. Jones FM, Hooper JA: *Brucella abortus* strain 19 calfhood vaccination – a review. Southwest Veterinary 1976, 29:219-225.

52. McDiarmid A: The Degree and Duration of Immunity in Cattle Resulting from Vaccination with S. 19 *Br. abortus* Vaccine and its Implication in the Future Control and Eventual Eradication of Brucellosis. Veternary Record 1957, 69:877-879.

53. Koh SH, Morley FH: The effect of calfhood vaccination with strain 19 on the serological diagnosis and eradication of bovine brucellosis. Australian Veterinary Journal 1981, 57:551-553.

54. Mingle CK, Manthei CA, Jasmin AM: The stability of reduced virulence exhibited by *Brucella abortus* strain 19. Journal of American Veterinary Medical Association 1941, 99:203-205.

55. Meyer ME, Nelson CJ: Persistence of *Brucella abortus*, strain 19 infection in immunized cattle. Proceedings, annual meeting of the United States Animal Health Association 1969, 73:159-165.

56. Cheville NF, Olsen SC, Jensen AE, Stevens MG, Palmer MV, Florance AM: Effects of age at vaccination on efficacy of *Brucella abortus* strain RB51 to protect cattle against brucellosis. American Journal of Veterinary Research 1996, 57:1153-1156.

57. Breitmeyer RE, Hird DW, Carpenter TE: Serologic and bacteriologic test results after adult vaccination with strain 19 in three dairy herds infected with brucellosis. Journal of the American Veterinary Medical Association 1992, 200:806-811.

58. Nicoletti P: Prevalence and persistence of *Brucella abortus* strain 19 infections and prevalence of other biotypes in vaccinated adult dairy cattle. Journal of the American Veterinary Medical Association 1981, 178:143-145.

59. Corner LA, Alton GG: Persistence of *Brucella abortus* strain 19 infection in adult cattle vaccinated with reduced doses. Research in veterinary science 1981, 31:342-344.

60. Lambert G, Manthei CA, Deyoe BL: studies on *Brucella abortus* infection in bulls. American Journal of Veterinary Research 1963, 24:1152-1157.

61. Schurig GG, Roop RM, 2nd, Bagchi T, Boyle S, Buhrman D, Sriranganathan N: Biological properties of RB51; a stable rough strain of *Brucella abortus.* Vet Microbiol 1991, 28:171-188.

62. Cheville NF, Stevens MG, Jensen AE, Tatum FM, Halling SM: Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus.* American Journal of Veterinary Research 1993, 54:1591-1597.

63. Olsen SC: Immune responses and efficacy after administration of a commercial *Brucella abortus* strain RB51 vaccine to cattle. Veterinary Therapeutics : research in applied veterinary medicine 2000, 1:183-191.

64. Lord VR, Schurig GG, Cherwonogrodzky JW, Marcano MJ, Melendez GE: Field study of vaccination of cattle with *Brucella abortus* strains RB51 and 19 under high and low disease prevalence. American Journal of Veterinary Research 1998, 59:1016-1020.

65. Dorneles EM, Santana JA, Alves TM, Pauletti RB, Mol JP, Heinemann MB, Lage AP: Genetic stability of *Brucella abortus* isolates from an outbreak by multiple-locus variable-number tandem repeat analysis (MLVA16). BMC Microbiology 2014, 14:186.

66. Palmer MV, Cheville NF, Jensen AE: Experimental infection of pregnant cattle with the vaccine candidate *Brucella abortus* strain RB51: pathologic, bacteriologic, and serologic findings. Veterinary Pathology 1996, 33:682-691.

67. Palmer MV, Olsen SC, Cheville NF: Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. American Journal of Veterinary Research 1997, 58:472-477.

68. Samartino LE, Fort M, Gregoret R, Schurig GG: Use of *Brucella abortus* vaccine strain RB51 in pregnant cows after calfhood vaccination with strain 19 in Argentina. Preventive veterinary medicine 2000, 45:193-199.

69. Edmonds MD, Schurig GG, Samartino LE, Hoyt PG, Walker JV, Hagius SD, Elzer PH: Biosafety of Brucella abortus strain RB51 for vaccination of mature bulls and pregnant heifers. American journal of veterinary research 1999, 60:722-725.

70. Ashford DA, di Pietra J, Lingappa J, Woods C, Noll H, Neville B, Weyant R, Bragg SL, Spiegel RA, Tappero J, Perkins BA: Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. Vaccine 2004, 22:3435-3439.

71. Olsen SC, Stevens MG, Cheville NF, Schurig G: Experimental use of a dot-blot assay to measure serologic responses of cattle vaccinated with *Brucella abortus* strain RB51. Journal of Veterinary Diagnostic Investigation 1997, 9:363-367.

72. Nielsen K, Smith P, Yu W, Nicoletti P, Elzer P, Vigliocco A, Silva P, Bermudez R, Renteria T, Moreno F, et al: Enzyme immunoassay for the diagnosis of brucellosis: chimeric Protein A-Protein G as a common enzyme labeled detection reagent for sera for different animal species. Vet Microbiol 2004, 101:123-129.

73. Stevens MG, Olsen SC, Cheville NF: Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. Veterinary Immunology and Immunopathology 1995, 44:223-235.

74. Luna-Martinez JE, Mejia-Teran C: Brucellosis in Mexico: current status and trends. Vet Microbiol 2002, 90:19-30.

75. Dorneles EM, Teixeira-Carvalho A, Araujo MS, Lima GK, Martins-Filho OA, Sriranganathan N, Lage AP: T lymphocytes subsets and cytokine pattern induced by vaccination against bovine brucellosis employing S19 calfhood vaccination and adult RB51 revaccination. Vaccine 2014, 32:6034-6038.

76. He Y, Vemulapalli R, Zeytun A, Schurig GG: Induction of Specific Cytotoxic Lymphocytes in Mice Vaccinated with *Brucella abortus* RB51. Infection and Immunity 2001, 69:5502-5508.

77. McEwen AD, Samuel JMD: *Brucella abortus*: heat stable protective antigen revealed by adjuvant and present in a rough variant strain 45/20: immunization experiments on guinea pigs. Veterinary Record 1955, 67:546-548.

78. McEwen AD: Experiments on contagious abortion. The immunity of cattle inoculated with vaccines of graded virulence. Veterinary Record 1940:815.

79. Cunningham B: The effect of immaturity of the calf on immunological responses to strain 19 and killed 45/20 adjuvant vaccines. The Veterinary Record 1977, 101:283-285.

80. Woodard LF, Jasman RL: Comparative efficacy of an experimental S45/20 bacterin and a reduced dose of strain 19 vaccine against bovine brucellosis. American Journal of Veterinary Research 1983, 44:907-910.

81. Schurig GG, Hammerberg C, Finkler BR: Monoclonal antibodies to *Brucella* surface antigens associated with the smooth lipopolysaccharide complex. American Journal of Veterinary Research 1984, 45:967-971.

82. Ivanov AV, Salmakov KM, Olsen SC, Plumb GE: A live vaccine from *Brucella abortus* strain 82 for control of cattle brucellosis in the Russian Federation. Animal health research reviews / Conference of Research Workers in Animal Diseases 2011, 12:113-121.

83. Salmakov KM, Fomin AM, Plotnikova EM, Safina GM, Galimova GM, Salmakova AV, Ivanov AV, Panin AN, Sklyarov OD, Shumilov KV, Klimanov AI: Comparative study of the immunobiological properties of live brucellosis vaccines. Vaccine 2010, 28 Suppl 5:F35-40.

84. Li X, Xu J, Xie Y, Qiu Y, Fu S, Yuan X, Ke Y, Yu S, Du X, Cui M, et al: Vaccination with recombinant flagellar proteins FlgJ and FliN induce protection against *Brucella abortus* 544 infection in BALB/c mice. Vet Microbiol 2012, 161:137-144.

85. Fu S, Xu J, Li X, Xie Y, Qiu Y, Du X, Yu S, Bai Y, Chen Y, Wang T, et al: Immunization of mice with recombinant protein CobB or AsnC confers protection against *Brucella abortus* infection. PloS One 2012, 7:e29552.

86. Onate AA, Vemulapalli R, Andrews E, Schurig GG, Boyle S, Folch H: Vaccination with live Escherichia coli expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. Infection and Immunity 1999, 67:986-988.

87. Vemulapalli R, Cravero S, Calvert CL, Toth TE, Sriranganathan N, Boyle SM, Rossetti OL, Schurig GG: Characterization of Specific Immune Responses of Mice Inoculated with Recombinant Vaccinia Virus Expressing an 18-Kilodalton Outer Membrane Protein of *Brucella abortus*. Clinical and Vaccine Immunology 2000, 7:114-118.

88. Edmonds M, Booth N, Hagius S, Walker J, Enright F, Roop RM, 2nd, Elzer P: Attenuation and immunogenicity of a *Brucella abortus* htrA cycL double mutant in cattle. Vet Microbiol 2000, 76:81-90.

89. Zhang M, Han X, Liu H, Tian M, Ding C, Song J, Sun X, Liu Z, Yu S: Inactivation of the ABC transporter ATPase gene in *Brucella abortus* strain 2308 attenuated the virulence of the bacteria. Vet Microbiol 2013, 164:322-329.

90. Trant CG, Lacerda TL, Carvalho NB, Azevedo V, Rosinha GM, Salcedo SP, Gorvel JP, Oliveira SC: The Brucella abortus phosphoglycerate kinase mutant is highly attenuated and induces protection superior to that of vaccine strain 19 in immunocompromised and immunocompetent mice. Infection and Immunity 2010, 78:2283-2291.

91. Wang Z, Niu J, Wang S, Lv Y, Wu Q: In vivo differences in the virulence, pathogenicity, and induced protective immunity of wboA mutants from genetically different parent *Brucella* spp. Clinical and Vaccine Immunology: CVI 2013, 20:174-180.

92. Rajasekaran P, Seleem MN, Contreras A, Purwantini E, Schurig GG, Sriranganathan N, Boyle SM: *Brucella abortus* Strain RB51 Leucine Auxotroph as an Environmentally Safe Vaccine for Plasmid Maintenance and Antigen Overexpression. Applied and Environmental Microbiology 2008, 74:7051–7055.

93. Vemulapalli R, He Y, Boyle SM, Sriranganathan N, Schurig GG: *Brucella abortus* strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infection and Immunity 2000, 68:3290-3296.

94. Ramamoorthy S, Sanakkayala N, Vemulapalli R, Jain N, Lindsay DS, Schurig GS, Boyle SM, Sriranganathan N: Prevention of vertical transmission of *Neospora caninum* in C57BL/6 mice vaccinated with *Brucella abortus* strain RB51 expressing *N. caninum* protective antigens. International Journal for Parasitology 2007, 37:1531-1538.

95. Sabio y Garcia JV, Bigi F, Rossetti O, Campos E: Expression of MPB83 from Mycobacterium bovis in *Brucella abortus* S19 induces specific cellular immune response against the recombinant antigen in BALB/c mice. Microbes and infection / Institut Pasteur 2010, 12:1236-1243.

96. Sabio y Garcia JV, Farber M, Carrica M, Cravero S, Macedo GC, Bigi F, Oliveira SC, Rossetti O, Campos E: Expression of *Babesia bovis* rhoptry-associated protein 1 (RAP1) in *Brucella abortus* S19. Microbes and Infection 2008, 10:635-641.

97. Avila-Calderón E, Araiza-Villanueva M, Cancino-Diaz J, López-Villegas E, Sriranganathan N, Boyle S, Contreras-Rodríguez A: Roles of bacterial membrane vesicles. Arch Microbiol 2014:1-10.

98. Pollak CN, Delpino MV, Fossati CA, Baldi PC: Outer membrane vesicles from *Brucella abortus* promote bacterial internalization by human monocytes and modulate their innate immune response. PloS One 2012, 7:e50214.

99. Lamontagne J, Butler H, Chaves-Olarte E, Hunter J, Schirm M, Paquet C, Tian M, Kearney P, Hamaidi L, Chelsky D, et al: Extensive cell envelope modulation is associated with virulence in *Brucella abortus*. Journal of Proteome Research 2007, 6:1519-1529.

100. Jain-Gupta N, Contreras-Rodriguez A, Vemulapalli R, Witonsky SG, Boyle SM, Sriranganathan N: Pluronic P85 enhances the efficacy of outer membrane vesicles as a subunit vaccine against *Brucella melitensis* challenge in mice. FEMS Immunology and Medical Microbiology 2012, 66:436-444.

CHAPTER 2 - IMMUNE RESPONSE TRIGGERED BY *Brucella abortus* FOLLOWING INFECTION OR VACCINATION

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ABSTRACT

Brucella abortus live vaccines have been used successfully to control bovine brucellosis worldwide for decades. However, due to some limitations of these live vaccines, efforts are being made for the development of new safer and more effective vaccines that could also be used in other susceptible species. In this context, understanding the protective immune responses triggered by *B. abortus* is critical for the development of new vaccines. Such understandings will enhance our knowledge of the host/pathogen interactions and enable to develop methods to evaluate potential vaccines and innovative treatments for animals or humans. At present, almost all the knowledge regarding *B. abortus* specific immunological responses comes from studies in mice. Active participation of macrophages, dendritic cells, IFN-γ producing $CD4^+$ T-cells and cytotoxic $CD8^+$ T-cells are vital to overcome the infection. In this review, we discuss the characteristics of the immune responses triggered by vaccination versus infection by *B. abortus*, in different hosts.

Keywords: bovine brucellosis; brucellosis vaccines; immune response; RB51; S19.

1. INTRODUCTION

Brucellosis is one of the major zoonosis in public and animal health, worldwide. Infection by *Brucella* spp. leads important economic losses and affects numerous livestock, wildlife and humans [1-3]. In cattle, infection is predominantly due to *B. abortus* and usually causes placentitis followed by abortion in pregnant cows, epididymitis and orchitis in bulls [1].

Vaccination is one of the most effective measures to reduce the prevalence of bovine brucellosis and has largely contributed to the success of many control programs [4]. S19 and RB51 are the two *B. abortus* vaccines more broadly used in the control of brucellosis in cattle, being effective in the prevention of abortion and infection, besides offering long lasting protection [5-8]. However, due some issues presented by these current vaccines, such as be pathogenic for humans, cause abortion in pregnant cows and, for S19, induce antibodies that interfere with the serological tests employed in the diagnosis, great effort have been

made to find a better and safer brucellosis vaccine. Characterization of the immune profile associated with resistance to *B. abortus* infection is critical, since the advances in genomics, proteomics and recombinant DNA technology have allowed the exploration of new vaccines, more effective and safer [9-11].

However, at the present, most of our understanding about protective immune response against B. abortus infection/vaccination comes from studies using mouse model. In contrast, there is a limited amount of information concerning the immune mechanism by which the *B. abortus* vaccines confer protection in cattle. Therefore, in this review, we opt to broaden the discussion on the host/*B. abortus* interaction, including vaccination and infection in the natural host, cattle, or in animal models of infection, in order to understand which immunological mechanisms and events are stimulated by this pathogen.

2. INNATE IMMUNE RESPONSE

In case of brucellosis as well as in other diseases, the innate immune system will act as the first line of host defense, responsible for preventing replication, reducing the initial number and killing of the microorganism, besides creating conditions for the generation of an effective adaptive immune response [12]. This first line of defense include phagocytosis of pathogens by cells such as neutrophils, macrophages and dendritic cells (DC), death by natural killer (NK)-cells, secretion of cytokines and chemokines, recognition of molecules typical of a microbe [pathogen-associated molecular patterns (PAMPs)] by pattern-recognition receptors (PRRs), and activation of the complement system [12].

2.1. CELLS

Macrophages, DCs, along with NK-cells and neutrophils are the first cells to respond against infection [13]. Natural killer-cells are activated by *B. abortus* or their antigenic fractions [14] and are thought to be important in the activation of B-cells and consequently to antibody production [15]. However, even though NK-cells may be activated following infection, they seem to be not crucial in controlling brucellosis in mice, since its depletion in vivo does not affect the course of infection [14]. Likewise, it has been shown that human NK-cells did not express IFN-γ mRNA or secrete IFN-γ protein in response to *B. abortus* [16] and have a significantly depressed cyitotoxicity in patients with acute infection, suggesting that NK-cells are also not critical to immune response against *B. abortus* in humans [17].

Neutrophils are the most numerous and important short-lived phagocytes in innate immune response, but in case of *B. abortus* infection, after phagocytosis the neutrophils are not stimulated to induce an effective level of degranulation [18-20]. Studies have demonstrated that neutrophils appear not to play a significant role in the clearance of *B. abortus* from infected mice [18]. On the contrary, later in the infection of mice, during the chronic phase (after 15 days post-infection), *B. abortus* is killed more efficiently in the absence of neutrophils than in their presence [21]. It was suggested that neutrophils limit and regulate the activation of adaptive immune response against intracellular *B. abortus* infection, mainly throughout decreasing T lymphocytes activation [21]. In addition, a response consistent with an activation profile, increase in the expression of CD35, CD11b and IL-8 and, decrease of CD62, has been observed in of human neutrophils has beenand associated with pathogenesis of brucellosis, contributing to localized tissue injury and inflammation (Fig. 1) [22]. Also, human neutrophils have been implicated in potential mechanisms of tissue damage during liver brucellosis, since hepatic cell apoptosis was significantly enhanced by stimulation with supernatants from *Brucella*-infected neutrophils [23]. Therefore, activation of neutrophils seems not to be associated with protective immunity against *B. abortus*, but rather, it appears to be related to tissue damage and down regulation of adaptive immune response.

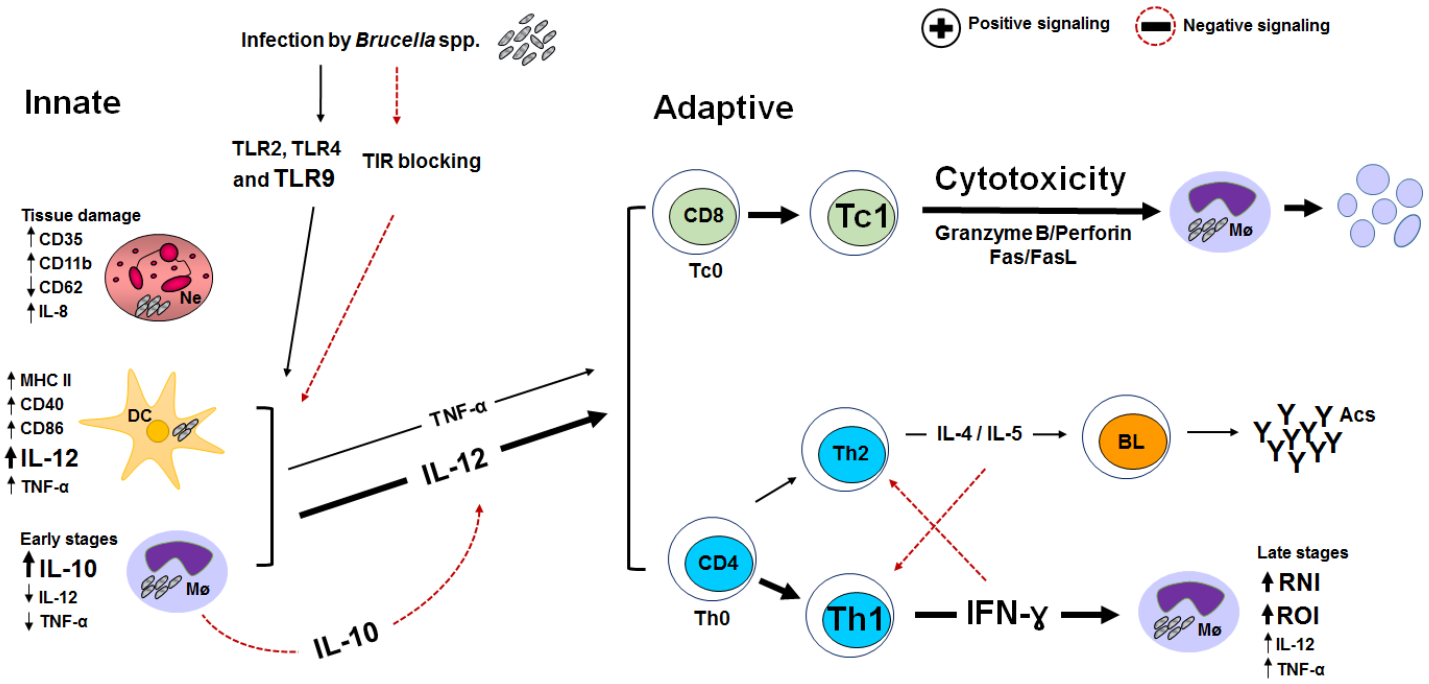

Figure 1 - The key mechanisms in different cells of the innate and adaptive immune system after B. abortus infection. The TLR2, TLR4 and TLR9 are the most important TLRs involved in the recognition of *B. abortus*. Signaling pathways activated by these TLRs mediated the secretion of IL-12 and TNF-α by macrophages (Mø) and mainly by dendritic cells (DCs), in early stages of infection. However, toll / interleukin-1 receptor (TIR) domain-containing proteins (Tcps) produced by *Brucella* spp. appear to be involved in the decrease of the TLR-dependent response to infection. Moreover, IL-10 Neutrophils (Ne) increase the expression of CD35, CD11b and IL-8, and decrease of CD62 after infection, a response consistent with neutrophil activation but that also leads to tissue damage. Macrophages in the earlier stages of infection allow the replication and survival of the *B. abortus*, mediated by the increase of IL-10 that down regulates production of proinflammatory cytokines (IFN-γ, IL-12 and TNF-α). Whereas in the later stages of infection, the bactericidal activity of activated macrophages are mainly due to reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs), which are induced by IFN-γ produced mainly by T helper type 1 (Th1) $CD4^+$ cells. The activation of DCs after *B. abortus* infection is characterized by IL-12 and TNF-α secretion, besides up-regulation in the expression of MHC class II (MHC II) and costimulatory molecules CD40, CD80 and CD86. The production of TNF-α and mainly IL-12 by macrophages and DCs stimulate $CD4^+$ and $CD8^+$ T-lymphocytes to secret IFN-γ and exert cytotoxic activities. Th1 cell response includes IFN-γ produced by $CD4^+$ T-cells. Th1 $CD4^+$ T-cells also increase the expression of CD28 and decrease the expression of CTLA-4, whereas the opposite is observed in T helper type 2 (Th2) $CD4^+$ T-cells. Th2 response is characterized mainly by secretion of IL-4 and IL-5 by $CD4^+$ T-cells, that stimulate an immune response mediated by antibody (Acs)-secreting B lymphocytes (BL) and is not very effective to overcome intracellular infections, in contrast it favors the chronic infection. The $CD8^+$ cytotoxic (Tc1) T-cells kill infected host cells by cytolytic activity. The font size indicates the importance of the mechanism to overcome the *B. abortus* infection.

In contrast to secondary involvement of neutrophils and NK-cells, macrophages plays a central role in *B. abortus* infection. In the earlier stages of infection in mice, before the development of an adaptive immune response by the host, macrophages allow the replication and survival of the microorganism, whereas in the later stages they are the main cells responsible for elimination of *B. abortus* [18, 24-26]. *B. abortus* enter in mouse macrophages, remodel their phagosomes and avoid the fusion of late endosomes and lysosomes, forming special phagosomes called *Brucella*-containing vacuoles (BCVs) at endoplasmic reticulum [27]. After internalized by macrophages, BCV interact with endoplasmic reticulum and stablish a replicative niche by mainly up-regulation of the virB type IV secretion system [28]. The mechanisms used by *B. abortus* to remodel the phagosome and successfully stablish a replicative compartment are promising targets to develop an attenuated mutant that could be explored as potential vaccine. Moreover, in these early stages of infection in mice, *B. abortus* induce the expression of low levels of proinflammatory cytokines and high levels of anti-inflammatory cytokines [18, 29-32]. All together, these mechanisms allow survival of *Brucella* in phagocyte cells. Once inside the mouse macrophages, *B. abortus* replicates extensively without inducing toxic effects to the cell and spreads throughout the host (lymph nodes, spleen, liver and bone marrow) via lymphatic and hematogenous [18, 33]. In later stages, after the establishment of antimicrobial mechanisms by adaptive immunity, activated macrophages are the primary source of *B. abortus* elimination in the infected mice [24, 26, 34]. The bactericidal activity of activated mouse macrophages are mainly due to reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs), which are induced by gamma interferon (IFN-γ) and tumor necrosis factor (TNF-α) and increased in the presence of iron (Fig. 1) [25, 35]. However, a small population of bacteria may still survive inside the macrophages, leading recurrence of the disease and chronic infection.

Dendritic cells form a key link between innate and adaptive immune systems. *B. abortus* down-modulates DC maturation in mice by interfering with the toll-like receptor 2 (TLR2) signaling pathway [36]. It has been shown that cattle DCs are resistant to *B. abortus* infection, in spite of exhibiting some signs of maturational and activation impairment and lack of up-regulation of co-stimulatory molecules and IL-12p40 after infection [37]. Mice and human DCs are susceptible to *B. abortus* infection [36, 38]. These differences in susceptibility of DCs among hosts may be related to the differences observed in the progression of the disease, since cattle have more ability in control the infection, showing less clinical signs compared to humans and some mice strains. However, rough *B. abortus* strains are able to induce higher phenotypic and functional maturation of human and murine DC cells, characterized by IL-12 and TNF-α secretion, and naive CD4 T-lymphocytes stimulation, compared to smooth strains [38-40]. Higher exposition of outer membrane proteins (Omp) in rough strains, compared to smooth strains, has been indicated as responsible by the stronger DC maturation in rough strains [39]. The maturation and activation of DCs along with cytokine secretion after *B. abortus* infection seem to be dependent of caspase-2 and TLR6 [38, 41]. Caspase-2 plays different roles in rough and smooth strains, being critical to mouse DC maturation and cytokine production in rough strains infection, whereas in infection by smooth strains promotes the cell death, favoring bacterial dissemination [38]. TLR6 is required by mouse DC to induce TNF-α and IL-12 [41]. Furthermore, rough *B. abortus* RB51-infected murine DCs show up-regulated expression of MHC class II and costimulatory molecules CD40, CD80 and CD86, suggesting that RB51 vaccine strain is capable of inducing significant innate immune response [38-40, 42].

2.2. CYTOKINES, CHEMOKINES AND PRRS / PAMPS

During bacterial infection the antimicrobial activity of macrophages is modulated by sequential production of cytokines, some of these secreted by the macrophages themselves (TNF-α and IL-12), and others produced by neighboring cells (IFN-γ). Tumor necrosis factor-α is one of the first cytokines released following *B. abortus* infection of macrophages, and its production results from direct interaction between *Brucella* and macrophages [31]. Experimental evidence shows that human macrophages activated by TNF-α inhibit the replication of *Brucella* spp. in vitro [31] and that TNF-α and IL-12 are directly involved in resistance to brucellosis in mice [43-45]. Furthermore, TNF-α release is increased by the phagocytosis of opsonized bacteria, indicating that the Fcγ receptor regulates the expression of TNF-α in a positive manner [31]. Nevertheless, it was observed that *B. abortus* actively prevents the release of TNF-α by human and mouse macrophages during infection, indicating that this is the basic mechanism of antibacterial activity of the host in the initial stage of infection [18, 30, 31]. The inhibition of TNF-α response in early stages of

infection in human and mouse macrophages, may be associated with the higher permissiveness of these cells to bacterial multiplication and, thereby with the ability of *B. abortus* to overcome the host innate immune response and establish infection. The low / lack of induction of TNF-α along with absence of host cell toxicity favors the establishment of a *Brucella* replication niche within the macrophage. Besides these mechanisms, *B. abortus* also appears to promote a reduction / modification of PAMPs expression that basically leads to hiding of the microorganism [18]. However, this low stimulation induced by *B. abortus* can be reversed by mutation of the gene wadC, whose disruption does not result in the loss of the O-side chain but results in an altered core, suggesting that LPS core acts as a shield against innate immune recognition [46].

Indeed, the recognition of PAMPs by pattern-recognition receptors (PRRs) is one of the first lines of host defense [47]. Toll-like receptors (TLRs), the earliest and better characterized PRRs [47], have been implicated in the resistance to *B. abortus* infection and in the induction of proinflammatory cytokines in mice [18, 42, 48-51]. Activation of PRRs by *B. abortus* PAMPs, as LPS, lipoproteins and DNA, leads the production of proinflammatory cytokines and is required for efficient clearance of the bacteria in mice [49]. However, *Brucella* efficiently induces parasitism and long lasting infection mainly due its reduced or absence of molecules with canonical PAMPs. In fact, *Brucella* does not have classical surface structures as capsules, fimbriae and pili and its LPS needs high concentrations to activate TLR4 [18]. Furthermore, toll / interleukin-1 receptor domain-containing proteins (Tcps) produced by *Brucella* spp. appear to be involved in the subversion of innate immune system, decreasing the TLR-dependent response to infection, thereby promoting intracellular bacterial survival and tissue pathology [52]. The most important TLRs involved in the recognition of *B. abortus* are TLR2, TLR4 and TLR9. Regarding resistance to *B. abortus* infection in mice, there is no consensus in the literature on the participation of TLR4, whereas TLR2 was reportedly to be not involved [18, 49, 53]. Even though possibly not involved in resistance to infection, it is well established that the recognition of *B. abortus* PAMPs by both TLR2 and TLR4 induce the secretion of TNF-α, IL-12 and IL-6 by murine cells [18, 42, 48-50, 53-55]. Interestingly, TLR4-linked signaling interacting with Janus kinase 2 (TLR4-JAK2) is also involved in *B. abortus* internalization by murine macrophages [56]. In contrast to TLR2 and TLR4, myeloid differentiation factor 88 (MyD88), an adapter molecule of all TLRs except TLR3, is critical for efficient clearance of *B. abortus* [53, 57], besides mediating induction of IL-12p40 and TNF-α in mice [50]. Likewise, TLR9 is necessary for the control of *B. abortus* infection in mice and partially responsible by the induction of IL-12p40 and TNF-α [42, 51, 57]. Susceptibility of MyD88 KO mice to *B. abortus* infection appears to be due to impaired DC maturation and lack of IL-12 synthesis [57].

Beyond cytokines, infected macrophages, DCs and other infected host cells also produce chemokines, chemoattractant peptides and proteins that induce directed chemotaxis in nearby responsive cells, which initiates an inflammatory response and have an important role in host defense. Heat-killed *B. abortus* (HKBA) or LPS stimulates high levels of MIP-1α and MIP-1β production in human monocytes and even higher levels in human macrophages [58]. However, it is not yet established what is the exact role of these chemokines in the control the infection, but it has been suggested that induction of chemokines could limit local bacterial spread. Monocytes infected by *B. abortus* are also capable of expressing several CXC (GRO-α, IL-8) and CC (MIP-1α, MIP-1β, MCP-1, RANTES) chemokines, however rough *Brucella* induces higher amounts than smooth *Brucella* [32]. Also, bovine placental cells experimentally infected or stimulated with HKBA showed up regulation in expression of CXCL6 (GCP-2) and CXCL8 (IL-8) genes [59].

Despite the response of countless components of the innate immune system to infection by *B. abortus*, it is known that they are not sufficient to overcome the infection and the development of an adaptive immune response is absolutely necessary to control the disease. *B. abortus* is able to evade host innate defenses by stealth strategy that ensures its replication in intracellular niche before activation of antimicrobial mechanisms of adaptive immunity [18].

3. ADAPTIVE CELL-MEDIATED IMMUNE RESPONSE

The desirable cell-mediated immune response against intracellular infections, such as brucellosis, is characterized by predominance of T helper type 1 (Th1) cell response that includes IFN-γ produced by T-

cells with αβ TCR receptor ($CD4^+$ or $CD8^+$), IgG2 antibody produced by B-cells and cytotoxic $CD8^+$ T-cells (cattle) [60]. On the other hand, T helper type 2 (Th2) response, characterized by secretion of substantial amounts of IL-4, IL-5 and IL-10 by $CD4^+$ T-cells, that stimulate an immune response mediated by antibody-secreting cells (IgG1 and IgE) and eosinophilia, is not effective to overcome intracellular infections [61].

3.1. CELLS

B. abortus antigens access both major histocompatibility complexes (MHC), I and II, for antigen presentation to $CD8^+$ and $CD4^+$ T lymphocytes, respectively [62-65]. The $CD4^+$ cells exert most of their helper functions by secreting cytokines, which act on modulating the response of other cells or producing autocrine action [61]. The IFN-γ is the principal cytokine produced by $CD4^+$ T- cells in brucellosis and has been shown to be crucial for resistance to infection in mice (Fig. 1) [14, 24, 25, 66-69]. Human $CD4^+$ and $CD8^+$ T-lymphocytes also produced IFN-γ in response to *B. abortus* stimulation [16, 58]. Recently, we showed that $CD4^+$ T-cells are also the main source of IFN- γ after *B. abortus* vaccination in cattle [70].

Infection by *B. abortus* promotes strong stimulus for Th1 response, which is able to suppress Th2 response against ovalbumin + alum in mice by inhibiting IL-4 production [71]. One of the mechanisms by which the microorganism directs the response of mice is by altering the expression of costimulatory molecules on T-cells, decreasing CD28 and increasing B7.2 [72]. Since B7.2 (CD86) evokes release of Th2 cytokines, CD28 provides a potent co-stimulatory signals and CTLA-4 (CD152) down regulates the immune response, the increase in B7.2 accompanied by the decrease of CD28 on T cells favors the interaction of B7.2 on antigen presenting cells with CTLA-4 on T cells, inhibiting the Th2 response [72].

Nonetheless, as well as demonstrated in innate immune response, *B. abortus* has mechanisms to impair the optimal establishment of an acquired immune response. Recently, it was showed that *B. abortus* negatively regulates Th1-mediated cell response induced in mice through the upregulation of lipid mediators leukotriene B4 and lipoxin A4 [73]. These lipids lead a decrease in the expression of proinflammatory cytokines, such as IFN-γ and IL-12, during the course of the infection [73].

Initially, the effective immunity against brucellosis was considered to be mediated exclusively by IFN-γ-secreting $CD4^+$ T-cells, however, today, the resistance to infection in mice is credited to coordinated action of two major subpopulations of T-cells ($CD4^+$ and $CD8^+$) (Fig. 1) [66, 74, 75]. Although the primary source of IFN-γ is $CD4^+$ T-cells, $CD8^+$ T-cells are also responsible for a fraction of IFN-γ produced in response to *B. abortus* infection in mice and cattle [63, 66, 70, 76]. In fact, passive transfer of $CD4^+$ and $CD8^+$ T-cells have proved to be equally protective against S19 infection in mice [74]. Also, experiment with $CD8^+$ and $CD4^+$ deficient mice showed that $CD8^+$ T-cells appears to be more crucial for the *B. abortus* infection control than the $CD4^+$ T-cells, given that MHC I knockout mice are much more susceptible to brucellosis, whereas MHC II knockout mice can eliminate the infection [63]. These results can be explained by taking into account that in the absence of $CD4^+$, $CD8^+$ T-lymphocytes increase the production of IFN-γ in a compensatory mechanism [63].

Confirming that both subsets have an important role in IFN-γ production, it has been shown that the depletion of $CD4^+$ or $CD8^+$ T-lymphocytes obtained from mice in culture results in significantly decrease in total IFN-γ production [66]. Nevertheless, the most important effector function of *B. abortus* antigen-specific $CD8^+$ T-cells is the killing of infected host cells. The $CD8^+$ cytotoxic T-cells kill infected host cells by cytolytic activity mediated by perforin/granzyme or by Fas-Fas ligand interaction [60]. Besides $CD8^+$ T-cells, murine $CD4^+$ T-cells has also demonstrated cytotoxic potential after in vivo *B. abortus* infection [77]. This population expressed high levels of granzyme B and IFN-γ and also exhibited specific cytolytic capacity against infected murine macrophages [77]. The $CD8^+$ lymphocytes are critical for the resistance to brucellosis in mice [63] and, coupled with $CD4^+$ T-cells, induce the death of infected cells [78]. Taking together, all these results indicate that acquired immunity to *B. abortus* infection is due to cooperative action of both $CD4^+$ and $CD8^+$ T cell subsets, mainly mediated by two effector functions: IFN-γ secretion and cytotoxicity (Fig. 1).

Other T-cell subset, bovine γδ T-lymphocytes respond rapidly to *B. abortus* infection upon co-culture with autologous macrophages via IFN-γ, even though it does not play a central role in protection in mice [79]. Mice deficient in γδ T-cells have impaired innate immunity to *B. abortus*, suggesting that the protective function of γδ T-cells may be limited to innate immunity [79]. In early stages of infection, murine γδ T-cells upregulate IL-8, MIP-1α (CCL3), GM-CSF, IL-1β, IL-17, and CD25 mRNAs expression. While at later time points, the same cells have an enhanced expression of granzyme B, RANTES, and IFN-γ mRNAs [79]. Therefore, it is presumable that δγ T-cells have an important role as effector cells and mediators in innate immunity following *B. abortus* infection, but its participation in acquired immunity is still unknown.

3.2. CYTOKINES

The crucial role of IFN-γ was recognized once it was shown that IFN-γ knockout mice died due to brucellosis [68] and IFN- γ producing $CD4^+$ T-cells from infected donor was able to protect the recipient mice against challenge with *B. abortus* [69]. In fact, experiments with knockout mice showed that IFN-γ deficiency is more crucial than the deficiency of $CD8^+$ T-cells or $CD8^+$ T-cells and IL-12 against *B. abortus* infection, despite IL-12 and CD8+ T-cells have been shown to be significant in the host immune response to brucellosis [68]. Moreover, various studies show that blocking by anti-IFN-γ antibodies or administration of exogenous IFN-γ result in exacerbation or greater control of infection in mice, respectively [14, 24]. Therefore, based on experiments in knockout mice it seems that IFN-γ comes first, followed by CD8+ T-cells and then by $CD4^+$ T-cells in the immune response generated by the host to overcome *B. abortus* infection. Although contradictory, the last place of $CD4^+$ T-cells in this ranking should be understood considering that in the absence of $CD4^+$ T-cells, $CD8^+$ T-cells sufficiently assume the IFN-γ production, besides exercise their cytotoxic activity.

IFN-γ confers protection against brucellosis in mice through the activation of cells of the innate immune system such as macrophages to become more efficient in killing and inhibiting the replication of intracellular pathogens (Fig. 1) [80]. This mechanism is particularly important if you consider that the establishment of chronic disease is related to the survival of bacteria inside macrophages. The bactericidal activity of macrophages and the secretion of IFN-γ by T-cells have been shown to be in mice primarily dependent on IL-12, but not TNF-α produced by macrophages [44, 45, 68, 81]. Depletion or absence (knockout mice) of IL-12 prior to *B. abortus* infection in mice leads a decrease in the IFN-γ and nitric oxide (NO) production and consequently an exacerbation of infection [45, 68, 81]. Although not directly related to IFN-γ, TNF-α depletion is closely related to decreased secretion of IL-12 in infected mice [44, 45]. These results suggest that IL-12 contributes to brucellosis resistance in mice mainly via an IFN-γ-dependent pathway, whereas TNF-α acts possibly via direct action on effector cells, participating only in innate response [44, 45, 81]. In other words, *B. abortus* induces the secretion of IL-12 by macrophages and therefore direct the immune response, by differentiation of Th0 naive cells into Th1 effector and memory cells. Additionally, IL-2 has also shown to be induced in infected cell and being related to brucellosis resistance, probably because of its essential role of promoting T-cell proliferation and differentiation into effector cells [25, 66, 69].

As expected, IL-4 seems to be not produced in response to *B. abortus* infection. IL-4-producing $CD4^+$ T-cells from infected mice fail to protect donors against infection [67, 69]. On the other hand, IL-10, an anti-inflammatory cytokine, is secreted in response to *B. abortus* infection in mice and is responsible for the down regulation of macrophage effector function and IFN-γ production [14, 29, 63, 67]. IL-10 produced by macrophages and mainly by $CD4^+$ T-cells in early stages of *B. abortus* infection in mice seems to support the intracellular replication of the bacteria, enhancing persistent infection due to down regulation of proinflammatory cytokines [29]. This complex balance between proinflammatory and anti-inflammatory cytokines should be comprehended considering the intricate interaction between the host and the pathogen.

4. ADAPTIVE HUMORAL IMMUNE RESPONSE

Regarding brucellosis, the exact contribution of humoral immunity in resistance is not quite established. The LPS O-side chain (OPS) appears to be the immunodominant antigen of smooth *B. abortus* strains, since greater proportion of the antibody response in human and animal infections, as well as after immunizations with smooth vaccines, is directed against OPS antigen [82]. Usually molecules like LPS activate B-cells in

a T-independent manner. However, *B. abortus* LPS is capable of binding to MHC class II molecules in B-lymphocytes, suggesting that they are eventually presented to T cells [64, 65]. Therefore, it is tempting to speculate that the strong humoral response to the OPS, which causes the main problems related to the serological diagnosis of bovine brucellosis, can be related to the participation of T helper cells in B-cell response against this antigen. However, it is important to take into account that the crucial role in the overcoming the *B. abortus* infection is played by cellular immunity, exercising the humoral immunity probably a secondary role, as well as it is observed in other intracellular infections. Moreover, considering that rough strains have showed higher induction of immune response and pro-inflammatory cytokine production than smooth strains [32, 38, 39, 42], the future targets for the development of new vaccines against brucellosis should be focused on attenuated strains devoid in OPS. The greater ability of rough strains of *B. abortus* in eliciting a host response appears to be related their low capacity to subvert host defenses. Furthermore, the diagnosis interference observed in smooth vaccines is another point to be balanced.

In mice, it has been broadly established that the OPS antibodies are related to some level of protection against *B. abortus* infection [83-85]. Passive transfer of anti-OPS monoclonal antibodies into mice results in a significant reduction in the number of viable bacteria recovered from the spleen and liver, following challenge with virulent *B. abortus* wild type [84, 85]. Likewise, antibodies towards lipopolysaccharide-A epitope (LPS-A), outer membrane proteins (OMPs) and polysaccharide surface antigens also reduced bacterial splenic counts post-challenge in recipient mice [85, 86]. Moreover, passive transfer of total serum from S19-vaccinated or 2308-infected, but not from RB51-vaccinated mice has been shown protection [74, 75, 83]. All these data confirm that OPS antibodies may have a role in host defense.

On the other hand, humoral immunity components may also contribute to the establishment of chronic infection. A recent study demonstrated that IgM and complement-opsonized *B. abortus* infect murine B-cells and establish an intracellular niche [87]. It has been suggested that intracellular compartment containing *B. abortus* inside murine B-lymphocytes is maintained by the capacity of the cell to produce transforming growth factor (TGF) β1 in vivo, a regulatory cytokine [87]. After entry under opsonizing conditions, the bacteria induce the activation of B-cells and thereby cannot replicate, but promotes a niche for chronic infection.

The data point to a possible participation of adaptive humoral immunity in resistance to infection at least in mice, especially against smooth strains. However, antibody response triggered by *B. abortus* seems to be secondary in the resolution of the infection and even may contribute for the establishment of chronic infection. Draw hypothesis on involvement of humoral immune response in other *B. abortus* hosts, such as humans and cattle, is very difficult. This kind of speculation deserves experimentally supported.

5. BRUCELLOSIS VACCINATION

Vaccination against brucellosis in cattle has been performed for several decades, employing mainly two successful attenuated strains, S19 and RB51. However, even today, the mechanisms involved in the protection conferred by theses vaccine strains are not completely understood. In mice, it was demonstrated that RB51 vaccine induces a strong Th1 cellular immune response with production of IFN-γ and $CD8^+$ specific cytotoxic cells, but not IL-4 [67, 88-91]. RB51 vaccinated mice challenged with 2308 also produce high levels of IL-10, probably to avoid an excessive proinflammatory response, more than offset the production of Th1 cytokines [67]. Moreover, RB51-vaccinated mice exhibit strong cytolytic response with cytotoxic activity mainly exerted by $CD8^+$ T-cells but not NK-cells, whereas $CD4^+$ T-cells are mainly responsible for the secretion of high levels of IFN-γ and exhibit some level of lytic activity [88, 91].

T helper type 17 (Th17) subset cells, characterized by producing a signature of cytokines, IL-17A, IL-17F and IL-22 [92], were observed to have a protective role in oral RB51 and recombinant unlipidated Omp19 mice vaccination, although IL-17Rα-/- mice appears to be not impaired in their ability to control *B. abortus* infection [79, 93]. These results suggest that Th17 cells may act synergistically with Th1 cells to achieve protection due to vaccination, mainly mucosal immunity, since their cytokines (IL-17 and IL-22) has been

detected after oral RB51 vaccination and nasal challenge in mice and IFN-γ knockout mice produces higher levels of IL-17 after RB51 oral vaccination [93].

Regarding S19, as expected, a strong Th1 immune response was also observed after vaccination in mice, with production of IL-2, TNF-α and IFN-γ, and high levels of antigen-specific $CD4^+$ and granzyme B-secreting $CD8^+$ T-cells, but no IL-4 or IL-10 secretion by murine cells [69, 94, 95].

For cattle, there is limited information about the immune mechanism by which the *B. abortus* vaccines confer protection. Subpopulations of T and B-cells or signature cytokine profile induced by S19 or RB51 vaccination are not fully known in their target species. The lymphocyte response after vaccination or infection has been extensively evaluated in cattle only by proliferation [96-99]. Lymph node cells of S19 or RB51 vaccinated cattle, as well as murine cells, exhibit a significant proliferation rate compared to unvaccinated animals, upon in vitro stimulation with protein fractions or γ-irradiated 2308 [97, 101, 102]. In cattle, there are already some evidences that specific cell mediate immune components are stimulated after S19 or RB51 vaccination, as IFN-γ production and increases in $CD4^+$ or $CD8^+$ T-cells [70, 103, 104].

Concerning humoral immune response, after vaccination with S19 cattle produced high titers of IgG1, IgG2 and IgM, and low concentration of IgA [105]. Whereas, S19-vaccinated mice developed substantial levels of anti-O-antigen-specific IgG1, IgG2b and IgM [106]. RB51-vaccinated mice have a predominance of antigen-specific IgG2a that are associated with Th1 immune response, effective against *B. abortus* infection [88, 90, 107]. Immunoglobulin-G2 is particularly effective in opsonization and subsequent phagocytosis of the organism due the ability of the antibody Fc region to bind to the Fc receptor of phagocytes [60].

6. FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

Most of the current knowledge on the protective immune response against *B. abortus* comes from studies carried in mice. These efforts have point that the resistance to *B. abortus* infection in mice is mainly due to secretion of IL-12 and TNF-α by macrophages and DCs via TLR2, TLR4 and TLR9 signaling pathways, in early stages, and by coordinated action of IFN-γ-producing $CD4^+$ T-cells and cytotoxic $CD8^+$ T-cells, in later stages of infection, being IFN-γ the central point. A lack of broadly studies in *B. abortus* natural hosts, such as humans and cattle, preclude a complete understanding about what would be the optimal immune response to overcome *B. abortus* infection in theses hosts. However, protection given by *B. abortus* vaccines appears to be related to a strong Th1 immune response, characterized mainly by secretion of high levels of IFN-γ by $CD4^+$ T-cells, and cytotoxic activity, mainly exerted by $CD8^+$ T-cells. Moreover, Th17 cells may act synergistically with Th1 cells to achieve protection after brucellosis vaccination in mice. The rational way of looking for a new brucellosis vaccine, safer and more efficient, should be focused in the understanding which mechanisms are used by the widely and successful used *B. abortus* vaccines to confer protection in cattle. Based on what we know so far, the search for a new vaccine should direct to reach a live attenuated rough vaccine strain able to elicit a complex immune response, chiefly characterized by a strong activation of innate response – production of high levels IL-12 and TNF-α – followed by a Th1 predominant profile with high amount of IFN-γ and $CD8^+$ cytotoxic cells, but also with fine balance of anti-inflammatory cytokines.

7. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

8. ACKNOWLEDGEMENTS

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9. REFERENCES

[1] Corbel MJE, S.S.; Cosivi O. Brucellosis in humans and animals. Geneva: World Health Organization; 2006.

[2] Bernues A, Manrique E, Maza MT. Economic evaluation of bovine brucellosis and tuberculosis eradication programmes in a mountain area of Spain. Preventive Veterinary Medicine. 1997;30:137-49.

[3] OIE. Bovine Brucellosis. Manual of diagnostic tests and vaccines for terrestrial animals: World Organisation for Animal Health; 2009.

[4] Olsen SC, Stoffregen WS. Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert Review of Vaccines. 2005;4:915-28.

[5] McDiarmid A. The Degree and Duration of Immunity in Cattle Resulting from Vaccination with S. 19 Br. abortus Vaccine and its Implication in the Future Control and Eventual Eradication of Brucellosis. Veternary Record. 1957;69:877-9.

[6] Manthei CA. Summary of controlled research with strain 19. Proceedings 63rd Ann Meet US Livestock Sanitary Association 1959. p. 91-7.

[7] Cheville NF, Stevens MG, Jensen AE, Tatum FM, Halling SM. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. American Journal of Veterinary Research. 1993;54:1591-7.

[8] Poester FP, Goncalves VS, Paixao TA, Santos RL, Olsen SC, Schurig GG, et al. Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. Vaccine. 2006;24:5327-34.

[9] Onate AA, Cespedes S, Cabrera A, Rivers R, Gonzalez A, Munoz C, et al. A DNA Vaccine Encoding Cu,Zn Superoxide Dismutase of *Brucella abortus* Induces Protective Immunity in BALB/c Mice. Infection and Immunity. 2003;71:4857-61.

[10] Vemulapalli R, He Y, Sriranganathan N, Boyle SM, Schurig GG. *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. Vet Microbiol. 2002;90:521-32.

[11] Li X, Xu J, Xie Y, Qiu Y, Fu S, Yuan X, et al. Vaccination with recombinant flagellar proteins FlgJ and FliN induce protection against *Brucella abortus* 544 infection in BALB/c mice. Vet Microbiol. 2012;161:137-44.

[12] Diacovich L, Gorvel JP. Bacterial manipulation of innate immunity to promote infection. Nature Reviews Microbiology. 2010;8:117-28.

[13] Golding B, Scott DE, Scharf O, Huang LY, Zaitseva M, Lapham C, et al. Immunity and protection against *Brucella abortus*. Microbes and Infection. 2001;3:43-8.

[14] Fernandes DM, Benson R, Baldwin CL. Lack of a role for natural killer cells in early control of *Brucella abortus* 2308 infections in mice. Infection and Immunity. 1995;63:4029-33.

[15] Gao N, Jennings P, Guo Y, Yuan D. Regulatory role of natural killer (NK) cells on antibody responses to *Brucella abortus*. Innate Immunity. 2011;17:152-63.

[16] Zaitseva MB, Golding H, Betts M, Yamauchi A, Bloom ET, Butler LE, et al. Human peripheral blood CD4+ and CD8+ T cells express Th1-like cytokine mRNA and proteins following in vitro stimulation with heat-inactivated *Brucella abortus*. Infection and Immunity. 1995;63:2720-8.

[17] Salmeron I, Rodriguez-Zapata M, Salmeron O, Manzano L, Vaquer S, Alvarez-Mon M. Impaired activity of natural killer cells in patients with acute brucellosis. Clinical Infectious Diseases 1992;15:764-70.

[18] Barquero-Calvo E, Chaves-Olarte E, Weiss DS, Guzman-Verri C, Chacon-Diaz C, Rucavado A, et al. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. PloS One. 2007;2:e631.

[19] Kreutzer DL, Dreyfus LA, Robertson DC. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infection and Immunity. 1979;23:737-42.

[20] Canning PC, Roth JA, Tabatabai LB, Deyoe BL. Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. The Journal of Infectious Diseases. 1985;152:913-21.

[21] Barquero-Calvo E, Martirosyan A, Ordonez-Rueda D, Arce-Gorvel V, Alfaro-Alarcon A, Lepidi H, et al. Neutrophils exert a suppressive effect on Th1 responses to intracellular pathogen *Brucella abortus*. PLoS Pathogens. 2013;9:e1003167.

[22] Zwerdling A, Delpino MV, Pasquevich KA, Barrionuevo P, Cassataro J, Garcia Samartino C, et al. *Brucella abortus* activates human neutrophils. Microbes and Infection. 2009;11:689-97.

[23] Delpino MV, Barrionuevo P, Scian R, Fossati CA, Baldi PC. *Brucella*-infected hepatocytes mediate potentially tissue-damaging immune responses. Journal of Hepatology. 2010;53:145-54.

[24] Zhan Y, Cheers C. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. Infection and Immunity. 1993;61:4899-901.

[25] Jiang X, Baldwin CL. Effects of cytokines on intracellular growth of *Brucella abortus*. Infection and Immunity. 1993;61:124-34.

[26] Jiang X, Leonard B, Benson R, Baldwin CL. Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. Cellular Immunology. 1993;151:309-19.

[27] Pizarro-Cerda J, Moreno E, Sanguedolce V, Mege JL, Gorvel JP. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. Infection and Immunity. 1998;66:2387-92.

[28] Comerci DJ, Martinez-Lorenzo MJ, Sieira R, Gorvel JP, Ugalde RA. Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. Cellular Microbiology. 2001;3:159-68.

[29] Xavier MN, Winter MG, Spees AM, Nguyen K, Atluri VL, Silva TMA, et al. CD4(+) T Cell-derived IL-10 Promotes *Brucella abortus* Persistence via Modulation of Macrophage Function. PLoS Pathogens. 2013;9.

[30] Caron E, Gross A, Liautard JP, Dornand J. *Brucella* species release a specific, protease-sensitive, inhibitor of TNF-alpha expression, active on human macrophage-like cells. Journal of Immunology. 1996;156:2885-93.

[31] Caron E, Peyrard T, Kohler S, Cabane S, Liautard JP, Dornand J. Live *Brucella* spp. fail to induce tumor necrosis factor alpha excretion upon infection of U937-derived phagocytes. Infection and Immunity. 1994;62:5267-74.

[32] Rittig MG, Kaufmann A, Robins A, Shaw B, Sprenger H, Gemsa D, et al. Smooth and rough lipopolysaccharide phenotypes of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. Journal of Leukocyte Biology. 2003;74:1045-55.

[33] Jimenez de Bagues MP, Dudal S, Dornand J, Gross A. Cellular bioterrorism: how *Brucella* corrupts macrophage physiology to promote invasion and proliferation. Clinical Immunology. 2005;114:227-38.

[34] Splitter GA, Everlith KM. Collaboration of bovine T lymphocytes and macrophages in T-lymphocyte response to *Brucella abortus*. Infection and Immunity. 1986;51:776-83.

[35] Baldwin CL, Jiang X, Fernandes DM. Macrophage control of *Brucella abortus*: influence of cytokines and iron. Trends in Microbiology. 1993;1:99-104.

[36] Salcedo SP, Marchesini MI, Lelouard H, Fugier E, Jolly G, Balor S, et al. Brucella control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. PLoS Pathogens. 2008;4:e21.

[37] Heller MC, Watson JL, Blanchard MT, Jackson KA, Stott JL, Tsolis RM. Characterization of *Brucella abortus* infection of bovine monocyte-derived dendritic cells. Veterinary Immunology and Immunopathology. 2012;149:255-61.

[38] Li X, He Y. Caspase-2-dependent dendritic cell death, maturation, and priming of T cells in response to *Brucella abortus* infection. PloS One. 2012;7:e43512.

[39] Surendran N, Hiltbold EM, Heid B, Sriranganathan N, Boyle SM, Zimmerman KL, et al. Live *Brucella abortus* rough vaccine strain RB51 stimulates enhanced innate immune response in vitro compared to rough vaccine strain RB51SOD and virulent smooth strain 2308 in murine bone marrow-derived dendritic cells. Vet Microbiol. 2011;147:75-82.

[40] Surendran N, Hiltbold EM, Heid B, Sriranganathan N, Boyle SM, Zimmerman KL, et al. Heat-killed and gamma-irradiated *Brucella* strain RB51 stimulates enhanced dendritic cell activation, but not function compared with the virulent smooth strain 2308. FEMS Immunology and Medical Microbiology. 2010;60:147-55.

[41] de Almeida LA, Macedo GC, Marinho FA, Gomes MT, Corsetti PP, Silva AM, et al. Toll-like receptor 6 plays an important role in host innate resistance to *Brucella abortus* infection in mice. Infection and Immunity. 2013;81:1654-62.

[42] Surendran N, Hiltbold EM, Heid B, Akira S, Standiford TJ, Sriranganathan N, et al. Role of TLRs in Brucella mediated murine DC activation in vitro and clearance of pulmonary infection in vivo. Vaccine. 2012;30:1502-12.

[43] Zhan Y, Cheers C. Differential induction of macrophage-derived cytokines by live and dead intracellular bacteria in vitro. Infection and Immunity. 1995;63:720-3.

[44] Zhan Y, Cheers C. Control of IL-12 and IFN-gamma production in response to live or dead bacteria by TNF and other factors. Journal of Immunology. 1998;161:1447-53.

[45] Zhan Y, Liu Z, Cheers C. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. Infection and Immunity. 1996;64:2782-6.

[46] Conde-Alvarez R, Arce-Gorvel V, Iriarte M, Mancek-Keber M, Barquero-Calvo E, Palacios-Chaves L, et al. The lipopolysaccharide core of *Brucella abortus* acts as a shield against innate immunity recognition. PLoS Pathogens. 2012;8:e1002675.

[47] Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34:637-50.

[48] Giambartolomei GH, Zwerdling A, Cassataro J, Bruno L, Fossati CA, Philipp MT. Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*. Journal of Immunology. 2004;173:4635-42.

[49] Campos MA, Rosinha GM, Almeida IC, Salgueiro XS, Jarvis BW, Splitter GA, et al. Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. Infection and Immunity. 2004;72:176-86.

[50] Huang LY, Aliberti J, Leifer CA, Segal DM, Sher A, Golenbock DT, et al. Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is Toll-like receptor 2 dependent. Journal of Immunology. 2003;171:1441-6.

[51] Huang LY, Ishii KJ, Akira S, Aliberti J, Golding B. Th1-like cytokine induction by heat-killed Brucella abortus is dependent on triggering of TLR9. Journal of Immunology. 2005;175:3964-70.

[52] Cirl C, Wieser A, Yadav M, Duerr S, Schubert S, Fischer H, et al. Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. Nature Medicine. 2008;14:399-406.

[53] Weiss DS, Takeda K, Akira S, Zychlinsky A, Moreno E. MyD88, but not toll-like receptors 4 and 2, is required for efficient clearance of *Brucella abortus*. Infection and Immunity. 2005;73:5137-43.

[54] Barrionuevo P, Cassataro J, Delpino MV, Zwerdling A, Pasquevich KA, Garcia Samartino C, et al. *Brucella abortus* inhibits major histocompatibility complex class II expression and antigen processing through interleukin-6 secretion via Toll-like receptor 2. Infection and Immunity. 2008;76:250-62.

[55] Ferrero MC, Hielpos MS, Carvalho NB, Barrionuevo P, Corsetti PP, Giambartolomei GH, et al. Key role of Toll-like receptor 2 in the inflammatory response and major histocompatibility complex class ii downregulation in *Brucella abortus*-infected alveolar macrophages. Infection and Immunity. 2014;82:626-39.

[56] Lee JJ, Kim DH, Kim DG, Lee HJ, Min W, Rhee MH, et al. Toll-like receptor 4-linked Janus kinase 2 signaling contributes to internalization of *Brucella abortus* by macrophages. Infection and Immunity. 2013;81:2448-58.

[57] Macedo GC, Magnani DM, Carvalho NB, Bruna-Romero O, Gazzinelli RT, Oliveira SC. Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. Journal of Immunology. 2008;180:1080-7.

[58] Zaitseva M, King LR, Manischewitz J, Dougan M, Stevan L, Golding H, et al. Human peripheral blood T cells, monocytes, and macrophages secrete macrophage inflammatory proteins 1alpha and 1beta following stimulation with heat-inactivated *Brucella abortus*. Infection and Immunity. 2001;69:3817-26.

[59] Carvalho Neta AV, Stynen AP, Paixao TA, Miranda KL, Silva FL, Roux CM, et al. Modulation of the bovine trophoblastic innate immune response by *Brucella abortus*. Infection and Immunity. 2008;76:1897-907.

[60] Baldwin CL, Goenka R. Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection? Critical Reviews in Immunology. 2006;26:407-42.

[61] O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science. 2010;327:1098-102.

[62] Splitter GA, Everlith KM. *Brucella abortus* regulates bovine macrophage-T-cell interaction by major histocompatibility complex class II and interleukin-1 expression. Infection and Immunity. 1989;57:1151-7.

[63] Oliveira SC, Splitter GA. CD8+ type 1 CD44hi CD45 RBlo T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice. European Journal of Immunology. 1995;25:2551-7.

[64] Forestier C, Moreno E, Méresse S, Phalipon A, Olive D, Sansonetti P, et al. Interaction *of Brucella abortus* Lipopolysaccharide with Major Histocompatibility Complex Class II Molecules in B Lymphocytes. Infection and Immunity. 1999;67:4048-54.

[65] Escola JM, Moreno E, Chavrier P, Gorvel JP. The O-chain of *Brucella abortus* lipopolysaccharide induces SDS-resistant MHC class II molecules in mouse B cells. Biochemical and biophysical research communications. 1994;203:1230-6.

[66] Zhan Y, Yang J, Cheers C. Cytokine response of T-cell subsets from *Brucella abortus*-infected mice to soluble *Brucella* proteins. Infection and Immunity. 1993;61:2841-7.

[67] Pasquali P, Adone R, Gasbarre LC, Pistoia C, Ciuchini F. Mouse cytokine profiles associated with *Brucella abortus* RB51 vaccination or *B. abortus* 2308 infection. Infection and Immunity. 2001;69:6541-4.

[68] Brandao AP, Oliveira FS, Carvalho NB, Vieira LQ, Azevedo V, Macedo GC, et al. Host susceptibility to *Brucella abortus* infection is more pronounced in IFN-gamma knockout than IL-12/beta2-microglobulin double-deficient mice. Clinical & Developmental Immunology. 2012;2012:589494.

[69] Zhan Y, Kelso A, Cheers C. Differential activation of *Brucella*-reactive CD4+ T cells by *Brucella* infection or immunization with antigenic extracts. Infection and Immunity. 1995;63:969-75.

[70] Dorneles EM, Teixeira-Carvalho A, Araujo MS, Lima GK, Martins-Filho OA, Sriranganathan N, et al. T lymphocytes subsets and cytokine pattern induced by vaccination against bovine brucellosis employing S19 calfhood vaccination and adult RB51 revaccination. Vaccine. 2014;32:6034-8.

[71] Scott DE, Agranovich I, Inman J, Gober M, Golding B. Inhibition of primary and recall allergen-specific T helper cell type 2-mediated responses by a T helper cell type 1 stimulus. Journal of Immunology. 1997;159:107-16.

[72] Agranovich I, Scott DE, Terle D, Lee K, Golding B. Down-regulation of Th2 responses by Brucella abortus, a strong Th1 stimulus, correlates with alterations in the B7.2-CD28 pathway. Infection and Immunity. 1999;67:4418-26.

[73] Fahel JS, de Souza MB, Gomes MT, Corsetti PP, Carvalho NB, Marinho FA, et al. 5-Lipoxygenase negatively regulates Th1 response during *Brucella abortus* infection in mice. Infection and Immunity. 2015;83:1210-6.

[74] Araya LN, Elzer PH, Rowe GE, Enright FM, Winter AJ. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. Journal of Immunology. 1989;143:3330-7.

[75] Araya LN, Winter AJ. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infection and Immunity. 1990;58:254-6.

[76] Weynants V, Walravens K, Didembourg C, Flanagan P, Godfroid J, Letesson JJ. Quantitative assessment by flow cytometry of T-lymphocytes producing antigen-specific gamma-interferon in *Brucella* immune cattle. Veterinary Immunology and Immunopathology. 1998;66:309-20.

[77] Martirosyan A, Von Bargen K, Arce Gorvel V, Zhao W, Hanniffy S, Bonnardel J, et al. In Vivo Identification and Characterization of CD4+ Cytotoxic T Cells Induced by Virulent *Brucella abortus* Infection. PloS One. 2013;8:e82508.

[78] Wyckoff JH, 3rd, Potts RD. Killing of *Brucella* antigen-sensitized macrophages by T lymphocytes in bovine brucellosis. Veterinary Immunology and Immunopathology. 2007;120:148-59.

[79] Skyberg JA, Thornburg T, Rollins M, Huarte E, Jutila MA, Pascual DW. Murine and bovine gammadelta T cells enhance innate immunity against *Brucella abortus* infections. PloS one. 2011;6:e21978.

[80] Jiang X, Baldwin CL. Iron augments macrophage-mediated killing of *Brucella abortus* alone and in conjunction with interferon-gamma. Cellular Immunology. 1993;148:397-407.

[81] Zhan Y, Cheers C. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. Infection and Immunity. 1995;63:1387-90.

[82] Diaz R, Jones LM, Leong D, Wilson JB. Surface antigens of smooth brucellae. Journal of Bacteriology. 1968;96:893-901.

[83] Jimenez de Bagues MP, Elzer PH, Jones SM, Blasco JM, Enright FM, Schurig GG, et al. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infection and Immunity. 1994;62:4990-6.

[84] Montaraz JA, Winter AJ, Hunter DM, Sowa BA, Wu AM, Adams LG. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infection and Immunity. 1986;51:961-3.

[85] Cloeckaert A, Jacques I, Bosseray N, Limet JN, Bowden R, Dubray G, et al. Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of *Brucella*. J Med Microbiol. 1991;34:175-80.

[86] Plommet M, Plommet AM. Immune serum-mediated effects on brucellosis evolution in mice. Infection and immunity. 1983;41:97-105.

[87] Goenka R, Guirnalda PD, Black SJ, Baldwin CL. B Lymphocytes provide an infection niche for intracellular bacterium *Brucella abortus*. The Journal of Infectious Diseases. 2012;206:91-8.

[88] He Y, Vemulapalli R, Zeytun A, Schurig GG. Induction of Specific Cytotoxic Lymphocytes in Mice Vaccinated with *Brucella abortus* RB51. Infection and Immunity. 2001;69:5502-8.

[89] Vemulapalli R, He Y, Boyle SM, Sriranganathan N, Schurig GG. *Brucella abortus* strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infection and Immunity. 2000;68:3290-6.

[90] Vemulapalli R, He Y, Cravero S, Sriranganathan N, Boyle SM, Schurig GG. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infection and Immunity. 2000;68:3286-9.

[91] Andrews E, Salgado P, Folch H, Onate A. Vaccination with live Escherichia coli expressing *Brucella abortus* Cu/Zn superoxide-dismutase: II. Induction of specific CD8+ cytotoxic lymphocytes and sensitized CD4+ IFN-gamma-producing cell. Microbiology and Immunology. 2006;50:389-93.

[92] Hirota K, Martin B, Veldhoen M. Development, regulation and functional capacities of Th17 cells. Seminars in Immunopathology. 2010;32:3-16.

[93] Clapp B, Skyberg JA, Yang X, Thornburg T, Walters N, Pascual DW. Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infection and Immunity. 2011;79:4165-74.

[94] Fu S, Xu J, Li X, Xie Y, Qiu Y, Du X, et al. Immunization of mice with recombinant protein CobB or AsnC confers protection against *Brucella abortus* infection. PloS One. 2012;7:e29552.

[95] Yu DH, Hu XD, Cai H. A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against *Brucella abortus* 2308 by inducing specific CTL responses. DNA and Cell Biology. 2007;26:435-43.

[96] Confer AW, Hall SM, Faulkner CB, Espe BH, Deyoe BL, Morton RJ, et al. Effects of challenge dose on the clinical and immune responses of cattle vaccinated with reduced doses of *Brucella abortus* strain 19. Vet Microbiol. 1985;10:561-75.

[97] Palmer MV, Olsen SC, Cheville NF. Safety and immunogenicity of Brucella abortus strain RB51 vaccine in pregnant cattle. American Journal of Veterinary Research. 1997;58:472-7.

[98] Smith R, 3rd, Adams LG, Ficht TA, Sowa BA, Wu AM. Immunogenicity of subcellular fractions of Brucella abortus: measurement by in vitro lymphocyte proliferative responses. Veterinary Immunology and Immunopathology. 1990;25:83-97.

[99] Wyckoff JH, 3rd, Howland JL, Confer AW. Comparison of *Brucella abortus* antigen preparations for in vitro stimulation of immune bovine T-lymphocyte cell lines. Veterinary immunology and immunopathology. 1993;36:45-64.

[100] Banks HT, Sutton KL, Thompson WC, Bocharov G, Roose D, Schenkel T, et al. Estimation of cell proliferation dynamics using CFSE data. Bulletin of Mathematical Biology. 2011;73:116-50.

[101] Stevens MG, Olsen SC, Cheville NF. Lymphocyte proliferation in response to immunodominant antigens of *Brucella abortus* 2308 and RB51 in strain 2308-infected cattle. Infection and Immunity. 1994;62:4646-9.

[102] Stevens MG, Olsen SC, Cheville NF. Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. Veterinary Immunology and Immunopathology. 1995;44:223-35.

[103] Singh R, Basera SS, Tewari K, Yadav S, Joshi S, Singh B, et al. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in cross bred cattle calves in India. Indian Journal of Experimental Biology. 2012;50:239-42.

[104] Hu XD, Yu DH, Chen ST, Li SX, Cai H. A combined DNA vaccine provides protective immunity against *Mycobacterium bovis* and *Brucella abortus* in cattle. DNA and Cell Biology. 2009;28:191-9.

[105] Hall SM, Confer AW, Patterson JM. *Brucella abortus*-specific immunoglobulin in isotypes in serum and vaginal mucus from cattle vaccinated with strain 19 and challenge exposed with virulent strain 2308. American Journal of Veterinary Research. 1988;49:840-6.

[106] Vemulapalli R, He Y, Buccolo LS, Boyle SM, Sriranganathan N, Schurig GG. Complementation of *Brucella abortus* RB51 with a functional wboA gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infection and Immunity. 2000;68:3927-32.

[107] Vemulapalli R, Cravero S, Calvert CL, Toth TE, Sriranganathan N, Boyle SM, et al. Characterization of Specific Immune Responses of Mice Inoculated with Recombinant Vaccinia Virus Expressing an 18-Kilodalton Outer Membrane Protein of *Brucella abortus*. Clinical and Vaccine Immunology. 2000;7:114-8.

CHAPTER 3 - CROSS-REACTIVITY OF ANTI-HUMAN CYTOKINE MONOCLONAL ANTIBODIES AS A TOOL TO IDENTIFY NOVEL IMMUNOLOGICAL BIOMARKERS IN DOMESTIC RUMINANTS

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ABSTRACT

Eleven commercially available PE-labeled mAbs anti-human (IL-1-β, IL-6, IL-8, TNF-α, IL-17A, IL-5, IL-10, IL-12 and IL-13) and anti-mouse cytokines (IL-10, TNF-α) were tested to cross-reactivity with cattle, goat and sheep cytokines. Cross-reactivity was assessed by comparative analysis with the standard reactivity of the target species. Our data demonstrated that anti-human IL-1-β, IL-6, IL-8, IL-17A and IL-10 mAbs cross-react with all ruminant species tested. Anti-human IL-5 mAb presented a strong cross-reactivity with cattle and goat IL-5, while anti-human TNF-α mAb showed a selective cross-reactivity with goat TNF-α. No cross-reactivity with the ruminant cytokines was observed for anti-human IL-12 and IL-13 mAbs neither for all anti-mouse cytokine mAbs tested. The present study elected a range of anti-human cytokine mAbs that cross-reacted with cattle, sheep and goat cytokines, increasing the universe of immunological biomarkers for studies in veterinary medicine.

Keywords: Cytokines; Cross-reactivity; Human; Cattle; Goat; Sheep.

1. INTRODUCTION

Cytokines, released from different cells, activate an entire network of interactions among cells and occupy a strategic position in the development of immune responses. Among the numerous physiologic roles played by cytokines are the development of cellular and humoral immune responses, induction of the inflammatory responses, regulation of hematopoiesis, control of cellular proliferation and differentiation and the healing of wounds (Kindt et al., 2007).

Analysis of cytokines in biological fluids could be a useful tool in the diagnosis and in understanding of pathological conditions in domestic animals (Dernfalk et al., 2004). Specific monoclonal antibodies (mAbs) are essential requirements for the assessment of intracytoplasmic produced cytokines by flow cytometry. In this context, studies on the role of cytokines and immune mechanisms involved in domestic ruminant diseases are severely hampered by the low availability or the lack of species-specific reagents.

The search for cross-reactivity of commercially available monoclonal antibodies (mAbs) among different species has been raised as a putative strategy to obtain valuable reagents for immunological studies in

veterinary medicine. Numerous studies have been done in order to identify the existence of cross-reactivity among mAbs of different species by flow cytometry, especially the ability of antibodies directed to cytokines or surface markers of animal cells (Davis et al., 1987; Naessens et al., 1993; Brodersen et al., 1998; Griebel et al., 2007). Scheerlinck (1999) suggests a higher probability of occurrence of cross-reactivity when amino acid sequence homology between cytokines from different species is at least 60%. The comparison of cytokine amino acid sequence of human/mouse and ruminant species have demonstrated up to 84% of homology for a selected set of pro-inflammatory and regulatory cytokines, suggesting high probability of cross-reactivity amongst several anti-cytokine mAbs (Table 1).

Pedersen et al. (2002), investigating the existence of cross-reactivity of anti-ovine, bovine and human IL-2, IL-4, IL-6, IL-8, IL-12, TNF-α, IFN-γ and GM-CSF mAbs with sheep, cow, goat, pig, horse, dog, mink and man found biological cross-reactivity for the majority of the species investigated with four mAbs specific for IL-4, IL-8, IFN-γ and TNF-α.

Considering that a better understanding of the immune response in diseases can lead to the development of new diagnostic methods and vaccines as well as that the development and production of new monoclonal antibodies are expensive and slow, the aim of this study was to evaluate, by flow cytometry, the cross-reactivity of commercial anti-human/mouse cytokine mAbs against cytokines from cattle, goat and sheep.

2. MATERIAL AND METHODS

2.1. MONOCLONAL ANTIBODIES (mAbs)

A total of eleven commercially available anti-cytokine mAbs conjugated with phycoerythrin (PE) were used in this study. The mAbs clone specification, host/target species, immunoglobulin isotype and manufacturer are provided in table 2.

2.2. ANIMALS AND CONTROLS

A total of fifteen healthy domestic ruminants were included in this investigation, including five cattle (*Bos taurus*), five goats (*Capra hircus*), and five sheep (*Ovis aries*). All animals were maintained at Fazenda Modelo (Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG)), located in the Pedro Leopoldo, Minas Gerais State, Brazil. Ten milliliter of heparinized peripheral whole blood was collected from each domestic ruminant and maintained at room temperature up to 24 hours prior processing.

Four healthy human subjects (*Homo sapiens*) living in Belo Horizonte, Minas Gerais State, Brazil and three health Swiss mice (*Mus musculus*), maintained in the animal facility at Centro de Pesquisas René Rachou, FIOCRUZ-Minas were included as control groups to evaluate the standard reactivity of anti-human and anti-mouse cytokine mAbs with the target species, respectively. Ten milliliter of human heparinized peripheral whole blood was collected by vein puncture, whereas mouse blood was collected from the orbital sinus with a glass Pasteur pipette, immediately transferred to a polypropylene conical vial containing sodium heparin as anticoagulant and pooled. Human and mice blood also were maintained at room temperature up to 24 hours prior processing.

This study was approved by the Ethical Committee for the use of Experimental Animals (CEUA) of the Fundação Oswaldo Cruz, Brazil, and by the Ethical Committee for the use of Experimental Animals of the Universidade Federal de Minas Gerais, Brazil (CETEA). All animal procedures were in accordance to the guidelines of the Brazilian Association for Laboratory Animal Science(COBEA). The inclusion of human subjects in this study complied with the regulations 196/1996 (Brasil, 1996) of Brazilian National Council on Research in Humans and was approved by the Ethical Committee of the Fundação Oswaldo Cruz, Brazil.

Table 1- Percentage of amino acid sequence homology among human (H) and Mouse (M) cytokines and domestic ruminants (cattle, sheep and goat) cytokines

Cytokine	Cattle (%)	Sheep (%)	Goat (%)
IL-1-β^{H}	71	70	72
IL-6^{H}	53	52	53
IL-8^{H}	79	81	-
IL-12^{H}	82	80	80
TNF-α^{H}	80	79	80
IL-17A^{H}	84	84	76
IL-5^{H}	66	65	66
IL-10^{H}	76	76	76
IL-13^{H}	66	65	-
TNF-α^{M}	73	72	73
IL-10^{M}	70	70	71

Source: <http://www.ncbi.nlm.nih.gov/BLAST/> Acessed in: 09.23.2010.
^{H}Human amino acid sequence; ^{M}Mouse amino acid sequence

Table 2 – Summary of cross-reactivity of anti-human and anti-mouse cytokines mAbs with ruminant cytokines

mAb[a]	Target Species	Clone	Host	Isotype	Cross-reactivity Pattern		
					Cattle	Goat	Sheep
Anti-IL-1β	Human	3643B314[b]	Mouse	IgG1	+	+	+
Anti-IL-6	Human	MQ2-13A5[b]	Rat	IgG1	+	+	+
Anti-IL-8	Human	G265-8[b]	Mouse	IgG2b	+	+	+
Anti-IL-12	Human	C11.5.14[b]	Mouse	IgG1	-	-	-
Anti-TNF-α	Human	Mab11[b]	Mouse	IgG1	-	+	-
Anti-IL-17A	Human	eBio64DEC17[c]	Mouse	IgG1	+	+	+
Anti-IL-5	Human	TRFK5[b]	Rat	IgG1	+	+	-
Anti-IL-10	Human	JES3-19F1[b]	Rat	IgG2a	+	+	+
Anti-IL-13	Human	JES10-5A2[b]	Rat	IgG1	-	-	-
Anti-TNF-α	Mouse	MP6-XT22[b]	Rat	IgG1	-	-	-
Anti-IL-10	Mouse	JES5-16E3[b]	Rat	IgG2b	-	-	-

[a]mAb –monoclonal antibody; [b]mAb purchased from BD Pharmingen (USA); [c]mAb purchased from eBiosciense (USA).

2.3. SHORT-TERM WHOLE BLOOD IN VITRO CULTURE AND IMMUNOSTAINING FOR INTRACELLULAR CYTOKINE ANALYSIS BY FLOW CYTOMETRY

Short-term whole blood in vitro culture and immunostaining for intracellular cytokine was adapted for ruminants, according to the protocol described by Teixeira-Carvalho et al. (2008) (Figure 1). Briefly, 1 mL aliquots of whole blood samples were cultured (4 hours at 37oC, 5% CO2) in 1 mL of RPMI 1640 (Invitrogen, USA), referred as Control cultures. Another 1 mL aliquots were incubated at the same condition, but cultured in 1 mL of RPMI 1640 supplemented with 50 µL of phorbol 12-myristate 13-acetate solution (1 µg/mL) (Sigma Aldrich, USA) plus 2 µL of ionomycin solution (1 mg/mL) (Sigma Aldrich), referred as PMA+IONO Stimulated cultures. Twenty microliters of brefeldin A (BFA) solution (1 mg/mL) (Sigma Aldrich) were added to all whole blood cultures. Following the short-term in vitro culture, 220 µL of 20 mM EDTA solution were added to each tube before incubation for 10 min at room temperature. Erythrocytes were then lysed and leukocytes fixed with 3 mL of FACS Lysing Solution (Becton Dickinson, USA). After centrifugation at 400 x g for 10 min, the fixed leukocytes were incubated with 3 mL of FACS permeabilizing buffer (permBuffer – PBS 0.01 M pH 7.2 supplemented with 0.5% of bovine serum albumin plus 0.5% of saponin and 0.1% of sodium azide, all from Sigma Aldrich) for 10 min at room temperature. Following this step, samples were incubated with 15 µL of permBuffer (Tube 1), 15 µL of PE-labeled anti-cytokine mAbs (Table 1) diluted 1:50 (dilution determined by previously experiments) in sterile permBuffer (Tube 2) or 15 µL of whole serum blocking buffer (permBuffer supplemented with 5% of mouse/rat sera, both from the animal facility at the Centro de Pesquisas René Rachou, FIOCRUZ-Minas) to monitor unspecific binding (Tube 3). The cells were incubated in the dark for 30 min at room temperature and then, washed once with 2 mL of permBuffer followed by a washing with washBuffer (PBS 0.01 M pH 7.2 supplemented with 0.5% of bovine serum albumin and 0.1% of sodium azide, all from Sigma Aldrich) by centrifugation at 400 x g for 10 min. After washing, the stained cells were fixed in 200 µL of FACS fix solution (10 g/L of paraformaldehyde, 10.2 g/L of sodium cacodilate, 6.63 g/L of sodium chloride, pH 7.2, all reagents from Sigma Aldrich) and the samples immediately used for flow cytometry acquisition or stored at 4 oC up to 24 hours prior acquisition.

The evaluation of cross-reactivity against ruminant cytokines was conducted in three rounds. First, the pattern of reactivity of mAbs with target species (human and mouse) was assessed. In a second step, the existence of cross-reactivity of these mAbss with ruminant cytokines was evaluated. And finally, we evaluated the effect of whole serum blocking on the cross-reactivity observed in the previous step.

2.4. FLOW CYTOMETRY DATA STORAGE AND ANALYSIS

Flow cytometric measurements were performed on a FACScan instrument (Becton Dickinson) interfaced to an Apple G3 FACStation. The FlowJo 7.6.1 (Tree Star, USA) software was used in data analysis. A total of 30,000 events were acquired for each sample.

Figure 1 summarizes the major steps of whole blood short-term in vitro culture, the immunophenotyping procedures, flow cytometry acquisition and data analysis. Lymphocyte gating was based on their selection by forward scatter (FSC) versus side scatter (SSC) properties on dot plot distributions, where they are confined into a region of low size and complexity (R1) (Figure 2). Cytokine-expressing lymphocyte cell subpopulations were quantified using Fluorescence 1 (FL1)/VOID versus Fluorescence 2 (FL2)/anti-cytokine-PE dot plots, by setting quadrants to segregate FL2 positive and negative cells based on the negative control immunostaining. The results are expressed as percentage of lymphocytes that express the cytokine of interest. Cross-reactivity for mAbs was evaluated comparatively to the reactivity observed for target species (human and mouse). Cytokines whose main sources are not the lymphocytes were also investigated in other populations of leukocytes (data not shown).

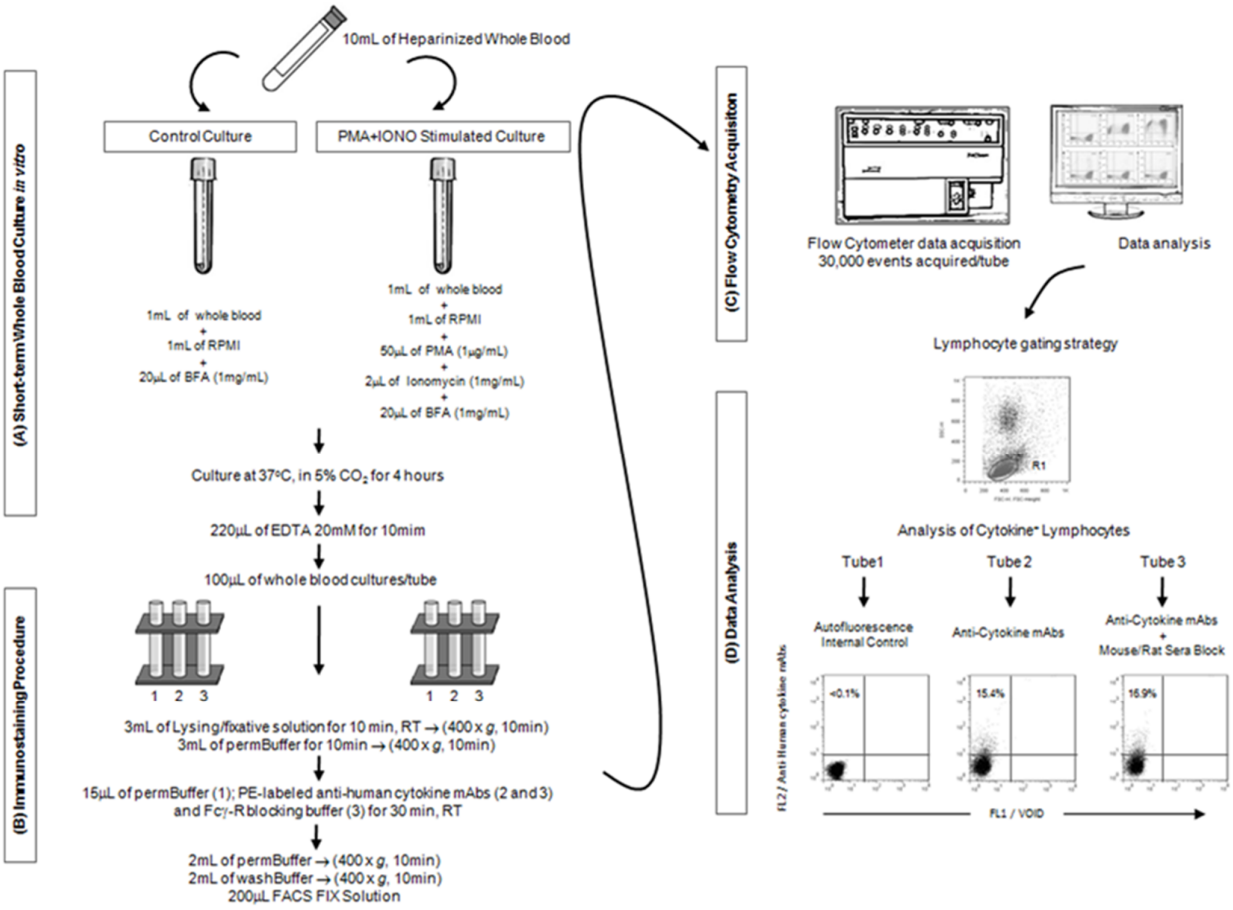

Figure 1. Short-term whole blood culture in vitro. Flowchart summarizing the major steps of (A) whole blood short-term culture in vitro, (B) the immunophenotyping procedures, (C) flow cytometry acquisition and (D) data analysis used to quantify the frequency of lymphocytes expressing intracytoplasmic cytokines

Figure 2 displays the gating strategy used to identify the lymphocyte population based on size and granularity morphometric features. After appropriated instrument settings in the flow cytometer (FSC – gain 1.5 in linear scale and SSC – 359 in linear scale and threshold around 200 in FSC parameter) the lymphocytes assumed a homogeneous distribution in both Control and PMA+IONO stimulated cultures. PMA+IONO induced neutrophil death leading to a typical change in the FSC versus SSC profile as compared to the Control cultures. Neutrophils, representing a large cellular cluster with SSChigh profile (>400) are clearly observed following short-term whole blood in vitro culture maintained under Control conditions but is almost absent in the cattle and goat PMA+IONO stimulated cultures (Figure 2).

3. RESULTS

Aiming to provide a range of valuable reagents for immunological studies in veterinary medicine, the present study accessed the patterns of reactivity of commercially available anti-cytokine mAbs with cytokines from domestic ruminants. For this purpose, a standard protocol for short-term whole blood in vitro culture was established and a flow cytometry-based immunofluorescence assay used to test the cross-reactivity pattern of eleven commercially available PE-labeled mAbs anti-human (IL-1-β, IL-6, IL-8, TNF-α, IL-17A, IL-5, IL-10, IL-12 and IL-13,) and anti-mouse cytokines (IL-10, TNF-α) with cattle, goat and sheep cytokines. Basal cytokine and PMA+IONO-induced cytokine profiles were evaluated and the cross-reactivity with ruminant cytokines assessed by comparative analysis with the standard reactivity of the target species (human and mouse). Data analysis demonstrated that five out of eleven anti-human cytokine mAbs (IL-1-β, IL-6, IL-8, IL-17A and IL-10) cross-react with all ruminant species tested. The anti-human IL-5 mAbs presented cross-reactivity with cattle and goat IL-5. On the other hand, the anti-human TNF-α mAb showed a selective cross-reactivity with goat TNF-α (Figure 3).

The overall pattern of cross-reactivity observed for the anti-human and anti-mouse mAbs besides the clone specification for each mAbs as well as the manufacturer of the commercially available reagent are summarized in Table 2. The selected set of mAbs with outstanding cross-reactivity with ruminant cytokines is highlighted by gray shading.

In order to further address whether the cross-reactivity of the select set of anti-human cytokine mAbs with ruminant cytokines would represent an unspecific binding via Fcγ -R, we have performed a parallel immunophenotyping staining in the “absence” or in the “presence” of mouse/rat sera reagent. This strategy was used to provide a major source of unlabeled IgG for species-specific whole serum blocking by competitive binding. Due to the lack of universal cross-reactivity, the anti-human TNF-α and the anti-human IL-5 mAbs were selectively used to monitor the unspecific binding with goat and cattle/goat lymphocytes, respectively. Our data demonstrated that no differences could be observed in the “presence” of “whole serum blocking” as compared with the reference immunophenotyping procedure performed in the “absence” of “whole serum blocking” (Figure 4).

4. DISCUSSION

Cross-reactivity studies using well-defined mAbs are an important and quick way to obtain reagents that will be valuable for studies of domestic animal immunology, since the development and production of new mAbs is time-consuming and expensive (Dernfalk et al., 2004). The present study identified seven mAbs against anti-human cytokines (Table 2) that cross-reacted with cattle, sheep or goat cytokines, which can improve the information on immunological status and host-parasite relationship in these species.

A general problem in cytokine flow cytometry concerns the establishment of cut-off levels between positive and negative cross-reactivity (Pedersen et al., 2002). To avoid this problem, cross-reactivity for each cytokine mAb was assessed by comparison with the staining profile in the target species, used as standard reactivity. Five healthy individuals of each domestic ruminant species were used in assays to give more reliability to the findings. Moreover, the lack of cells producing a particular cytokine was minimized through the use of short-term whole blood culture that results in smaller loss of cells due the cell adherence or during cell isolation, which occurs in assays with peripheral blood mononuclear cells (PBMC).

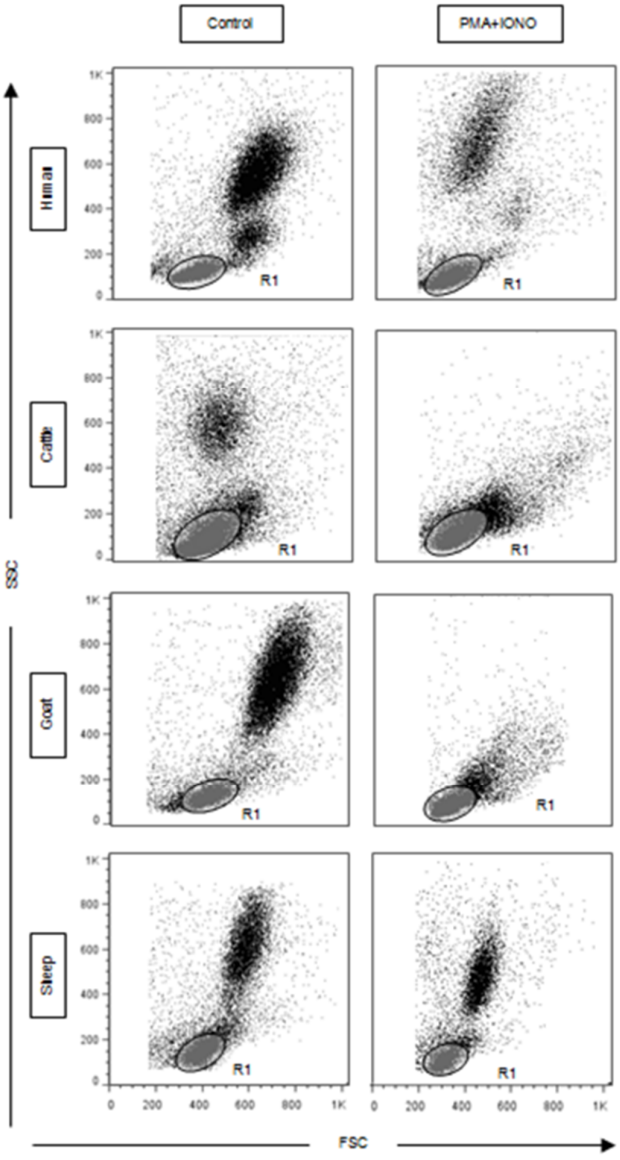

Figure 2. Gating strategy. Details of gating strategy used to select the lymphocyte population (R1) based on their size (Forward scatter –FSC) and granularity (Side scatter – SSC) flow cytometric features prior the analysis of cytokine+ events. The lymphocytes assume a homogeneous distribution (gray ellipse) in Control cultures and PMA+IONO stimulated cultures. PMA+IONO stimuli (right panels) induced neutrophils death leading to a typical change in the FSC versus SSC profile as compared to the Control cultures (left panels).

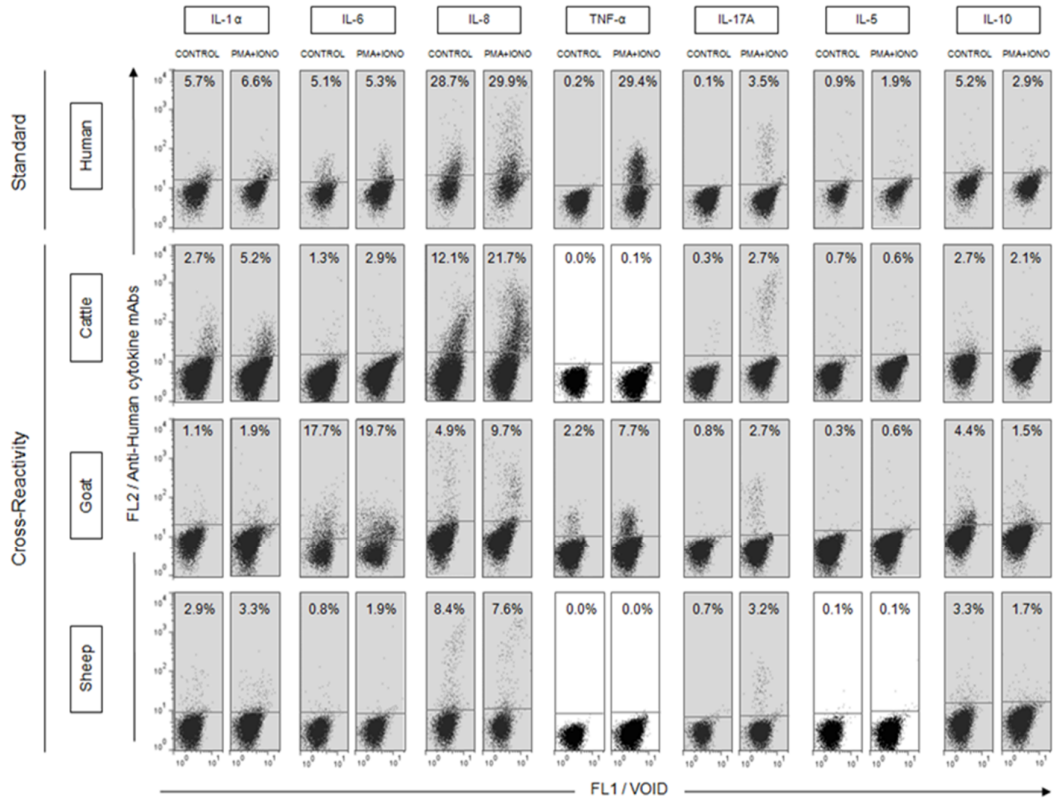

Figure 3. Cross-reactivity of anti-human cytokine mAbs with cattle, goat and sheep lymphocytes. Flow cytometric charts illustrating the standard reactivity of anti-human cytokine mAbs (IL-1-β, IL-6, IL-8, IL-17A, TNF-α, IL-5, IL-10) with human lymphocytes and the cross-reactivity with cattle, goat and sheep lymphocytes. Short-term whole blood culture in vitro was performed in the absence (Control) and in the presence of PMA plus Ionomycin (PMA+IONO) as described in material and methods. After the immunostaining procedures and flow cytometric acquisition, lymphocytes were first selected based on their homogeneous morphometric profile on size versus granularity dot plot distribution. Following gating strategies, dual-quadrant statistics were used to quantify the frequency of cytokine-expressing lymphocytes on FL1 versus FL2 dot-plot distribution, using the autofluorescent internal control as the baseline for the cutoff definition (gray line). The results are expressed as percentage of cytokine+ events within gated lymphocytes. The standard anti-human cytokine mAbs profile and the cross-reactivity patterns are highlighted by gray rectangles. The dot plot distributions are representative of five independent experimental batches. Standard reactivity with human samples was monitored by four independent experimental batches.

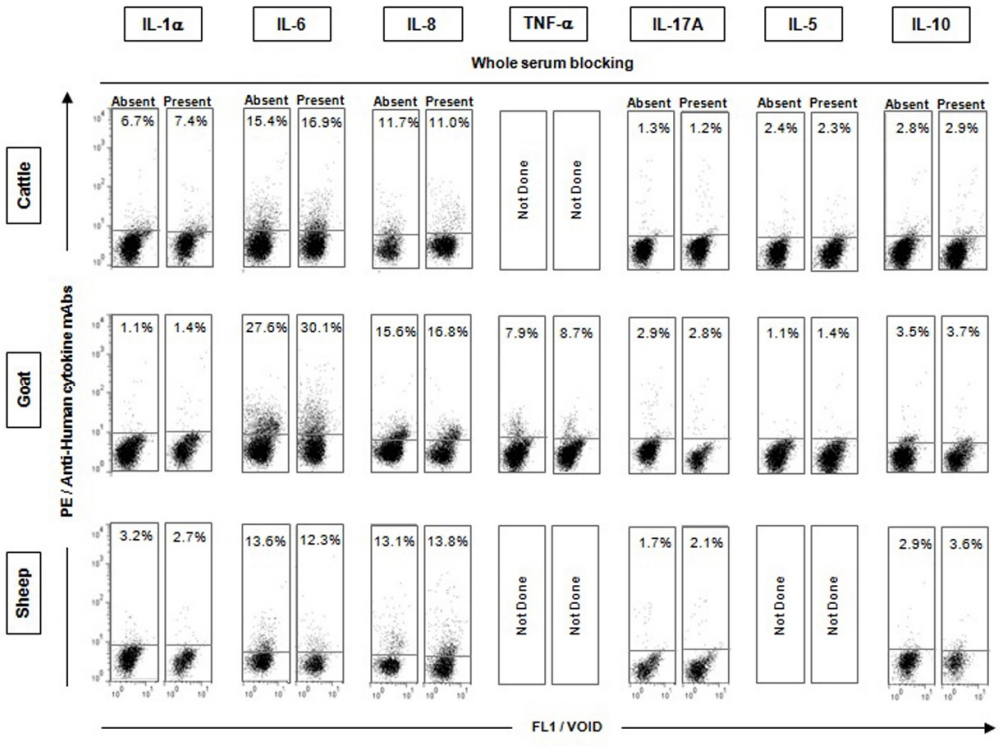

Figure 4. Whole serum blocking. Flow cytometric charts illustrating no of "Whole serum blocking" with mouse/rat sera reagent on the binding profile of anti-human cytokines mAbs (IL-1-β, IL-6, IL-8, TNF-α, IL-17A, IL-10 and IL-5) with cattle, sheep and goat lymphocytes. Comparative analysis of FL1 versus FL2 dot plot distribution showed that the blocking procedure led to no changes in the binding profile of anti-human cytokines mAbs observed after short-term whole blood culture in vitro. The results illustrated the cytokine profile of gated cattle, goat and sheep lymphocytes, following in vitro stimulation with PMA+IONO. The results are expressed as percentage of cytokine+ events within gated lymphocytes. Due to the lack of universal cross-reactivity, the anti-human TNF-α and the anti-human IL-5 mAbs were selectively used to monitor the unspecific binding with goat and cattle/goat lymphocytes, respectively. No significant differences were observed in the "presence" of "whole serum blocking" (right panels) as compared with the reference immunophenotyping procedure performed in the "absence" of "whole serum blocking" (left panels). The dot plot distributions are representative of two independent experimental batches.

Furthermore, the observed cross-reactivity patterns were very consistent for all animals from the same species studied. Additionally, the time of culture in this study (4 hours) was chosen on the basis of previously reported kinetic studies that observed a decrease in cytokine production after long incubation with brefeldin A (Sewell et al., 1997; Mascher et al., 1999). The choice of PMA, a know activator of protein kinase C, and ionomycin as stimulants was because the stimulus is deliberately supra-physiological in an attempt to activate as many cells as possible, mainly primed cells, capable of producing a given cytokine. However, the PMA intensity of cytokine generation does vary between cytokines and depends on the cell source (Schauer et al., 1996).

Our results show that anti-human IL-1α, IL-8, IL-17A and IL-10 mAbs cross-reacted with the three ruminant species studied, whereas anti-IL-5 mAb presented cross-reactivity with bovine and goat cytokines and TNF-α mAb showed cross-reactivity only with goat cytokines. These results are supported by a high degree of amino acid sequence homology found among human, cattle, sheep and goat IL-1β, IL-5, IL-8, IL-17A, IL-10 and TNF-α (Table 1), which showed greater than 65% of amino acid sequence similarity. Similarly, anti-human IL-6 mAb also presented cross-reactivity with all domestic ruminant studied, however, the degree of similarity found to IL-6 amino acid sequence among human, cattle, sheep and goat were smaller. Despite the low similarity (around 50%) it is probably that the epitope recognized by this mAb remained evolutionarily conserved among these species, justifying the cross-reactivity observed. The findings with "whole serum blocking", confirms the potential of this cross-reactivity study to enhance the tools available for immunological assays in veterinary medicine.

These cross-reactivity are broadly supported by the findings in "whole serum blocking" testing, that confirmed the link of the mAbs tested by the Fab portion. Moreover, the cross-reactivity found in the present study are an important tools to understanding the contribution of different cells to cytokine production in heterogeneous cell populations, since surface markers are more widely available than anti-cytokines for domestic ruminants species. Additionally, IL-1α, IL-6, IL-8 and TNF-α are important pro-inflammatory cytokines, and the clear cross-reactivity found in this study provides new tools to evaluate the production of these cytokines in domestic ruminants. Also the cross-reactivity of anti-human IL-17A mAb opens a new horizon to investigate the TH17 cells, which requires a unique combination of cytokines and depends on distinct intracellular events (Hirota et al., 2010), in the immune response of domestic ruminants. Additionally, IL-5 and IL-10, a TH2 and a regulatory cytokine respectively, are also very important for understanding the immunological status in domestic ruminants, especially under pathological conditions. These cytokines presented lowest frequency of positive cells, and for IL-10 this percentage is even lower under the stimulus of PMA + IONO. Both results can be easily understood, not because of difficulty finding a suitable mAb, but because these cytokines need restimulation in vitro to increase their frequencies of responses (Sander et al., 1993; Schauer et al., 1996; Caraher et al., 2000). This was especially evident for IL-10 secreting cells, which increased 30-80 times in secondary responses (Sander et al., 1993; Caraher et al., 2000) and may have inhibited their secretion in the presence of PMA (Boehringer et al., 1999).

Corroborating the present findings, Pedersen et al. (2002) found cross-reactivity between anti-ovine IL-8 and cattle leukocytes. Furthermore, the identification of cross-reactivity for mAbs against TNF-α between evolutionarily related species has been difficult (Dernfalk et al., 2004; Kwong et al., 2010). The findings of cattle and sheep cytokines against anti-human TNF-α were similar to those observed by Dernfalk et al. (2004). Using the same clone for TNF-α (Mab 11), they were also unable to detect ovine or bovine TNF-α, although an 80% homogeneity is found between human and bovine TNF-α and ovine and bovine TNF-α shows more than 90% homogeneity of nucleotide sequences (Dernfalk et al., 2004). Kwong et al. (2010) produced by immunizing mice two mAbs, CC327 and CC328, which were used to develop a sandwich ELISA capable of detecting both native and recombinant bovine TNF-α; however, only CC328 detected intracytoplasmic ovine TNF-α Thus, high homology of nucleotide or amino acid sequence alone does not define the occurrence of cross-reactivity, which need that the recognition epitope of mAb tested has remained between the species.

In this sense, the absence of cross-reactivity between anti-human IL-12 and IL-13 with the respective cytokines produced by cells of the domestic ruminants tested are easily understood. The deficient evolutionary conservation of epitopes among species probably also caused the absence of cross-reactivity observed for anti-mouse IL-10 and TNF-α in all ruminant species tested. Since the amino acid identity among mouse IL-10 and TNF-α and the ruminant species are greater than 70% (Table 1) and that both anti-mouse and anti-human IL-10 were produced in the same host (rat), the cross-reactivity differences can be attributed only to differences in the specificities of each mAb.

Maintenance of epitopes on molecules may reflect functional importance of the region of the molecule recognized and an evolutionary pressure to conserve those regions (Sopp and Howard, 2001). Comparison of the cytokine amino acid sequences (http://www.ncbi.nlm.nih.gov/BLAST/) among control (human/mouse) and ruminant species studied (Table 1) showed that they can share up to 84% of homology, which suggests a higher probability of occurrence of cross-reactivity (Scheerlinck, 1999). However, some results found in this study and in others (Dernfalk et al., 2004; Pedersen et al., 2002) do not completely support this theory, probably because the epitopes recognized by mAbs are not in regions that have been conserved evolutionarily. Comparative studies of the reactivity with cells from several species have shown that each antibody recognizes a different epitope (Davis et al., 1987). Weynants et al. (1998) tested various monoclonal antibodies towards ovine IL-4, but found only one antibody with satisfactory reactivity. Thus, it is often necessary that several antibodies against the same antigen should be tested, which could suggest that a cross-reacting mAb could be found if other different clones were searched for.

The present study elected a range of anti-human cytokine mAbs that cross-reacted with cattle, sheep and goat cytokines, expanding the universe of immunological biomarkers for studies about pathological conditions in veterinary medicine. The identification of mAbs that cross-reacted with cattle, sheep and goat cytokines in flow cytometry provided very useful tools that could be use to understand the regulation of immune response in healthy and unhealthy animals, and additionally allows not only the identification and quantification of cytokine produced but also the characterization of the cell population that produced it.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

Boehringer N, Hagens G, Songeon F, Isler P, et al. (1999). Differential regulation of tumor necrosing factor-alpha (TNF-alpha) and interleukin-10 (IL-10) secretion by protein kinase and phosphatase inhibitors in human alveolar macrophages. Eur. Cytokine Netw. 10: 211-218.

Brasil (1996). Ministério da Saúde, Conselho Nacional de Saúde, Resolução nº 196 de 10 de outubro de 1996. Aprova as seguintes diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos.

Brodersen R, Bijlsma F, Gori K, Jensen KT, et al. (1998). Analysis of the immunological cross reactivities of 213 well characterized monoclonal antibodies with specificities against of human and 11 animal species. Vet. Immunol. Immunopathol. 64: 1-13.

Caraher EM, Parenteau M, Gruber H and Scott FW (2000). Flow cytometric analysis of intracellular IFN-gamma, IL-4 and IL-10 in CD3(+)4(+) T-cells from rat spleen. J. Immunol. Methods. 244: 29-40.

Davis WC, Marusic S, Lewin HA, Splitter GA, et al. (1987). The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. Vet. Immunol. Immunopathol. 15: 337-376.

Dernfalk J, Waller KP and Johannisson A (2004). Commercially available antibodies to human tumour necrosis factor-α tested for cross-reactivity with ovine and bovine tumour necrosis factor-α using flow cytometric assays. Acta Vet. Scand. 45: 9-107.

Griebel PJ, Entrican G, Rocchi M, Beskorwayne T, et al. (2007). Cross-reactivity of mAbs to human CD antigens with sheep leukocytes. Vet. Immunol. Immunopathol. 119: 115-122.

Hirota K, Martin B and Veldhoen M (2010). Development, regulation and functional capacities of Th17 cells. Semin. Immunopathol. 32: 3-16.

Kindt TJ, Goldsby RA and Osborne BA (2007). Kuby Immunology. 6th ed. W.H. Freeman and Company, New York.

Kwong LS, Thom M, Sopp P, Rocchi M, et al. (2010). Production and characterization of two monoclonal antibodies to bovine tumour necrosis factor alpha (TNF-a) and their cross-reactivity with ovine TNF-a . Vet. Immunol. Immunopathol. 135: 320-324.

Mascher B, Schlenke P and Seyfarth M (1999). Expression and kinetics of cytokines determined by intracellular staining using flow cytometry. J. Immunol. Methods. 223: 115–121.

Naessens J, Olubayo RO, Davis WC and Hopkins J (1993). Cross-reactivity of workshop antibodies with cells from domestic and wild ruminants. Vet. Immunol. Immunopathol. 39: 283-290.

Pedersen LG, Castelruiz Y, Jacobsen S and Aasted B (2002). Identification of monoclonal antibodies that cross-react with cytokines from different animal species. Vet. Immunol. Immunopathol. 88: 111-112.

Sander B, Höidén I, Andersson U, Möller E, et al. (1993). Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen Cytokine detection by immunoassay and intracellular immunostaining. J. Immunol. Methods. 166: 201-214.

Schauer U, Jung T, Krug N and Frew A (1996). Measurement of intracellular cytokines. Immunol. Today. 17: 305-306.

Scheerlinck JPY (1999). Functional and structural comparison of cytokines in different species. Vet. Immunol. Immunopathol. 72: 39-44.

Sewell WAC, North MO, Webster ADB and Farrant J (1997). Determination of intracellular cytokines by flow-cytometry following whole-blood culture. J. Immunol. Methods. 209: 67–74.

Sopp P and Howard CJ (2001). INF-γ and IL-4 production by CD4, CD8 and WC1 γδ TCR+ T cells from cattle lymph nodes and blood. Vet. Immunol. Immunopathol. 81, 85-96.

Teixeira-Carvalho A, Martins-Filho OA, Peruhype-Magalhães V, Silveira-Lemos D, et al. (2008). Cytokines, chemokine receptors, CD4+CD25HIGH+ T-cells and clinical forms of human schistosomiasis. Acta Trop. 108: 139-149.

Weynants V, Gilson D, Furger A, Collins RA, et al. (1998). Production and characterization of monoclonal antibodies specific for bovine interleukin-4. Vet. Immunol. Immunopathol. 66: 99-112.

CHAPTER 4 - T LYMPHOCYTES SUBSETS AND CYTOKINE PATTERN INDUCED BY VACCINATION AGAINST BOVINE BRUCELLOSIS EMPLOYING S19 CALFHOOD VACCINATION AND ADULT RB51 REVACCINATION

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ABSTRACT

The aims of this study were to address the protective immune response induced by S19 vaccination (n=10) and RB51 revaccination, in pregnant (n=9) and non-pregnant (n=10) S19 calfhood-vaccinated cattle as follows: evaluate the *in vitro* $CD4^+$ and $CD8^+$ T-lymphocytes specific proliferation, and *in vitro* expression of IFN-γ by $CD4^+$ and $CD8^+$ T-cells and IL-4 by $CD4^+$, $CD8^+$ and $CD21^+$ lymphocytes subset. Upon *in vitro* stimulation with γ-irradiated *B. abortus* 2308, blood mononuclear cells from S19 vaccinated and RB51 revaccinated cows exhibited significantly higher proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes and $CD4^+$IFN-γ^+ T-cells compared to non-vaccinated animals. RB51 revaccination, regardless of the pregnancy status, did not enhance the proliferation of $CD4^+$ or $CD8^+$ T-cells nor IFN-γ or IL-4 production. Data from the present study suggest that cattle's cellular immune response induced after brucellosis vaccination and revaccination is due to $CD4^+$ and $CD8^+$ T-lymphocytes, being $CD4^+$ T-cells the main source of IFN-γ.

Keywords: S19, RB51, calfhood vaccination, revaccination of pregnant cattle, cellular immunity, brucellosis.

1. INTRODUCTION

Currently, S19 and RB51 are the two *Brucella abortus* vaccine strains widely used for prevention of brucellosis in cattle. S19 is an attenuated stable smooth strain employed in brucellosis control for several decades [1]. RB51 vaccine is a rough mutant that does not induce antibodies detectable by routine serological tests [2].

Despite their good outcome on the control of bovine brucellosis, very scant information is available concerning the mechanisms involved in protection induced by S19 and RB51 vaccines in cattle. Understanding the protective immune response triggered in cattle by S19 and RB51 may help to develop more effective and safer vaccines, and new methods for their evaluation.

Thus, the aims of this study were to evaluate *in vitro* $CD4^+$ and $CD8^+$ T-lymphocytes specific proliferation, and *in vitro* expression of IFN-γ by $CD4^+$ and $CD8^+$ T-cells and IL-4 by $CD4^+$, $CD8^+$ and $CD21^+$ lymphocytes subset, induced by S19 vaccination and RB51 revaccination, in pregnant or non-pregnant cattle.

2. MATERIAL AND METHODS

Thirty-nine crossbred cows (first and second parturition), divided into four groups: non-vaccinated (n=10); S19-vaccinated (n=10); pregnant S19+RB51 vaccinated (n=9); and non-pregnant S19+RB51 vaccinated (n=10), were used. Ten non-vaccinated cows were selected from a brucellosis-free herd from Santa Catarina State, Brazil, which has the lowest prevalence of brucellosis in the country and vaccination with S19 is prohibited [3,4]. S19-V group was composed of ten cows previously vaccinated with S19 ($0.6\text{-}1.2x10^{11}$ CFU) (aged 3 to 8 months), selected from a herd in Minas Gerais State, Brazil. Nineteen cows selected from a dairy farm in Minas Gerais were revaccinated with RB51 ($1.3x10^{10}$ CFU). At the time of revaccination, nine cows were at the 9^{th} month of pregnancy (PregS19+RB51) and ten cows were not pregnant (Non-pregS19+RB51). All cows were randomly selected into the herds and serologically negative for brucellosis by rose Bengal test, standard tube agglutination test, and 2-mercaptoethanol test [5].

Six months after the revaccination of PregS19+RB51and Non-pregS19+RB51 groups, 14 mL of heparinized peripheral blood was collected from all cows. Blood samples were maintained at room temperature (25°C) up to 24 hours prior to processing. This study was approved by the Ethical Committee for the use of Experimental Animals of the Universidade Federal de Minas Gerais, Brazil (protocol 139/2010).

Monoclonal antibodies against bovine molecules including anti-CD4 (CC8), anti-CD8 (CC63) and anti-CD21 (CC21) cell surface markers labeled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), and anti-IFN-γ (CC302) and anti-IL-4 (CC303) mAbs labeled with phycoerithrin (PE) were purchased from AbD Serotec (USA).

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples using Ficoll-Paque™ density gradient (GE Healthcare, Sweden) as previously described [6], stained with Carboxyfluorescein Diacetate Succinimidyl Ester CFSE (Life Techonologies, USA) according to manufacturer, and cultured in 96-well cell culture plates for 120 hours (1×10^6 cells/well). The cell viability was monitored by trypan blue staining on light microscopy. Antigen stimulated cultures were incubated with γ-irradiated (1.4×10^6 rads) *B. abortus* strain 2308 (10^8 CFU/mL), control cultures with RPMI 1640 and positive control cultures with phytohaemagglutinin-P (PHA-P) (Medicago, Sweden) (5 μg/mL). Following the incubation, cells were stained with anti-bovine PE-conjugated anti-CD4 or anti-CD8 mAbs. A minimum of 20,000 cells per sample was analyzed in FACScan (Becton Dickinson, USA).

Whole blood *in vitro* culture and immunostaining for intracellular cytokine was performed as previously described [7]. γ-irradiated *B. abortus* (10^8 CFU/mL) was used in antigen stimulated cultures, whereas RPMI 1640 was used in control cultures and phorbol-12-myristate-13-acetate (PMA) (25 ng/mL) plus ionomycin (1 μg/mL) were used in positive control cultures. Following 20 hours of incubation, 10 μg/mL brefeldin A (BFA) (Sigma, USA) were added to all whole blood cultures that were incubated for an additional 4 hours in 5% CO_2 at 37°C.

Following *in vitro* stimulation, the cell cultures were stained with previously standardized amount of anti-CD4, anti-CD8 and anti-CD21-FITC-conjugated mAbs, and then with PE-labeled anti-cytokine mAb (anti-IL-4 or anti-IFN-γ). A minimum of 30,000 cells per sample was analyzed in FACScan.

FlowJo 7.6.1 (Tree Star, USA) software was used in all flow cytometry data analysis. The level of lymphocyte proliferation was determined as described in Figure 1 (Panel A). Specific lymphocyte proliferation was calculated taking the percentage of lymphocytes that express CD4 or CD8 that proliferated divided by the percentage of the surface marker of interest expressing-lymphocytes [(Q1/Q1+Q2) *100] (Fig. 1, A).

The steps in data analysis used to quantify the frequency of lymphocytes expressing intracytoplasmic cytokines are summarized (percentage of lymphocytes expressing the cytokine) in Fig. 1B.

Data were evaluated for independence, normality and homogeneity of variance (SAEG 9.1, UFV, Brazil). Intergroup analyses were performed by ANOVA followed by Student-Newman-Keuls test (SNK) and the analyses within groups (control culture vs. antigen-stimulated culture) were performed by paired *t*-test [8] (Graphpad PRISM 5.0, GraphPad Software, USA). Significance was defined in all cases at $P<0.05$.

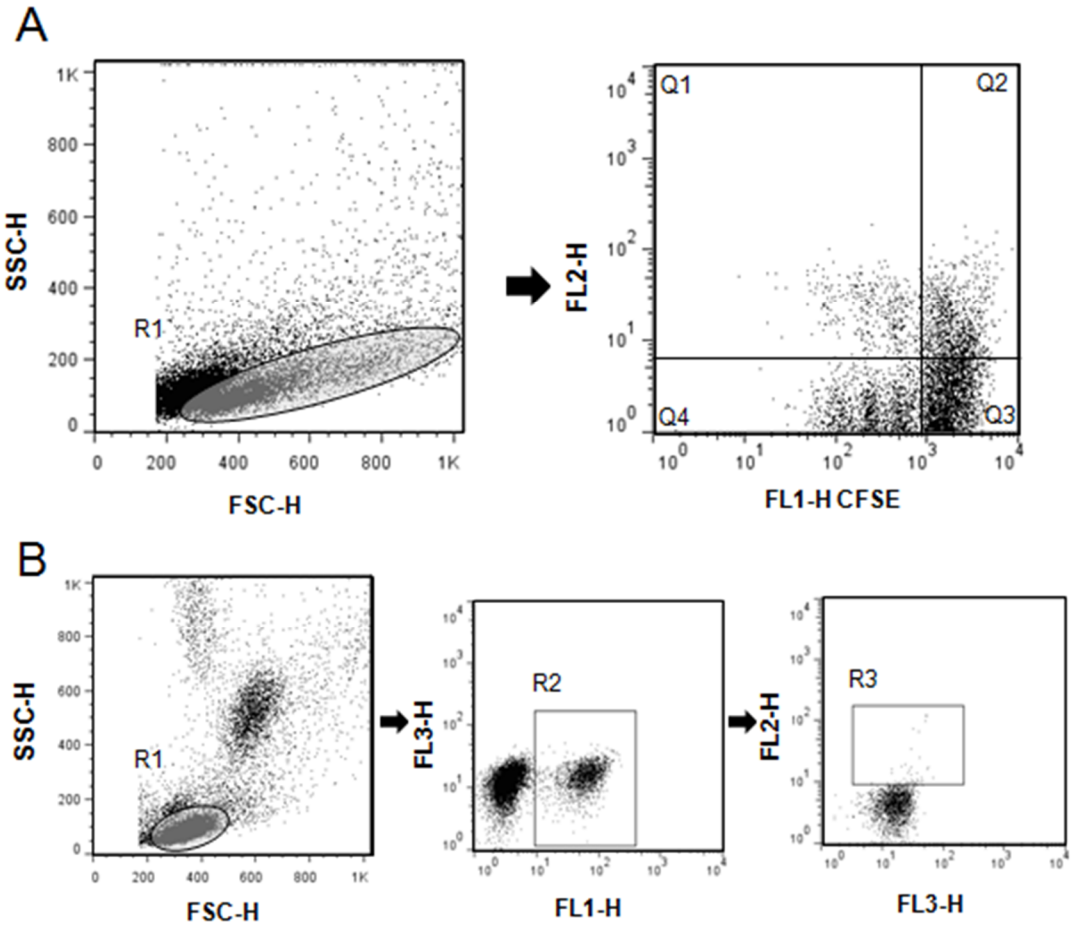

Figure 1. Gating strategies used to select specific leukocytes subpopulation. The lymphocytes were identified as R1 based on their size and granularity flow cytometric features prior to the analysis of proliferation (A) and cells expressing cytokines (B). For proliferation assay, the phenotypic analysis was carried to determine the percentage of divided cells using CFSE/anti-bovine surface marker-PE (anti-CD4 or anti-CD8) dot plots (A). To quantify the frequency of lymphocytes expressing intracytoplasmic cytokines, lymphocytes subpopulations were analyzed based on their selective staining with FITC-labeled anti-CD4, anti-CD8 and anti-CD21 mAbs (R2) and cytokine-expressing lymphocyte cell subpopulations were quantified based on R3 (B).

3. RESULTS

S19 vaccination and RB51 revaccination induce a specific $CD4^+$ and $CD8^+$ blastogenic response upon *in vitro* stimulation with γ-irradiated *B. abortus* (Fig. 2). There were no significant differences in percentages of $CD4^+$ and $CD8^+$ T-lymphocytes specific proliferation between S19-vaccinated and revaccinated groups. The proliferation patterns observed in positive control cultures (PHA-P stimulated cultures) confirmed the cell viability of all samples, as demonstrated by high levels of both $CD4^+$ and $CD8^+$divided cells when compared with the control cultures (data not shown).

$CD4^+$ T-cells were the most relevant source of IFN-γ following S19 vaccination or RB51 revaccination, irrespective of pregnancy status (Fig. 2). Furthermore, comparison of control cultures and antigen-stimulated cultures for $CD4^+IFN\text{-}\gamma^+$ cells showed statistical difference in all vaccinated groups. In contrast, the results demonstrated no difference in the $CD8^+IFN\text{-}\gamma^+$ cytokine pattern among the groups. High levels of IFN-γ expression in PMA-stimulated cultures demonstrated the cell viability of all samples (data not shown).

IL-4 was not associated with immune response induced by vaccination against brucellosis, as no significant differences were observed in the $CD4^+IL\text{-}4^+$, $CD8^+IL\text{-}4^+$ and $CD21^+IL\text{-}4^+$ among the groups (Table 1).

RB51 revaccination, regardless of pregnancy status, did not enhance the specific proliferation of $CD4^+$ or $CD8^+$ T-cells nor IFN-γ or IL-4 production upon *in vitro* stimulation with *B. abortus* (Fig. 2, Table 1). No abortion was recorded in RB51 revaccinated-pregnant-animals.

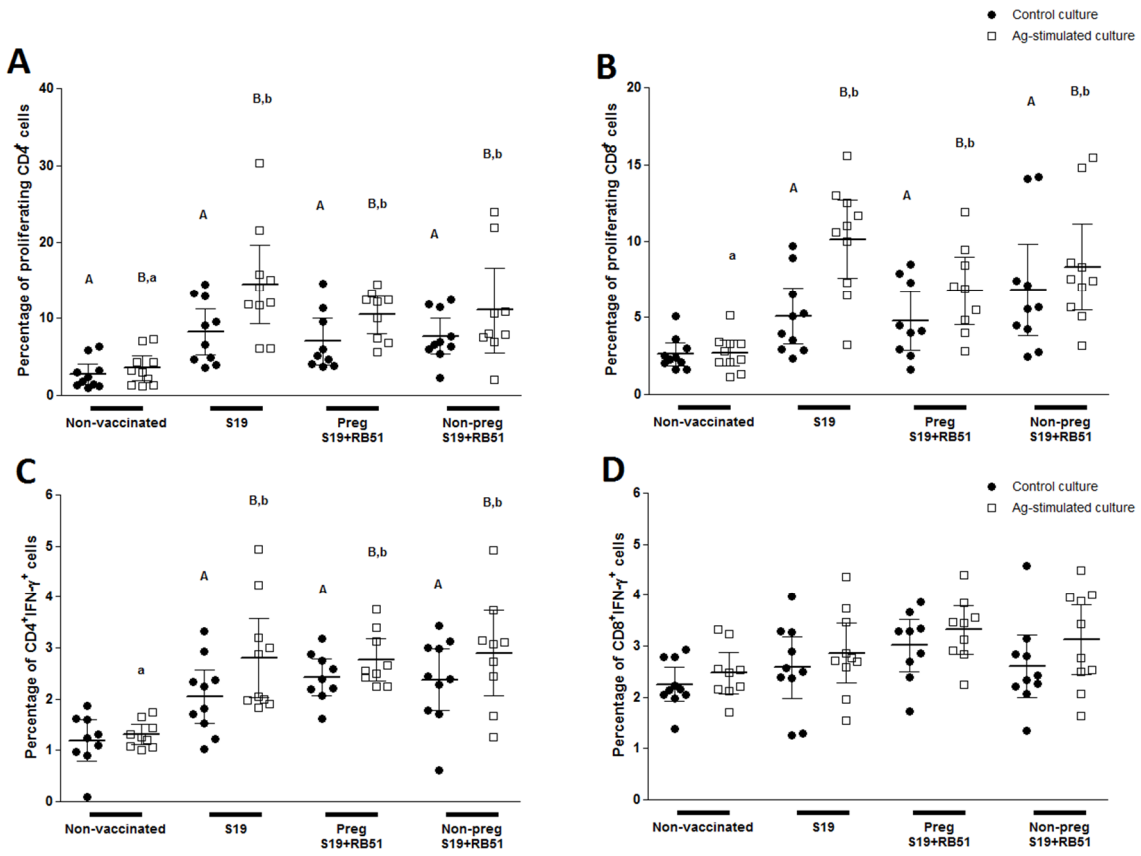

Figure 2. CFSE proliferation analysis of CD4+ and CD8+ T-cell subsets in peripheral blood mononuclear cells (PBMC) of non-vaccinated, S19 vaccinated (S19), RB51 pregnant revaccinated (PregS19+RB51) and RB51 non-pregnant revaccinated (Non-pregS19+RB51) cattle upon in vitro stimulation with γ-irradiated B. abortus 2308. The results are expressed as mean and 95% of confidence interval. Significant differences ($P<0.05$) between control culture and Ag-stimulated culture (in same group) are indicated by uppercase letters (Paired t-test), and lowercase letters indicate intergroup statistical difference based on antigen stimulated cultures (One-way analysis of variance followed by SNK). CFSE proliferation data are shown in graphs A ($CD4^+$) and B ($CD8^+$). Expression of IFN-γ are shown in graphs C ($CD4^+$) and D ($CD8^+$).

4. DISCUSSION

B. abortus specific blastogenic response is usually demonstrated in S19 and RB51 vaccinated cattle [6, 9-11] indicating cellular immune response induced by vaccination. However, only proliferation does not allow differentiation between lymphocyte subsets with different biological functions. Therefore, the sensitive CFSE proliferation assay, which allows subsequent evaluation of phenotype of cells proliferating in response to specific stimulus, was used. Hence, we found that bovine $CD4^+$ and $CD8^+$ T-lymphocytes responded to S19 vaccination and RB51 revaccination (Fig. 2). To our knowledge, this is the first report describing that these two major lymphocyte subsets proliferate in response to brucellosis vaccination and revaccination in cattle. Mouse model experiments widely corroborate these results, showning that the protective response induced by vaccination with S19 or RB51 is due to $CD4^+$ and $CD8^+$ T-cells [12-16]. Furthermore, considering that S19 and RB51 are proven effective vaccines [1,6,17] and that the evaluation of blastogenic response promotes experimental evidence of stimulation of components of cell-mediated immune response, it is possible to infer that both $CD4^+$ and $CD8^+$ T-cells subsets might be involved in the protection afforded by brucellosis vaccination in cattle.

Table 1 – Statistic data of immunologic evaluation of S19 vaccinated and RB51 revaccinated cattle in peripheral mononuclear cells upon *in vitro* stimulation with γ-irradiated *B. abortus* 2308.

Parameter	**Non-vaccinated[a]**		**S19[b]**		**PregS19+RB51[c]**		**Non-pregS19-RB51[d]**	
	CC[e]	**Ag[f]**	**CC**	**Ag**	**CC**	**Ag**	**CC**	**Ag**
CD4 proliferation								
Mean (SD[g])	2.7 (1.9)	3.5 (2.2)	8.3 (4.2)	14.4 (7.2)	6.9 (3.9)	10.5 (3.4)	7.6 (3.2)	12.2 (6.8)
Median (IQR[h])	2.1 (2.6)	3.1 (3.7)	7.8 (8.6)	13.1 (6.9)	5.1 (6.6)	12.3 (6.1)	6.7 (5.7)	9.3 (11.5)
CD8 proliferation								
Mean (SD)	2.6 (1.0)	2.7 (1.2)	5.1 (2.6)	10.1 (3.6)	4.8 (2.5)	6.7 (2.9)	6.8 (4.2)	8.3 (3.9)
Median (IQR)	2.3 (1.2)	2.5 (1.4)	4.5 (4.2)	10.8 (5.5)	4.1 (4.9)	6.8 (4.5)	5.6 (5.2)	7.4 (4.6)
$CD4^+IFN\text{-}\gamma^+$								
Mean (SD)	1.4 (0.3)	1.4 (0.2)	2.0 (0.7)	2.8 (1.2)	2.4 (0.5)	2.9 (0.6)	2.3 (0.9)	2.9 (1.1)
Median (IQR)	1.4 (0.5)	1.4 (0.5)	2.0 (1.1)	2.5 (2.0)	2.5 (0.7)	2.6 (0.9)	2.4 (1.3)	3.1 (1.4)
$CD8^+IFN\text{-}\gamma^+$								
Mean (SD)	2.2 (0.5)	2.5 (0.5)	2.6 (0.9)	2.9 (0.9)	3.0 (0.7)	3.3 (0.6)	2.6 (0.8)	3.1 (0.9)
Median (IQR)	2.1 (0.7)	2.5 (0.8)	2.6 (1.4)	2.8 (1.3)	3.3 (0.9)	3.4 (0.8)	2.4 (0.7)	3.1 (1.6)
$CD4^+IL\text{-}4^+$								
Mean (SD)	1.7 (0.4)	1.8 (0.7)	1.4 (0.5)	1.7 (0.9)	1.9 (0.7)	2.3 (0.4)	1.7 (0.7)	2.2 (0.7)
Median (IQR)	1.7 (0.7)	1.6 (1.0)	1.4 (1.0)	1.5 (1.8)	1.9 (0.6)	2.3 (0.6)	1.9 (1.0)	2.0 (0.9)
$CD8^+IL\text{-}4^+$								
Mean (SD)	1.7 (0.6)	2.1 (0.5)	2.0 (0.7)	1.9 (0.7)	2.3 (0.6)	2.4 (0.4)	2.0 (0.6)	2.1 (0.7)
Median (IQR)	1.8 (1.3)	2.2 (0.9)	2.1 (1.0)	1.8 (1.2)	2.4 (1.1)	2.4 (0.6)	1.9 (0.9)	2.2 (1.2)
$CD21^+IL\text{-}4^+$								
Mean (SD)	1.8 (0.8)	1.9 (0.5)	1.2 (0.3)	1.4 (0.4)	1.2 (0.4)	1.7 (0.6)	1.5 (0.5)	1.6 (0.5)
Median (IQR)	2.0 (0.9)	1.7 (1.0)	1.3 (0.4)	1.3 (0.8)	0.9 (0.7)	1.6 (0.8)	1.5 (0.7)	1.6 (0.6)

[a]Non-vaccinated group
[b]S19 calfhood vaccinated group
[c]RB51 revaccinated group (pregnant)
[d]RB51 revaccinated group (non-pregnant)
[e]Control culture
[f]Antigen stimulated culture
[g]Standard deviation
[h]Interquartile range

In mice, it is largely reported that the role of $CD4^+$ and $CD8^+$ T-lymphocytes subsets in protective immunity is mediated by the cooperative interaction between them, and some studies have shown that $CD4^+$ T-cells producing IFN-γ and $CD8^+$ cytotoxic T-cells are essential for survival of infected animals [12-16]. For cattle, our findings showed that, as previously demonstrated in mice, the $CD4^+$ T-cells induced by *B. abortus* vaccination and revaccination differentiate into Th1 IFN-γ-producing cells (Fig. 3). Moreover, the present results confirm $CD4^+$ T-cells as the most relevant source of IFN-γ following cattle S19 vaccination or RB51 revaccination, since no significant differences in IFN-γ-expressing cells were found within $CD8^+$ T-cells between non-vaccinated and vaccinated groups (Fig. 3). To our knowledge, this is the first comprehensive report showing that $CD4^+$ T-cells are the major source of IFN-γ in cattle after S19 vaccination and RB51 revaccination. The absence of difference in IFN-γ production by $CD8^+$ T-cells indicates that these cells play another effector role in protective immunity, since $CD8^+$ T-cells exhibited significant proliferative response after S19 vaccination or RB51 revaccination (Fig. 2). Indeed, previous studies performed in mice showed that following RB51 vaccination mice $CD8^+$ T-cells secreted low levels of IFN-γ but demonstrated strong specific *Brucella* spp. cytolytic activity, despite the high levels of IFN-γ secretion by $CD4^+$ T-cells [18]. Additionally, confirming the impact of MHC I dependent $CD8^+$ T-cells on the acquisition of resistance to infection by *B. abortus*, it was demonstrated that MHC I knockout mice are more susceptible to brucellosis, while MHC II knockout mice can quickly mount an effective immune response and eliminate the infection [14].

Regarding RB51 revaccination, our findings from blastogenic response of $CD4^+$ and $CD8^+$ T-cells and the production of IFN-γ and IL-4 by the lymphocyte subsets indicate that there was no increase or improvement in the immunological response resulting from revaccination of adult cattle (Fig. 2 and 3). This result is particularly important taking into account that, despite no experimental evidence of improvement in immune response or protection, RB51 revaccination has been widely recommended and performed, especially in areas with high prevalence of brucellosis [19]. The absence of significant different between S19 vaccinated and RB51 revaccinated animals in relation to immunological markers evaluated in this study corroborates previous studies that estimate a long duration of immunity conferred by S19 calfhood vaccination (around five pregnancies) [1]. Although the present results indicate that RB51 booster does not enhance the immunological response acquired by primary S19 vaccination, RB51 revaccination may still be considered as a tool for increasing herd immunity, since not all animals are completely protected after primary immunization [20].

In conclusion, our data showed that S19 vaccination and RB51 revaccination induces in cattle a strong proliferative response by both $CD4^+$ and $CD8^+$ T-lymphocytes, whereas $CD4^+$ T-cells are the main source of IFN-γ and IL-4 is not associated with the protective immunity induced by brucellosis vaccination.

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6. REFERENCES

[1] McDiarmid A. The degree and duration of immunity in cattle resulting from vaccination with S19 *Br. abortus* vaccine and its implication in the future control and eventual eradication of brucellosis. Vet Rec 1957; 69: 877-79.

[2] Schurig GG, Roop RM, Bagchi T, Boyle S, Buhrman D, Sriranganathan N. Biological properties of RB51: a stable rough strain of *Brucella abortus*. Vet Microbiol 1991; 28: 171-188.

[3] Brasil. Ministério da Agricultura, Pecuária e Abastecimento. Portaria nº 11, de 26 de janeiro de 2004. Exclui Santa Catarina da vacinação contra brucelose. Diário Oficial da União, Brasília, DF, 29 jan. 2004. Seção 1, p. 3.

[4] Sikusawa S, Amaku M, Dias RA, Ferreira Neto JS, Martins C, Gonçalves VSP, Figueiredo VCF, Lôbo JR, Ferreira F. Situação epidemiológica da brucelose bovina no Estado de Santa Catarina. Arq Bras Med Vet Zootec 2009; 61: suppl. 1, 103-8.

[5] Brasil. Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal (Manual Técnico). Ministério da Agricultura, Pecuária e Abastecimento – MAPA, Brasília; 2006.

[6] Palmer MV, Olsen SC, Cheville NF. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. Am J Vet Res 1997; 58: 472-7.

[7] Dorneles EMS, Araújo MSS, Teixeira-Carvalho A, Martins-Filho OA, Lage AP. Cross-reactivity of anti-human cytokine monoclonal antibodies as a tool to identify novel immunological biomarkers in domestic ruminants. Genet Mol Res *In Press*.

[8] Sampaio IBM. Estatística Aplicada à Experimentação Animal. Belo Horizonte: Fundação de Ensino e Pesquisa em Medicina Veterinária e Zootecnia; 2002.

[9] Stevens MG, Olsen SC, Cheville NF. Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. Vet Immunol Immunopathol 1995; 44: 223-35.

[10] Wyckoff III JH, Howland JL, Confer AW. Comparison of *Brucella abortus* antigen preparations for in vitro stimulation of immune bovine T-lymphocyte cell lines. Vet Immunol Immunopathol 1993; 36: 45-64.

[11] Wyckoff III JH, Potts RD. Killing of *Brucella* antigen-sensitized macrophages by T lymphocytes in bovine brucellosis. Vet Immunol Immunopathol 2007; 120: 148-59.

[12] Araya LN, Elzer PH, Rowe GE, Enright FM, Winter AJ. Temporal development of protective cell mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. J Immunol 1989; 143: 3330-37.

[13] Agranovich I, Scott DE, Terle D, Lee K, Golding B. Down-Regulation of Th2 Responses by *Brucella abortus*, a Strong Th1 Stimulus, Correlates with Alterations in the B7.2-CD28 Pathway. Infect Immun 1999; 67: 4418-26.

[14] Oliveira SC, Splitter GA. CD8+ type I CD44hi CD45RBlo T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I and class II deficient mice. Eur J Immunol 1995; 25: 2551-57.

[15] Pasquali P, Adone R, Gasbarre LC, Pistoia C, Ciuchini F. Mouse Cytokine Profiles Associated with *Brucella abortus* RB51 Vaccination or *B. abortus* 2308 Infection. Infect Immun 2001; 69: 6542-44.

[16] Zhan Y, Cheers C. Endogenous Gamma Interferon Mediates Resistance to *Brucella abortus* Infection. Infect Immun 1993; 61: 4899-901.

[17] Poester FP, Gonçalves VSP, Paixão TA, Santos RL, Olsen SO, Schurig GG, Lage AP. Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. Vaccine 2006; 24: 5327-34.

[18] He Y, Vemulapalli R, Zeytun A, Schurig GG. Induction of specific cytotoxic lymphocytes in mice vaccinated with *Brucella abortus* RB51. Infect Immun 2001; 69: 5502-08.

[19] Sanz C, Sáez JL, Alvarez J, Cortés M, Pereira G, Reyes A, Rubio F, Martín J, García N, Domínguez L, Hermoso-de-Mendoza M, Hermoso-de-Mendoza J. Mass vaccination as a complementary tool in the control of a severe outbreak of bovine brucellosis due to *Brucella abortus* in Extremadura, Spain. Prev Vet Med 2010; 97: 119–25.

[20] Jones FM, Hooper JA. *Brucella abortus* strain 19 calfhood vaccination – a review. Southwest Vet 1976; 29: 219-25.

CHAPTER 5 - CHARACTERIZATION OF COMPARATIVE IMMUNE RESPONSE OF *Brucella abortus* S19 OR RB51 VACCINATED CALVES AND RB51-REVACCINATED HEIFERS

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ABSTRACT

Brucella abortus S19 and RB51 have been successfully used to control bovine brucellosis worldwide, however, at the present, most of our understanding of the protective immune response induced by vaccination comes from studies in mice. The aim of this study was to characterize and compare the immune responses induced in cattle prime immunized with *B. abortus* S19 or RB51 and revaccinated with RB51. Female calves aged 4 to 8 months were immunized with either vaccine S19 or RB51 on day 0, and revaccinated with RB51 on day 365 of the experiment. The characterization of the immune response was performed using serum and peripheral blood mononuclear cells. Blood samples were collected on days 0, 28, 210, 365, 393 and 575 post-immunization. Results showed that S19 and RB51 vaccination induced an immune response characterized by proliferation of $CD4^+$ and $CD8^+$ T-cells; IFN-γ and IL-17A production by $CD4^+$ T-cells; cytotoxic $CD8^+$ T-cells; IL-6 secretion; $CD4^+$ and $CD8^+$ memory cells; immunoglobulins of IgG1 class; and expression of the phenotypes of activation in T-cells. The differences in the immune response stimulated by S19 compared to RB51 were the higher persistency of IFN-γ and $CD4^+$ memory cells, induction of $CD21^+$ memory cells and higher secretion of IL-6. After RB51 revaccination, the immune response was chiefly characterized by increase in IFN-γ expression, proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells, cytotoxic $CD8^+$ T-cells and decrease in IL-6 production in both groups. However, a different polarization of the immune response, CD4- or CD8-dominant, was observed after the booster with RB51, for S19 and RB51 prime vaccinated animals, respectively. Our results indicate that after first vaccination both vaccine strains (S19 and RB51) induce a strong and complex immune response dominated by Th1 profile, although after RB51 revaccination the differences between immune profiles induced by prime vaccination become more accentuated.

Keywords: *B. abortus*; immune response; brucellosis vaccination; S19; RB51; cattle.

1. INTRODUCTION

The genus *Brucella* causes brucellosis, one of the major zoonosis in public and animal health, that affects livestock and wildlife animal species as well as human [1,2]. Cattle are the preferred host of *Brucella abortus* [1] and the economic importance attributed to bovine brucellosis is based on direct losses caused by abortions, stillbirths, weight loss, decreased milk production and the establishment of sanitary barriers to international trade of animals and their products [3].

Vaccination is the most effective measure to reduce the prevalence of brucellosis and it has contributed enormously to the success of many programs of the disease control [4]. Currently, S19 and RB51 are the

B. abortus vaccine strains more widely used to control brucellosis in cattle. Both vaccines are effective in the prevention of abortion and infection, besides offering long lasting protection [5-12]. *B. abortus* S19 is a stable smooth attenuated organism with high immunogenicity and antigenicity. It has been used to prevent brucellosis for more than seven decades. RB51 vaccine is a lipopolysaccharide O-antigen deficient naturally occurring rough mutant derived from the virulent smooth strain, *B. abortus* 2308 [13]. Therefore, RB51 does not induce antibodies against smooth lipopolysaccharide (LPS) detectable by routine serological tests [13]. This feature allows RB51 vaccination to be performed at any age, while vaccination with S19 is normally restricted to calves between 3 and 8 months of age to avoid the interference in the routine serological tests [2,14].

At present, almost all the knowledge available about the protective response induced by both *B. abortus* vaccines comes from research using the mouse model [15-18]. Studies in mice have shown that S19 and RB51 induces a strong Th1 cell response with production of INF-γ but not IL-4 in immunized animals, besides $CD8^+$ specific citotoxic T-cells [16,17,19-29]. In contrast, the immune mechanism used by *B. abortus* vaccines to confer protection in cattle is unclear. The T lymphocyte response induced by *B. abortus* vaccination in cattle has been extensively evaluated, but only through proliferation assays [30-35]. Blastogenic test promotes experimental evidence of the stimulation of cell-mediated immune response components [36], but it is not able to differentiate among the various biological functions of the lymphocyte subpopulations. Recently, studies have also showed that IFN-γ is induced after RB51 vaccination in cattle [37,38] and that immunization with S19 and RB51 stimulate both $CD4^+$ and $CD8^+$ T cell–responses [39,40]. However, the whole understanding of the immune response triggered by the worldwide used *B. abortus* vaccines in cattle is still undefined.

Characterize protective immunity conferred by *B. abortus* vaccines in cattle is critical for the development of new vaccines, more effective and safer, besides new methods to assess these potential vaccines. The incomplete characterization of *B. abortus*-specific T and B lymphocytes subsets preclude a definitive conclusion about the exact role of the immune cells subpopulations in protective response. Furthermore, it is unknown whether calves vaccinated with RB51 or S19 have equal profile and persistence of the immune response. Likewise, there is limited information on the immune response induced after RB51-revaccination.

Additionally, as several studies have shown promising results using RB51 and S19 as vaccine vector for heterologous antigens [19,20,22,23,41-44], the detailed understanding of the immune response generated by these strains could maximize their use as vectors. Therefore, the aims of the present study were to characterize and compare the adaptive immune response induced following primary vaccination with S19 or RB51 in calves and after RB51-revaccination in heifers.

2. MATERIAL AND METHODS

2.1. LOCALE, ANIMALS AND EXPERIMENTAL DESIGN

The experiment was conducted in a brucellosis-free dairy herd localized in Baldin, Minas Gerais State, Brazil. Forty crossbred females calves aged between 4 and 8 months were randomly selected and serologically confirmed as brucellosis-negative by standard tube agglutination test (STAT) and 2-mercaptoethanol test (2ME) [45]. These animals were divided into two experimental groups: group S19 - composed of 20 calves vaccinated with S19 vaccine strain at day 0 of the experiment; and group RB51 - composed of 20 calves vaccinated with RB51 vaccine strain at day 0 of the experiment (Fig. 1). Animals from both groups were revaccinated with RB51 at the day 365 of the experiment. The distribution of animals of different ages between groups was random and proportional (mean and median = 5.5 months). All animals were raised semi-intensively and fed a balanced diet of concentrate, mineral salt mixture and pasture.

The experimental design, as well as the number of animals tested at each time point, is shown in the Figure 1. For both experimental groups, the evaluation of the immune response was performed at days 0, 28, 210, 365, 393 and 575 after prime vaccination (Figure 1). The characterization of the immune response was performed in cells isolated from peripheral blood, which was collected by venipuncture from all animals in each time point. This study was approved by the Ethical Committee for the use of Experimental Animals of the Universidade Federal de Minas Gerais, Brazil (CETEA) under protocol 139/2010.

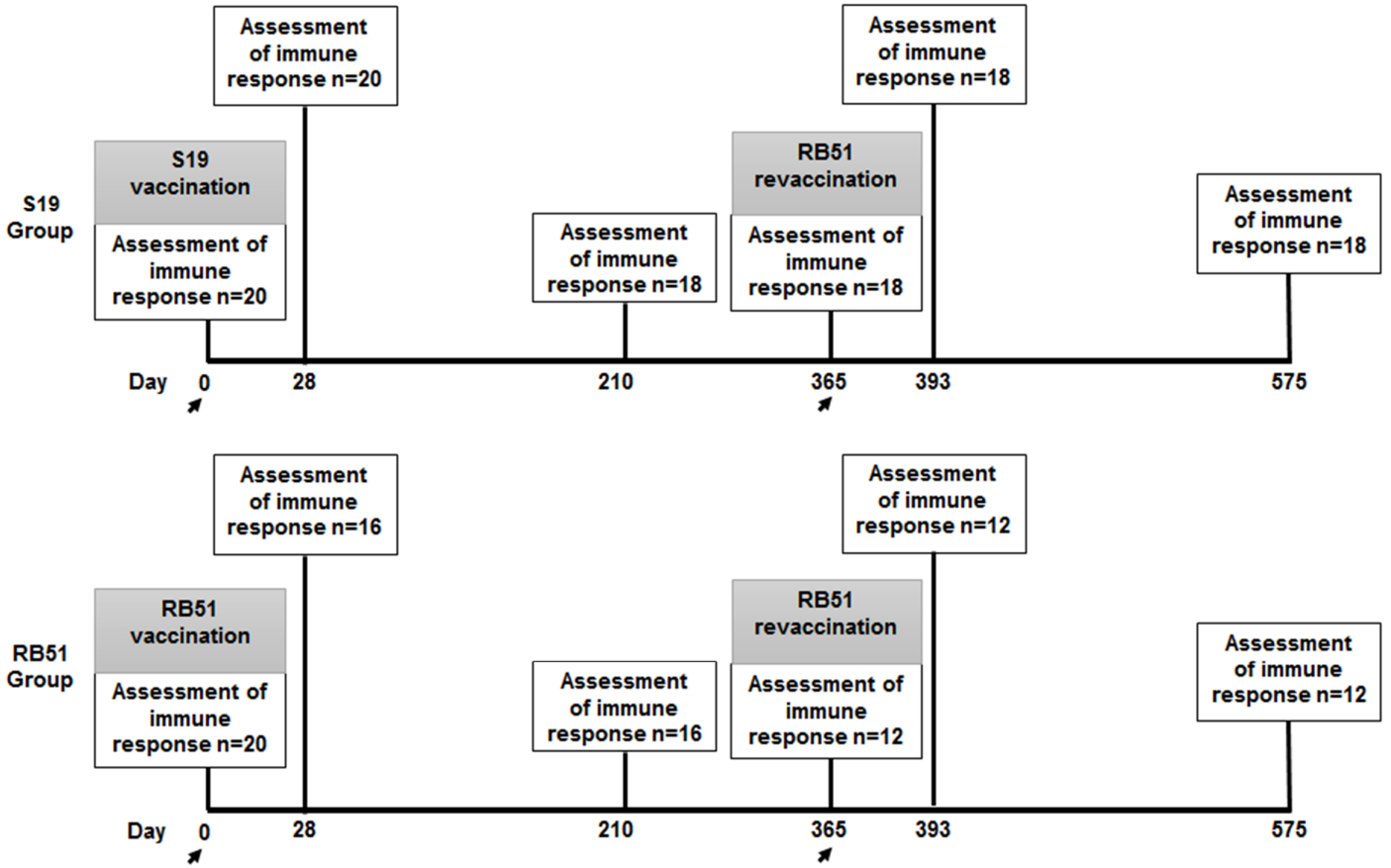

Figure 1. Experimental design. Forty crossbred females calves aged between 4 and 8 months were divided in two experimental groups: group S19 - composed of 20 calves vaccinated with S19 vaccine strain at day 0 of the experiment; and group RB51 - composed of 20 calves vaccinated with RB51 vaccine strain at day 0 of the experiment. Both groups were revaccinated with RB51 at day 365 of the experiment. The number of animals tested in each immunological assessment (0,28, 210, 365, 393 and 575) are shown in the rectangles. The days when the vaccinations occurred are highlighted with arrows.

2.2. VACCINES AND VACCINATIONS

On day 0 of the experiment, all calves from S19 group were subcutaneously vaccinated with S19 commercial vaccine (0.6-1.2 x 10^{11} CFU) [46]. RB51 group and RB51 revaccinated animals received subcutaneously 1.3 x 10^{10} CFU of viable *B. abortus* RB51 [47], on days 0 and 365 of the experiment, respectively. *B. abortus* RB51 vaccine strain was provided by Dr. Schurig (Virginia Tech, USA) and the bacterial suspensions for vaccination were prepared according to OIE [2]. Exact doses inoculated were assessed retrospectively [48].

2.3. MONOCLONAL ANTIBODIES (mAbs)

Monoclonal antibodies (mAbs) against bovine molecules, secondary mAbs and mAbs that cross-react with bovine cytokines [49] used in the present study are summarized in the Table 1. All mAbs had titration pre-determined before each time point.

2.4. PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) ISOLATION, CULTURE AND IMMUNOPHENOTYPING

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples using Ficoll-Paque™ density gradient (GE Healthcare, Sweden), as previously described [33]. Cells were cultured in 48-well cell culture plates (1×10^6 cells / well) (Corning, USA) for 144 h at 37 °C and 5% CO_2. The cell viability was monitored by trypan blue staining on light microscopy. Antigen stimulated cultures were incubated with γ-irradiated (1.4 x 10^6 rads) *B. abortus* strain 2308 (10^8 CFU / mL), control cultures with RPMI 1640 (Sigma, USA) and positive control cultures with phytohaemagglutinin-P (PHA-P) (Medicago, Sweden) (5 μg / mL). Brefeldin A (BFA) (Sigma, USA) was added (10 μg / mL) only in wells for intracellular immunostaining and cultures were submitted to an additional period of 4 h of incubation in 5% CO_2 at 37 °C. Following the incubation, cells were stained with mAbs (Table 1) in four, three or two-colour flow cytometric assays according with cell profile investigated (CD4-IFN-γ; CD8-IFN- γ; CD4-IL-17A; CD8-IL17A; CD4-IL-4; CD8-IL-4; CD8-Perforin; CD8-Granzyme B; CD4-CD45RO; CD8-CD45RO; CD21-CD45RO; CD4-MHCII; CD8-MHCII; CD21-MHCII; CD4-FoxP3-CD25; CD8-FoxP3-CD25) (Table 1). For intracellular immunostaining assay the cells were first stained with surface mAbs. Then, PBMC were fixed and permeabilized with permeabilizing buffer (Becton Dickinson, USA), before staining with intracellular mAbs, as previously described [39].

2.5. CELL PROLIFERATION ASSAY

PBMC were stained with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) (Life Techonologies, USA), according to manufacturer. The cells were cultured in 48-well cell culture plates for 144 hours (1×10^6 cells / well) at 37 °C and 5% CO_2. The cell viability was monitored by trypan blue staining on light microscopy. Antigen stimulated cultures were incubated with γ-irradiated (1.4 x 10^6 rads) *B. abortus* strain 2308 (10^8 CFU / mL), control cultures with RPMI 1640 and positive control cultures with PHA-P (5 μg / mL). Following the incubation, cells were stained with anti-bovine CD4 and anti-bovine CD8 mAbs conjugated with phycoerythrin (PE) or Alexa-Flour 647 (Table 1).

2.6. FLOW CYTOMETRY ACQUISITION AND ANALYSIS

A minimum of 30,000 cells per sample was analyzed in FACSCalibur (Becton Dickinson, USA) in all assays. The FlowJo 7.6.1 (Tree Star, USA) software was used in all flow cytometry data analysis. Distinct gating strategies were used to analyze the different lymphocytes subpopulation and cytokine-expressing lymphocytes subsets as shown in the Figure 2.

Selective analysis of T-cell subsets ($CD4^+$ and $CD8^+$) and B-cells ($CD21^+$) was performed by initially gating the lymphocytes on forward scatter (FSC) versus side scatter (SSC) dot plot distribution (R1), followed by individual or collective analysis on anti-CD4, anti-CD8 and anti-CD21 (R2) (Figure 2). These subpopulation of lymphocytes were then screened to expression of CD45RO (R3), MHC class II (mean of fluorescence intensity) (R4), cytokines (IFN-γ, IL-17A and IL-4), FoxP3 and CD25, and cytotoxicity markers (perforin and granzyme B) (Figure 2). For intracellular immunostaining assay following the selection of lymphocytes subset, the frequency of cytokine$^+$, $FoxP3^+CD25^+$ or cytotoxicity marker$^+$ cells was determined using quadrant statistics over anti-cell surface marker versus anti-cytokine / FoxP3-CD25 / cytotoxicity marker dot plot distribution. The results were expressed as percentages of cytokine$^+$, $FoxP3^+CD25^+$ or cytotoxicity marker$^+$ cells for different gated leucocytes subpopulations analyzed ($CD4^+$ and $CD8^+$) (Figure 2).

Table 1 – Monoclonal antibodies (mAbs) for intracytoplasmic and cell surface staining used in this study.

mAb	Conjugated	Target specie	Clone	Host	Isotype	Binding site	Concentration or dilution
Anti-CD4[a]	Alexa Flour® 647 / FITC[g] / PE[h]	Bovine	CC8	Mouse	IgG2a	Surface	0.25 to 0.5 µg/mL
Anti-CD8[a]	Alexa Flour® 647 / FITC / PE	Bovine	CC63	Mouse	IgG2a	Surface	0.25 to 0.5 µg/mL
Anti-CD21[a]	FITC / PE	Bovine	CC21	Mouse	IgG1	Surface	0.5 to 1.0 µg/mL
Anti-WC1[a]	FITC	Bovine	CC15	Mouse	IgG2a	Surface	2 µg/mL
Anti-MHC II[a]	FITC	Bovine	IL-A21	Mouse	IgG2a	Surface	0.5 to 1.0 µg/mL
Anti-CD25[a]	PE	Bovine	IL-A111	Mouse	IgG1	Surface	0.5 to 1.0 µg/mL
Anti-CD45 RO[b]		Bovine	GC42A1	Mouse	IgG1	Surface	2 to 5 µg/mL
Anti-IgG1[c]	PE-Cy®5.5	Mouse		Goat	IgG1	Surface	1 : 10
Anti-FoxP3[a]	Alexa Flour® 647	Bovine	7627	Human	HuCAL Fab bivalent	Intracellular	1 : 25
Anti-IL-4[a]	PE	Bovine	CC303	Mouse	IgG2a	Intracellular	1 : 50
Anti-IFN-γ[a]	PE	Bovine	CC302	Mouse	IgG1	Intracellular	1 : 50
Anti-IL-17A[d]	PE	Human	eBio64DEC17	Mouse	IgG1	Intracellular	0.25 to 0.5 µg/mL
Anti-Granzime B[e]	PE	Human	351927	Mouse	IgG2a	Intracellular	1 µg/mL
Anti-Perforin[f]	PE	Human	δG9	Mouse	IgG2b	Intracellular	1 : 50
Anti-Total IgG[a]	HRP[i]	Bovine	IL-A2	Mouse	IgG1	ELISA	1 : 5000
Anti-IgG1[a]	HRP	Bovine	IL-A60	Mouse	IgG1	ELISA	1 : 2500
Anti-IgG2[a]	HRP	Bovine	IL-A73	Mouse	IgG1	ELISA	1 : 2500

[a]mAb purchased from AbD Serotec (Raleigh, USA); [b]mAb purchased from VMRD (Pullman, USA); [c]mAb purchased from Life Technologies (Carlsbad, USA); [d]mAb purchased from eBioscience (San Diego, USA); [e]mAb purchased from R&D Systems (Minneapolis, USA); [f]mAb purchased from BD Pharmingen (San Diego, USA); [g]fluorescein isothiocyanate (FITC); [h]phycoerythrin (PE); [i]horseradish peroxidase.

The level of lymphocyte proliferation was quantified setting quadrants to segregate the fraction of lymphocytes that have divided and to segregate FL2 / PE or FL4 / Alexa Fluor 647 positive and negative cells based on the negative control immunostaining (Figure 2). Specific lymphocyte proliferation was calculated taking the percentage of lymphocytes that express CD4 or CD8 that proliferated divided by the percentage of the surface marker of interest expressing-lymphocytes [(Q1 / Q1 + Q2) × 100].

2.7. IL-4, IL-6, IL-10 AND IFN-Γ DETECTION

Supernatants of 6-days-old cultures were tested for the presence of IL-4, IL-6, IL-10 and IFN-γ by antigen-capture enzyme-linked immunosorbent assays (ELISA). The assays were performed according to manufacturer's recommendations (Thermo Fisher Scientific, USA) for IL-4, IL-6 and IFN-γ. The ELISA for detection of IL-10 was performed according Kwong et al. [50], using anti-bovine IL-10 (clone CC318) (AbD Serotec, USA) as capture antibody, anti-bovine IL-10-biotin (clone CC320) (AbD Serotec, USA) as detection antibody and recombinant bovine IL-10 (Kingfisher, USA) for build a standard curve.

2.8. SEROLOGIC ASSAYS

Sera collected on each time point (0, 28, 210, 365, 393 and 575) were centrifuged, separated in aliquots, and stored at -20 ºC. To detect S19 and RB51 vaccine antibodies two kinds of antigens, whole-cell [51] and lysed heat-killed [52], produced from *B. abortus* S19 and *B. abortus* RB51 were used in an indirect ELISA (I-ELISA). The antigens produced from *B. abortus* S19 were used to test serum samples from S19 group. Whereas, the antigens produced from *B. abortus* RB51 were used to test serum samples from RB51 group. Serum samples from S19 group on days 365, 393 and 575 were also tested using the two kinds of antigens produced from *B. abortus* RB51. All I-ELISA assays were performed similarly. Briefly, the antigens were adsorbed into the polystyrene plates (Nunc Maxisorp, Thermo Fisher, USA) at a concentration of 1.0 μg / well in bicarbonate buffer (0.06 M, pH 9.6). Plates were blocked with phosphate buffered saline (0.01 M, pH 7.4) with 5% of non-fat dry milk. Serum samples at 1 : 100 (S19 group) and 1 : 50 (RB51 group) dilution were added to the wells in duplicate. Isotype-specific mouse anti-bovine horseradish peroxidase conjugates (Total IgG, IgG1 and IgG2) (Table 1) were added. The substrate solution, 3.3', 5.5'-tetrametilbenzidina-peroxidase (TMB) (Sigma, USA) was added and the absorbance of the developed color was measured at 450 nm.

2.9. QUANTITATIVE REAL TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (qRT-PCR)

After six days of culture, total RNA extraction from PBMC samples was carried out with Trizol Reagent® (Life Technologies, Carlsbad, USA) following the manufacturer's instructions. Strand cDNAs were synthesized from 1.5 μg of total RNA using the TaqMan Reverse Transcription kit (Applied biosystems, Foster City, SA) with oligodT primers according to the manufacturer's instructions.

Primers used to amplify Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F- 5' ATGGTGAAGGTCGGAGTGAACG 3' and R- 5' TGTAGTGAAGGTCAATGAAGGGGTC 3') gene and IL-10 (F- 5' TGCTGGATGACTTTAAGGG 3' and R- 5' AGGGCAGAAAGCGATGACA 3') and TGF-β (F- 5' GCCATCCGCGGCCAGATTTTGT 3' and R- 5' AGGCTCCGTTTCGGCACTT 3') were designed from sequences deposited in GenBank, with the help of Primer Express 3.0 Software (Applied Biosystems, Foster City, USA). As a housekeeping / control gene the GAPDH gene was chosen. Quantitative RT-PCR was performed using using SYBR Green PCR Master Mix (Applied Biosystems 7500 Real Time PCR System, Foster City, USA). qPCR was carried out in a final volume of 25 μL containing 1 μM of forward and reverse primers, SYBR Green PCR Master Mix, and cDNA diluted at 1 : 3. The efficiency of each pair of primers was evaluated by serial dilution of cDNA according to the protocol developed by Applied Biosystems. Melting point analysis was done after the last cycle to verify the amplification specificity. In order to evaluate gene expression, two replicate analyses were performed and the amount of target RNA was normalized with respect to the control (housekeeping) gene GAPDH and expressed according to relative quantitation of gene expression method. The results are expressed as fold-difference of expression levels (fold-change).

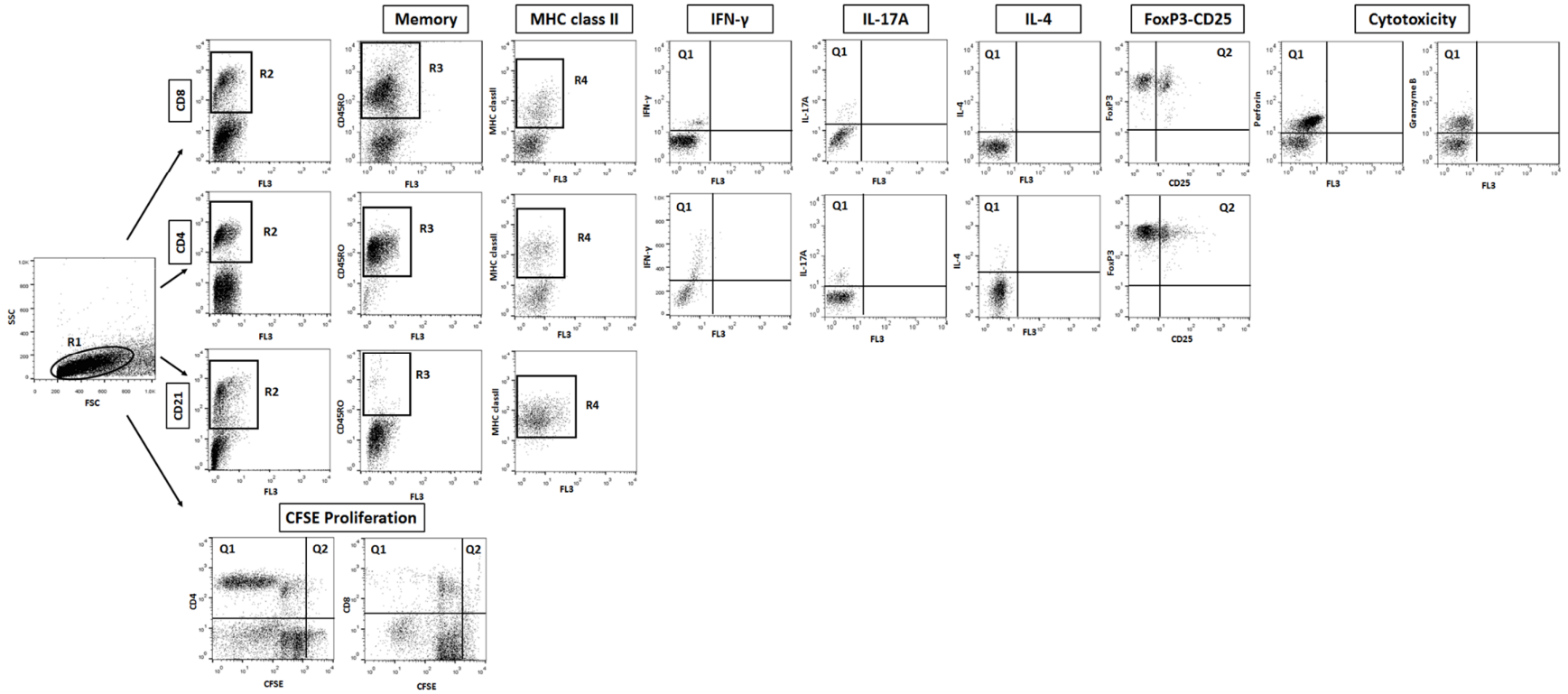

Figure 2. Gating strategies used to select specific leukocytes subpopulation**.** The lymphocytes were identified as R1 based on their size and granularity flow cytometric features prior to the analysis of $CD8^+$, $CD4^+$, $CD21^+$ lymphocytes subsets identified as R2 and proliferation. Lymphocytes subpopulations expressing the memory marker (CD45RO) were quantified based on R3. The mean of fluorescence intensity of MHC class II on lymphocytes subpopulations were quantified based on R4. Percentage of lymphocytes subsets expressing intracytoplasmic cytokines (IFN-γ, IL-17A and IL-4) or cytotoxic markers (perforin and granzyme B) were quantified based on Q1. Percentage of lymphocytes subsets expressing FoxP3-CD25 was determined using quadrant statistics over anti-Foxp3 versus anti-CD25 marker dot plot distribution (Q2). For proliferation assay, the phenotypic analysis was carried to determine the percentage of divided cells using CFSE / anti-bovine surface marker (anti-CD4 or anti-CD8) dot plots.

2.10. STATISTICAL ANALYSIS

The normalization of the data from subtraction of values of Ag-stimulated culture by values of control cultures was adopted to keep the homogeneity of the variance (homoscedasticity), since this was a long-time experiment. This procedure was performed for all flow cytometry and cytokine ELISA data.

Data were first tested to normality and variance of data sets using Epicalc package [53] of R software version 3.0.1 [54]. Considering the nonparametric nature of flow cytometry and cytokines ELISA data, analyses among days within the same vaccination regimen were perfomed by Skillings-Mack test followed by Wilcoxon signed rank test [55], using Skillings.Mack [56] and Stats packages of R software [54], respectively. Analyses between vaccination regimens within the same day were performed by Mann-Whitney test, also using the Stats package of R software [54]. For I-ELISA data considering its parametric nature, analyses among days within the same vaccination regimen were perfomed by ANOVA followed by paired t-test (Graphpad PRISM 5.0, GraphPad Software, USA). Significance was defined in all cases at $P < 0.05$ [57].

3. RESULTS

The main focus of results presentation was comparisons between: pre-vaccinated and vaccinated animals (day 0 vs. 28); peak and mid-term vaccination immune responses (day 28 vs. 210 and day 28 vs. 365); mid-term vaccination immune response and revaccination (day 365 vs. 393); and peak and mid-term revaccination immune response (day 393 vs. 575).

3.1. S19 AND RB51 VACCINATION, AS WELL AS RB51 REVACCINATION INDUCED SIGNIFICANT INCREASE IN PROLIFERATION OF ANTIGEN-SPECIFIC $CD4^+$ AND $CD8^+$ T-CELLS

Comparison between pre-vaccinated animals (day 0) and calves at 28 days post-vaccination showed a significant increase in proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells in both, S19 and RB51-vaccinated calves (Figure 3). However, on days 210 and 365 followed S19 prime vaccination, a decrease in $CD8^+$ T-cell proliferation was observed comparing to data on day 28, in which S19 showed a superior $CD8^+$ T-cell proliferation than RB51. Likewise, on day 210 there was a significant decline in $CD4^+$ T-cell proliferation compared to day 28 in RB51 group. After RB51 revaccination (day 393), for both groups, a significant increase in $CD4^+$ and $CD8^+$ T-cell proliferation was observed in comparison to days 0 and 365. Nevertheless, followed RB51 revaccination (day 393), S19 prime-vaccinated animals exhibited higher $CD4^+$ T-cell proliferation compared to RB51 prime-vaccinated animals, whereas RB51 prime-vaccinated group showed significant higher $CD8^+$ T-cell proliferation compared with S19 group. Comparison between day 393 (peak of immune response after revaccination) and day 575 showed a decrease in $CD4^+$ T-cell proliferation for S19 group and a decrease in $CD8^+$ T-cell proliferation for RB51 group.

3.2. S19 VACCINATION SIGNIFICANTLY INCREASE $CD8^+$GRANZYME B^+ T-CELLS, WHEREAS RB51 VACCINATION AND REVACCINATION SIGNIFICANTLY INCREASE BOTH $CD8^+$GRANZYME B^+ AND $CD8^+$PERFORIN$^+$ T-CELLS

Comparison between pre-vaccinated calves (day 0) and animals 28 days after vaccination showed that S19 induce $CD8^+$Granzyme B^+ T-cells and RB51 induce $CD8^+$Granzyme B^+ and $CD8^+$Perforin$^+$ T-cells (Figure 4). However, for RB51 vaccinated animals the levels of $CD8^+$Granzyme B^+ T-cells significantly decreased on days 210 and 365 in comparison to day 28. Followed RB51 revaccination, on day 393, both vaccination regimens exhibited significant increase in $CD8^+$Granzyme B^+ and $CD8^+$Perforin$^+$ T-cells in comparison to day 365, even though RB51 prime-vaccinated animals had shown a higher level of $CD8^+$Granzyme B^+ and $CD8^+$Perforin$^+$ T-cells than S19 group on day 393. Comparin to day 393, both groups exhibited lower levels of $CD8^+$Granzyme B^+ and $CD8^+$Perforin$^+$ T-cells on day 575.

3.3. $CD4^+$ T-CELLS ARE THE MAIN SOURCE OF IFN-γ AND IL-17A FOLLOWING S19 OR RB51 VACCINATION, AND RB51 REVACCINATION

S19 and RB51 vaccination as well as RB51 revaccination induced the production of significant levels of IFN-ɣ and IL-17A, whose main source was $CD4^+$ T-cells (Figure 5). Comparison between pre-vaccinated calves (day 0) and the same animals 28 days after vaccination showed a significant increase in $CD4^+$IFN-$ɣ^+$ T-cells for both vaccination regimens. In comparison to day 28, $CD4^+$IFN-$ɣ^+$ T-cells also showed a significant increase on day 365 and on days 210 and 365 for S19 group and RB51 group, respectively.

Significant levels of $CD8^+IFN\text{-}\gamma^+$ T-cells were induced later after vaccination, on day 365 and 210, for S19 and RB51, respectively.

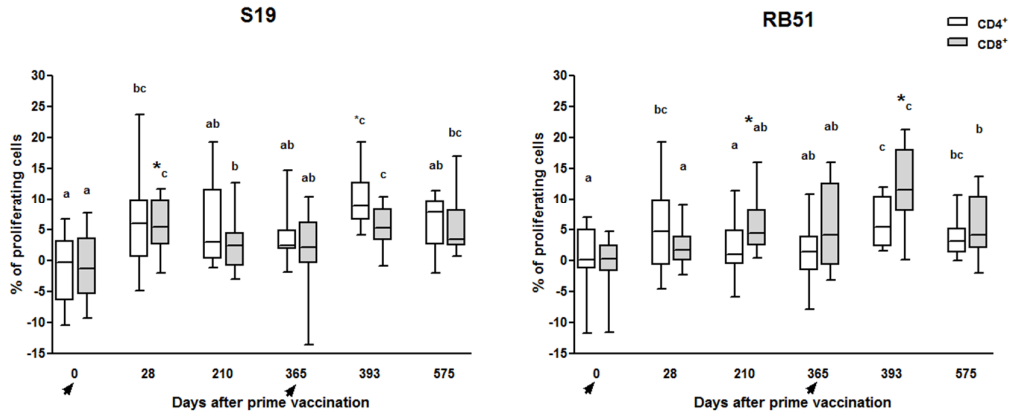

Figure 3. CFSE proliferation analysis of $CD4^+$ (white box plots) and $CD8^+$ (light gray box plots) T-cells subsets in peripheral blood mononuclear cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with γ-irradiated *B. abortus* 2308. The results are expressed in box plot chart. The days when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

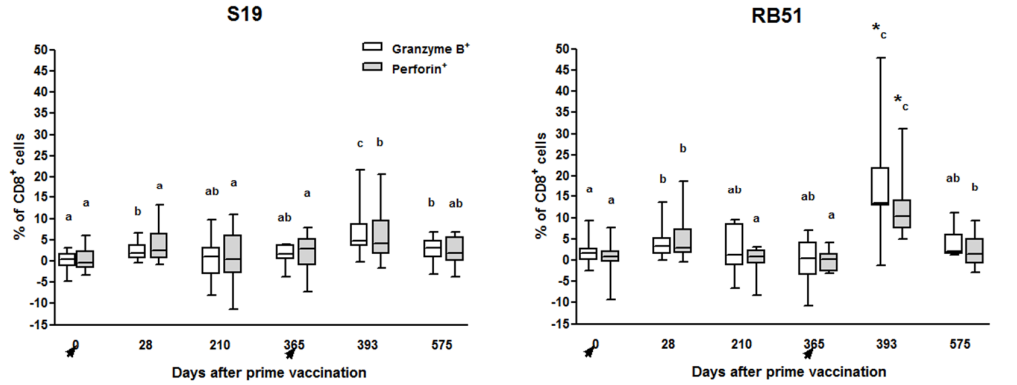

Figure 4. Granzyme B (white box plots) and perforin (light gray box plots)-expressing $CD8^+$ T-cells in peripheral blood mononuclear cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with γ-irradiated *B. abortus* 2308. The results are expressed in box plot chart. The day when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

3.4. $CD4^+IFN\text{-}\gamma^+$, $CD8^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}17A^+$ T-CELL RESPONSE WAS SIGNIFICANT HIGHER IN RB51-PRIME-VACCINATED ANIMALS AFTER RB51 REVACCINATION, COMPARED TO S19-PRIME VACCINATED CATTLE

After RB51 revaccination (day 365 vs 393), $CD4^+IFN\text{-}\gamma^+$ T-cells decreased only in S19 group. Moreover, comparison between the two vaccination regimens on day 393 showed higher levels of $CD4^+IFN\text{-}\gamma^+$ T-cells and $CD8^+IFN\text{-}\gamma^+$ T-cells in RB51 prime-vaccinated group (Figure 5). Seven months after RB51 revaccination (day 575), only RB51 group exhibited decrease in $CD4^+IFN\text{-}\gamma^+$ T-cells compared to day 393. Furthermore, after RB51 revaccination (day 393), only RB51 prime-vaccinated animals increased IFN-γ production by $CD8^+$ T-cells, in comparison to animals before revaccination (day 365). In contrast, comparison between days 365 and 393 for S19 group showed a decrease in $CD8^+IFN\text{-}\gamma^+$ T-cells. In addition, RB51 group exhibited higher levels of $CD8^+IFN\text{-}\gamma^+$ T-cells than S19 group on days 210 and 393. Seven

months after RB51 revaccination (day 575), only RB51 group exhibited decrease in $CD8^+IFN\text{-}\gamma^+$ T-cells compared to day 393.

Production of IL-17A increased after S19 and RB51 vaccination peaking one year after vaccination (day 365) (Figure 5). However, only for S19 group, on day 393, $CD4^+IL\text{-}17A^+$ T-cells showed lower levels than on day 365. At the last immune assessment (day 575), S19 group showed higher levels of $CD4^+IL\text{-}17A^+$ T-cells comparing to RB51 group at the same day. Although presenting lower levels than $CD4^+$ T-cells, $CD8^+$ T-cells also showed significant increase in IL-17A production after S19 and RB51 vaccination.

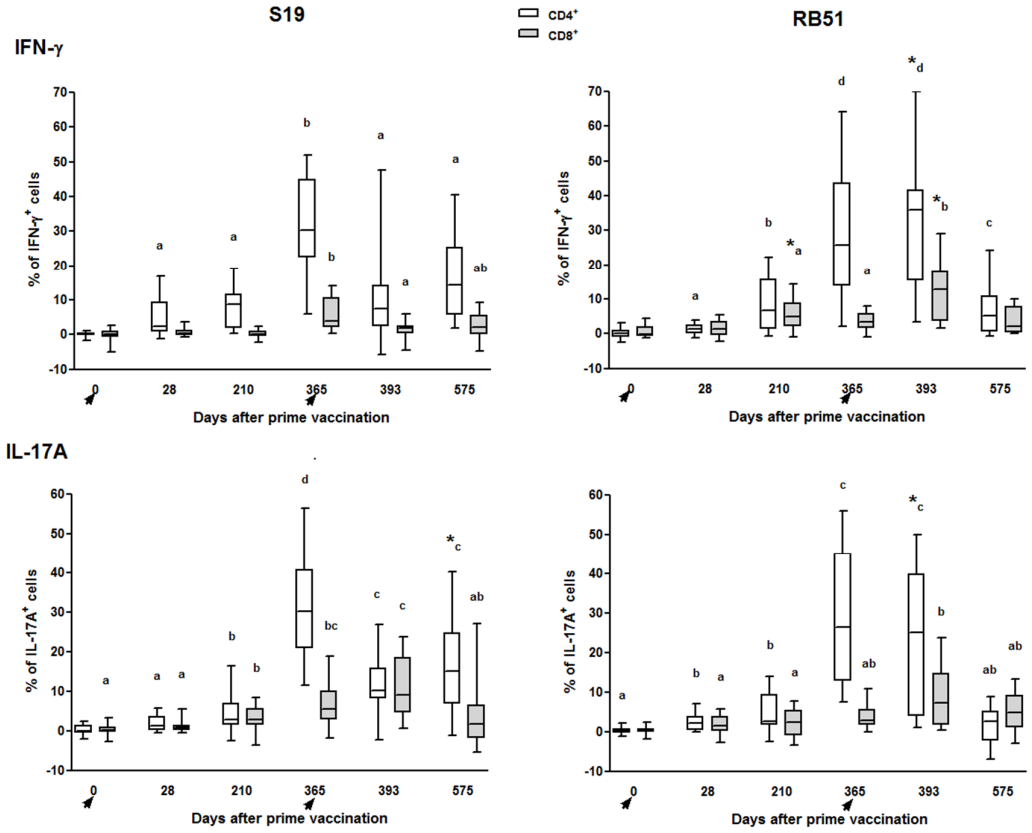

Figure 5. IFN-γ and IL-17A production by $CD4^+$ (white box plot) and $CD8^+$ (light gray box plot) T-cell subsets in peripheral blood mononuclear cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with γ-irradiated *B. abortus* 2308. The results are expressed in box plot chart. The day when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

3.5. S19 AND RB51 VACCINATION, AS WELL AS RB51 REVACCINATION INDUCED IFN-γ RESPONSES

Significant antigen-specific IFN-γ responses were observed in calves vaccinated with S19 or RB51 on 28 day after vaccination compared to pre-vaccinated animals (day 0) (Figure 6). However, only S19 vaccinated animals presented significant IFN-γ accumulation in culture supernatant seven months (day 210) after immunization comparing to pre-vaccinated animals (day 0). In addition, the antigen-specific IFN-γ responses of the S19 and RB51 vaccinated cattle decreased one year (day 365) post-vaccination compared to animals on day 28. After RB51 revaccination (day 393), both vaccination regimens exhibited a significant increase in IFN-γ responses compared to day 365. On day 575, a decrease in IFN-γ responses was observed in both groups compared to the response on day 393 (Figure 6).

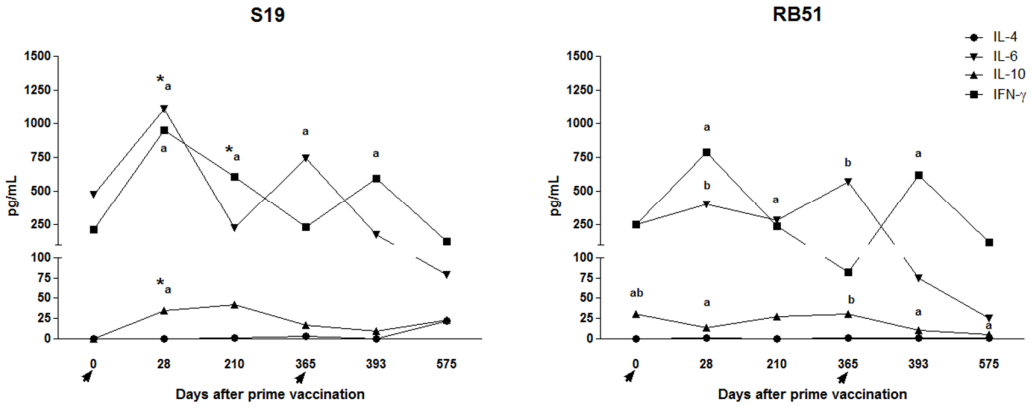

Figure 6. IFN-ɣ, IL-6, IL-4 and IL-10 accumulated in cell culture supernatant of peripheral blood mononuclear cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with ɣ-irradiated *B. abortus* 2308. The results are expressed as median. The day when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

3.6. S19 OR RB51 VACCINATION, AS WELL AS RB51 REVACCINATION, DID NOT INDUCE SIGNIFICANT LEVELS OF IL-4 NOR $CD4^+IL\text{-}4^+$ OR $CD8^+IL\text{-}4^+$ CELL RESPONSE

No significant levels of IL-4 were observed in cell culture supernatant on any time for both vaccination regimens or between the vaccination regimens at the same time point (Figure 6). Likewise, there was no significant difference in the intracellular expression of IL-4 by $CD4^+$ or $CD8^+$ T-cells among any time for both vaccination regimens or between the vaccination regimens at the same time point (data not shown).

3.7. S19 INDUCED HIGHER IL-6 SECRETION THAN RB51 FOLLOWING VACCINATION, BUT IL-6 LEVELS DECREASED AFTER RB51 REVACCINATION IN BOTH GROUPS

Following vaccination with S19 or RB51 there was a significant increase in IL-6 production (day 0 vs. 28), which was higher in S19-vaccinated calves than RB51-vaccinated animals (Figure 6). For RB51-prime vaccinated group the levels of IL-6 remain high on days 210 and 365, compared to day 28. Similarly, for S19 group production of IL-6 was still high on day 365, compared to day 28. However, IL-6 response decreases significantly after revaccination with RB51 for both S19 and RB51-prime-vaccinated cattle (day 365 vs. 393). This reduction was even greater comparing day 393 and 575 for both vaccination regimens.

3.8. ONLY CELLS FROM CALVES VACCINATED WITH S19 PRODUCED SIGNIFICANT LEVELS OF IL-10 FOLLOWING VACCINATION

Comparison between days 0 and 28 revealed that cells from calves vaccinated with S19, but not vaccinated with RB51, produced significant levels of IL-10 (Figure 6). This IL-10 secretion for S19 group significantly decrease on days 210 and 365 compared to day 28. RB51-prime vaccinated animals exhibited an increase in IL-10 production only on day 365 compared to day 28 (peak of immune response after vaccination). However, this high IL-10 secretion did not persist and decreased after RB51 revaccination (day 365 vs. 393). On day 575, IL-10 production was not significantly different from day 393, for both groups.

3.9. S19 AND RB51 VACCINATION INDUCE $CD4^+$ AND $CD8^+$ MEMORY CELLS, BUT ONLY S19 STIMULATE THE DEVELOPMENT OF $CD21^+$ MEMORY CELLS

Assessment of immune response 28 days after S19 and RB51 vaccination showed a substantial increase in $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ T-cells compared to pre-vaccinated animals (day 0) (Figure 7). On day 210 post-vaccination, only S19 group still exhibited high levels of $CD8^+CD45RO^+$ T-cells. However on day 365 after vaccination both groups showed a significantly reduction in $CD8^+CD45RO^+$ T-cells compared with day 28, being these reduction higher in RB51 prime-vaccinated animals.

Vaccination with S19, but not RB51, induced significant levels of $CD21^+CD45RO^+$ B-cells 28 days after immunization. $CD21^+CD45RO^+$ B-cells were significantly higher in S19 prime-vaccinated animals on days 28 and 210 post-vaccination comparing to RB51 prime-vaccinated animals at the same days. After RB51 revaccination, there was no induction of $CD21^+CD45RO^+$ B-cells in both groups. The induction of $CD8^+$ memory cells in RB51 group was still higher on day 575, compared to day 393.

After S19 prime-vaccination, the level of $CD4^+CD45RO^+$ T-cells significantly increased on day 28 and was kept high until one year post-vaccination. RB51 vaccinated calves, although having a significant increase of $CD4^+CD45RO^+$ T-cells on day 28 showed a significantly decrease of these cells on days 210 and 365 (Figure 7). Compared with RB51, S19 group showed significant higher levels of $CD4^+CD45RO^+$ T-cells on days 210 and 365 post-vaccination.

3.10. FOLLOWING RB51 REVACCINATION, RB51 BUT NOT S19 PRIME-VACCINATED ANIMALS SHOWED INCREASE IN $CD4^+$ AND $CD8^+$ MEMORY CELLS

After RB51 revaccination (day 393), the level of $CD4^+CD45RO^+$ T-cells in RB51 group significantly increased compared to day 365, but it decreased again between day 393 and 575. In contrast, for S19 group the level of $CD4^+CD45RO^+$ T-cells was not increased by RB51 revaccination (365 vs 393) and decreased between day 393 and 575 (Figure 7). Followed RB51 revaccination (day 393), only RB51 prime-vaccinated group had a significant increase in $CD8^+CD45RO^+$ T-cells compared to animals before revaccination (day 365).

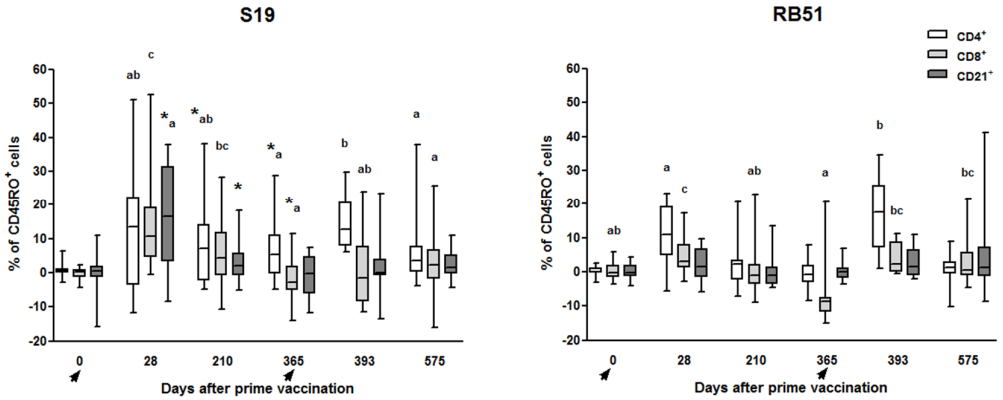

Figure 7. Subsets of memory ($CD45RO^+$) lymphocytes in peripheral blood mononuclear cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with ɣ-irradiated *B. abortus* 2308. The results are expressed in box plot chart. Data of $CD45RO^+$ lymphocytes are shown for $CD4^+$ (white box plots), $CD8^+$ (light gray box plots) and $CD21^+$ (dark gray box plots) cells. The day when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

3.11. IgG1 WAS THE MAIN IMMUNOGLOBULIN PRODUCED FOLLOWING S19 AND RB51 VACCINATION, AND AFTER RB51 REVACCINATION

In the two vaccination regimens and throughout all time points assessed there was a predominance of the IgG1isotype over IgG2 (Figure 8). S19 as well as RB51 prime-vaccination induced significant levels of total IgG, IgG1 and IgG2 in cattle (day 0 vs. 28). Comparisons between day 28 and days 210 and 365 showed a significant decrease in all antibody isotypes tested for both, S19 and RB51 groups. After RB51 revaccination (day 393), RB51 group and S19 group (B), tested with RB51 antigen, exhibited a significant increase in all IgG isotypes tested comparing to day 365. S19 group tested with S19 antigen only showed an increase of IgG2 after RB51 revaccination (day 365 vs. 393). Comparison between days 393 and 575 showed a decrease in IgG1 and IgG2 for RB51 group. Likewise, S19 group tested with S19 antigen exhibited a decrease of total IgG and IgG1 between days 393 and 575. However, S19 group tested with RB51 antigen kept the levels of all IgG isotypes tested, between days 393 and 575.

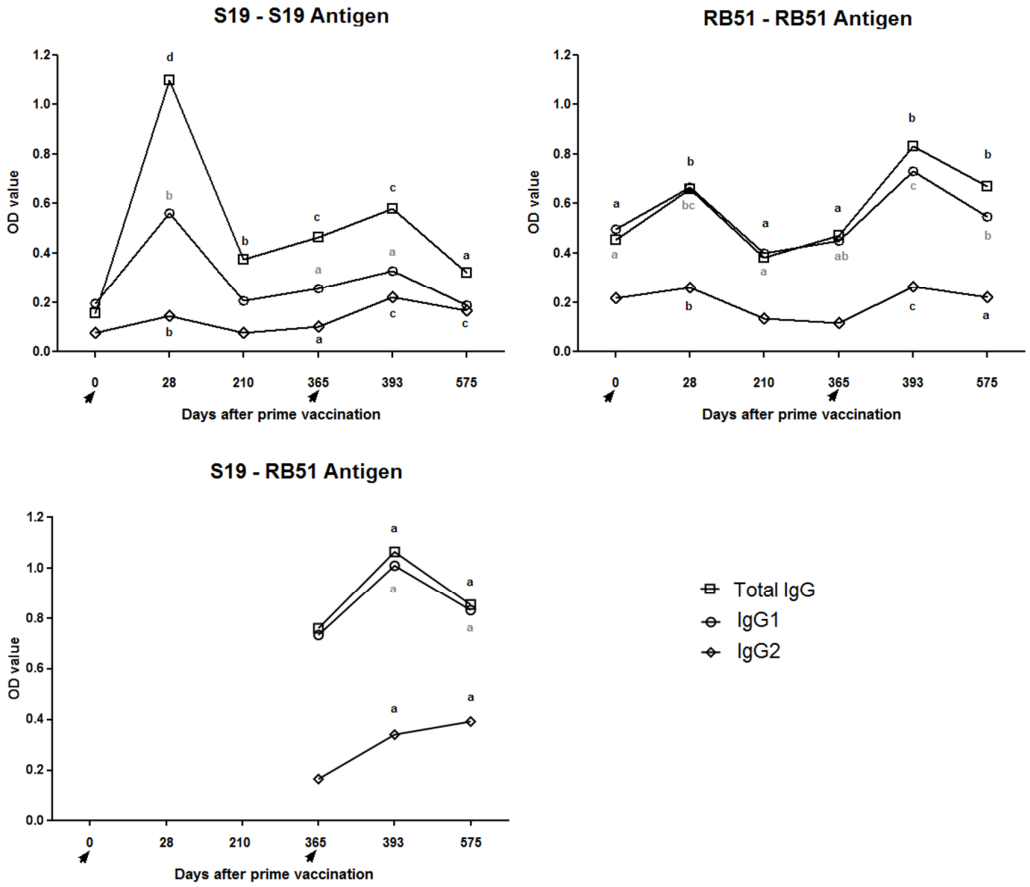

Figure 8. Immunoglobulin profile of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle measured by I-ELISA using S19 and RB51 whole-cell antigens. The results are expressed as mean. Data for total IgG are shown by (⊸), for IgG1 by (⊟) and for IgG2 by (⊸). The day when the vaccinations occurred are highlighted with arrows. Lowercase letters indicate statistical difference between days in same group (ANOVA followed by paired t-test).

3.12. IMMUNE RESPONSE FOLLOWING S19 OR RB51 VACCINATION, AS WELL AS AFTER RB51 REVACCINATION WAS PREDOMINANTLY TH1

Immune response after S19 or RB51 vaccination, as well as after RB51 revaccination is chiefly Th1, with great participation of IFN-ɣ, IL-6, $CD4^+IFN\text{-}ɣ^+$ T-cells, cytotoxic $CD8^+$ T-cells, $CD4^+$ and $CD8^+$ memory cells (Figures 4, 5, 6 and 7).

4. DISCUSSION

So far, it is not quite established whether calfhood vaccination with S19 or RB51 induces equivalent immune response and whether there are and which would be the effects of RB51 revaccination on heifers, despite S19 and RB51 being successful vaccine strains worldwide used in the control of bovine brucellosis. The present study addressed some of these questions and showed that prime vaccination of calves with S19 or RB51 as well as RB51 revaccination induce a strong and complex immune response dominated by Th1 profile, although after RB51 revaccination the differences between immune profiles induced by prime-vaccination become more accentuated.

Our results showed that vaccination with S19 or RB51, and RB51 revaccination induce a significant blastogenic response of both major T lymphocytes subpopulation, CD4 and CD8, indicating that both subsets are involved in the protection conferred by these *B. abortus* vaccines in cattle (Figure 3). Indeed, the resistance to *B. abortus* infection in mice has been credited to coordinated action of $CD4^+$ and $CD8^+$ T-cells [58-61]. After brucellosis vaccination in cattle, $CD4^+$ T-cells have been implicated as the main source of IFN-γ, whereas $CD8^+$ T-cells which were proliferating differentiate into cytotoxic effectors cells (Figure 4 and 5) [39]. However, a different polarization of the immune response, $CD4^+$- or $CD8^+$-dominant, was observed after the booster with RB51, for S19 and RB51 prime-vaccinated animals, respectively. These

results suggest that the vaccine strain used in the calfhood immunization directs the profile of the immune response observed after RB51 booster performed on heifers, which is CD4 directed in S19 prime-vaccinated animals and CD8 directed in RB51 prime-vaccinated animals. This CD8-dominant blastogenic response following the RB51 revaccination in RB51 group is supported considering that, the RB51 prime-vaccinated animals also showed a significant higher expression of both perforin and granzyme B by $CD8^+$ T-cells compared to the S19 group. Perforin, a pore-forming protein, and granzyme B, a serine protease, are upregulated and synergistically involved in the lytic activity triggered chiefly by $CD8^+$ T-cells after CD3 / TCR activation [62,63]. Beyond RB51 booster, vaccination with S19 or RB51 also elicited a significant up regulation in expression of granzyme B on $CD8^+$ T-cells, while expression of perforin was significantly increased only in RB51 group. These results indicate that both vaccines induce specific cytotoxic activity exercised by $CD8^+$ T-cells, however, this appears to be slightly stronger following RB51 vaccination. Similarly to the present findings, it was also previously demonstrated that RB51 vaccination induced specific cytotoxic activity, mainly by $CD8^+$ T lymphocytes, in mice [17]. Furthermore, studies in gene-disrupted mice also showed that MHC I dependent $CD8^+$ T-cells has a great impact on the acquisition of resistance to infection by *B. abortus* [64]. Nevertheless, to our knowledge, this is the first report describing the role of $CD8^+$ T-cells in the immune response induced in cattle by brucellosis vaccination employing S19 and RB51. Our findings, as well as previous results using mouse model, indicate that the protective immune response induced by vaccination with S19 or RB51, and by RB51 revaccination is characterized primarily by synergistic activity of $CD8^+$ cytotoxic T-cells and IFN-γ-producing $CD4^+$ T-cells.

Definitely, $CD4^+$ T-cells are the main source of IFN-γ following brucellosis vaccination in cattle. Data from the present study on intracellular expression of IFN-γ by $CD4^+$ and $CD8^+$ T-cells confirms our previous report [39]. Besides, IFN-γ, $CD4^+$ T-cells also demonstrated to be the main source of IL-17A, a key cytokine in the development of a Th17 immune response, which has been implicated in autoimmune and autoinflammatory diseases, but also has proven to be significant in overcoming several infectious diseases immune response [65]. The pattern of expression of IFN-γ and IL-17A by $CD4^+$ T-cells was similar between both vaccination regimens until day 365, in which the peak of expression was observed (Figure 5). We speculate that this apparent higher expression of IFN-γ and IL-17A by $CD4^+$ T-cells on day 365, in fact it is a reflex of the increased number of IFN-γ- or IL17A-expressing $CD4^+$ T-cells due to clonal expansion of memory cells, rather than a reflex of the amount of cytokine produced by those cells. This hypothesis is widely supported taking into account that the IFN-γ accumulated in the cell culture supernatants measured by ELISA did not show this increased production on day 365 (Figure 6). Additionally, the evaluation of the mean of fluorescence intensity of IFN-γ or IL-17A on CD4 T-cells also showed lower values at day 365 compared to the other time points assessed (data not shown).

In contrast to the similar IFN-γ profile on T-cells post prime-vaccination, following revaccination, only the group vaccinated with RB51 twice exhibited increase in IFN-γ expression, which was significantly higher compared to S19 group on both $CD4^+$ and $CD8^+$ T-cells. Similarly, the response of $CD4^+$IL-$17A^+$ T-cells was significantly higher in RB51 revaccinated animals compared to S19 group (day 393) (Figure 5). In addition, the results of memory markers on $CD4^+$ and $CD8^+$ T-cells, after revaccination with RB51, also exhibited a significant increase only in RB51 group (day 365 vs. 393) (Figure 7). These differences in the immune profile between the vaccination regimens observed post-revaccination could be attributed to the dose of vaccine used or to individual aspects of both brucellosis vaccines tested. Since the dose of S19 (0.6-1.2 x 10^{11} CFU) used was higher than that of RB51 (1.3 x 10^{10} CFU) [46,47], it is tempting to speculate that the significant increase in $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ response observed in animals vaccinated twice with RB51 compared with S19 group, may have occurred due the lower dose of RB51 used in both vaccinations. This also could explain the absence of significant increase of $CD4^+$CD45RO^+ and $CD8^+$CD45RO^+ response in S19 group following the RB51 revaccination. It seems that, as result of the larger dose of S19 used compared to RB51, after prime-vaccination there was a high stimulation of the immune system in S19 group that could not be enhanced by the RB51 revaccination, different from that observed for the RB51 group. This impression is supported considering that RB51 is more attenuated than S19, as several studies have demonstrated that S19 persist more time than RB51 in spleen of infected mice and in lymph node of cattle after immunization [11,66], besides causing severe placentitis and fetal death in pregnant mice [67]. Moreover, analysis of the IFN-γ accumulated in the cell supernatant culture confirming the longer persistence of immune stimulation given by vaccination with S19, as only S19 prime-vaccinated animals exhibited significant production of IFN-γ on day 210 compared to day 0 (Figure 6). Likewise, data on the evaluation of the mean of fluorescence intensity of MHC class II on CD4 T-cells also showed significant increase only in S19 group in comparison of day 0 with day 210 (Figure S1). The expression of MHC class II on T-cells is an important marker of activation of these cells, besides being functional, as it can present peptide antigens to other T-cells [68]. Furthermore, comparing to day 0, a significant higher expression of memory marker by $CD4^+$ and $CD8^+$ T-cells was observed on day 210 only

in S19 group (Figure 7), suggesting that S19, but not RB51 vaccination induced long-live $CD4^+$ memory cells.

However, it is noteworthy that although we have observed a greater persistence of immune stimulation in animals vaccinated with S19 evidenced by prolonged IFN-γ, MHC Class II^+CD4^+ cells and $CD4^+$ memory cells response, both vaccination regimens were able to evoke a significant IFN-γ response after vaccination and revaccination (Figure 6). Corroborating these findings, Singh et al. [38] also observed that RB51 vaccinated cattle have an IFN-γ response in the peripheral blood up to 60 days after vaccination, which was not detected at 90 days post-vaccination. Moreover, the significant induction of $CD4^+$IFN-γ^+ T-cells after S19 or RB51 vaccination and RB51 revaccination (RB51 group), as well as the absence of an IL-4 response, characterize the development of a predominant Th1 immune response following brucellosis vaccination in cattle. The central role of IFN-γ in the protection against brucellosis is recognized once IFN-γ knockout mice died due to brucellosis and IFN-γ deficiency is more severe than $CD8^+$ T-cells or IL-12 deficiency to overcome the infection in mice [69,70]. Besides Th1 immune response, our results also showed that Th17 subset cells were significantly stimulated by S19 and RB51 vaccination (Figure 5). Th17 cells appears to act synergistically with Th1 cells, being suggested that they may have a protective role in oral RB51 and recombinant unlipidated Omp19 mice vaccination, mainly by mucosal immunity [18,71]. Despite protection has not been assessed, the induction of Th1 and Th17 cell subsets observed after brucellosis vaccination in cattle suggests that these cells are involved in the protective immunity conferred by vaccination.

As expression of IFN-γ and IL-17A, $CD4^+$ and $CD8^+$ memory cells were also elicited by S19 and RB51 vaccination, although only S19 vaccination stimulated the development of $CD21^+$ memory cells. Memory cells are a critical parameter to be assessed in the long-term immune response to a vaccine, as *B. abortus* vaccines, since the desirable long-term protection requires generation of immune memory cells capable of rapid and effectively reactivation upon subsequent microbial exposure [72]. Therefore, the increase in $CD4^+$ and $CD8^+$ memory cells following S19 and RB51 vaccination and RB51 revaccination (RB51 group) suggest that this may be one of the mechanism used by these classical *B. abortus* vaccines to induce protection in cattle, as Tc1 $CD8^+$ and Th1 $CD4^+$ T-cells are major immune defenses against *B. abortus*. Differently, only S19 vaccinated animals induced B memory cells post-vaccination, which could be explained taking into account that differences in LPS composition between S19 and RB51. Lipopolysaccharide O-side chain is an immunodominant antigen of *B. abortus*, to which the majority of antibodies resulting from immunization or natural infection are directed, being expressed in S19 and absent in RB51 [13,73]. This highly deficient expression of the LPS O-side chain by RB51 is probably also the explanation to the markedly lower immunoglobulin production after the first vaccination in RB51 group compared to animals vaccinated with S19 (Figure 8). In fact, when animal sera were tested against antigens from the cell-lysed vaccine strains the difference between the two vaccines after vaccination was expressively reduced (Figure S2). Interestingly, the immunoglobulin profile observed in both vaccination regimens was similar following vaccination and revaccination and predominantly IgG1 than IgG2. This result was in contrast to the profile observed in cellular immune response assessed, which was predominantly Th1, given that in cattle as well as human and mouse IgG1 isotype is associated to a Th2 response, whereas IgG2 isotype is more related to a Th1 response [74]. The almost opposite findings observed to cellular and humoral immune response after brucellosis vaccination and revaccination should be understood considering that the exact contribution of humoral immunity in resistance to *B. abortus* infection is not quite established, while the response mediated by cells have been proved to be crucial to overcome the infection [59,60]. Moreover, the intricate interaction between the host and the pathogen usually demands a balance between Th1 and Th2 response.

Also between proinflammatory and anti-inflammatory cytokines, a balance is required, so that an optimal immunological response is established. IL-10, an anti-inflammatory cytokine, has been implicated in offset the production of Th1 cytokines and in the downregulation of macrophage effector functions after *B. abortus* infection or RB51 vaccination in mice [16,64,75]. However, our results showed that only S19 vaccinated calves exhibited a significant increase in IL-10 production, which was even statistically superior to RB51 vaccinated group at the same point (Figure 6). We speculate that, as result of the slight higher immunogenicity of S19, demonstrated by the significant production of proinflammatory cytokines as IFN-γ and IL-6 compared to RB51, higher levels of IL-10 are necessary probably to avoid an excessive proinflammatory response. Evidences in the literature showed that the phenotype of bovine regulatory T-cells (Treg), the main source of IL-10, may be different of Treg cells from mice and humans, being $WC1^+$ γδ-cells instead of $\alpha\beta^+CD4^+FoxP3^+CD25^+$ [76]. Despite the cell source of this cytokine being not assessed, the overlap of the results of IL-10 accumulated in the cell culture supernatant (Figure 6) with the results of $CD4^+FoxP3^+CD25^+$ T-cells (Figure S3) suggests that there was no association between the

$CD4^+FoxP3^+CD25^+$ T-cells and IL-10 production, corroborating the hypothesis that the source of IL-10 in cattle probably is another cell subset. As the $FoxP3^+CD25^+$-expressing $CD4^+$ or $CD8^+$ T-cells seems to be proliferating and CD25 is an IL-2 receptor, it is possible to infer that these cells may represent activated T-cells. Analysis of TGF-β, another anti-inflammatory cytokine, and IL-10 mRNA showed an increase in gene transcription over the experiment for both vaccination regimens evaluated (Figure S4). However, IL-10 gene transcription seemed not to be related to protein expression, indicating mRNA processing, since results of IL-10 ELISA and qPCR were widely disagreeing. On the other hand, as the time required for the detection of mRNA and protein are very different and IL-10 mRNA and protein were both assessed after six days of culture, this could explain the different results observed. For TGF-β, the mRNA levels observed need to be broadly investigated as this cytokine has pleiotropic effects, especially in the regulation of effector and regulatory $CD4^+$ T-cell responses, and can be secreted by many cell types [77].

Regarding IL-6, our findings revealed a significant increase in the secretion of this cytokine after both S19 and RB51 vaccination, suggesting that the secretion of IL-6 in response to brucellosis vaccination may assist the development of a Th1 and Th17 response and consequently favor the elimination of the pathogen. Nonetheless, the level of IL-6 showed significantly decreased after the RB51 revaccination in both vaccination regimens, although the increase in IFN-γ. As IL-6 is pro-inflammatory cytokine that plays a pivotal role during the transition from innate to acquired immunity, it possible to infer that the reduction in IL-6 observed after RB51 revaccination may be the reflect of the higher number of memory cells instead of naïve cells at on the moment of revaccination [78].

The present data showed that RB51 revaccination promote an increase in the immune response regardless if the primary vaccination was performed with S19 or RB51, with some of the parameters assessed being even higher in animals prime-vaccinated with RB51 compared to animals prime-vaccinated with S19. These results strengthen the argument in favor of use of RB51 revaccination in regions where brucellosis is present. However, more studies are necessary to determine which should be the minimum or better interval between the vaccinations and how many vaccinations can or should be performed.

Overall, the present results showed that in cattle the immune response to S19 or RB51 vaccination is characterized by proliferation of specific $CD4^+$ and $CD8^+$ T-cells; IFN-γ and IL-17A production, mainly by $CD4^+$ T-cells; cytotoxic activity exercised by $CD8^+$ T-cells; IL-6 secretion; induction of $CD4^+$ and $CD8^+$ memory cells; production of immunoglobulin, mainly of IgG1 class; and expression of the phenotypes of activation in T-cells. The main differences in the immune response stimulated by S19 compared to RB51 were the higher persistency of the IFN-γ response and $CD4^+$ memory cells, induction of $CD21^+$ memory cells and higher secretion of IL-6. After RB51 revaccination, the immune response was chiefly characterized by increase in IFN-γ expression, proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells, cytotoxic $CD8^+$ T-cells and decrease in IL-6 production in both groups. However, a different polarization of the immune response, $CD4^+$- or $CD8^+$-dominant, was observed after the booster with RB51, for S19 and RB51 prime-vaccinated animals, respectively. Compared to S19 group after the RB51 booster, RB51 prime -accinated animals exhibited significantly higher proliferation of $CD8^+$ T-cells, cytotoxic phenotype on $CD8^+$ T-cells, expression of IFN-γ by $CD4^+$ and $CD8^+$ T-cells and expression of IL-17A by $CD4^+$ T-cells. Our results indicate that after first vaccination both vaccine strains (S19 and RB51) induce a strong and complex immune response dominated by Th1 profile, although after RB51 revaccination the differences between immune profiles induced by prime-vaccination become more accentuated.

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6. REFERENCES

1. Corbel MJE, S.S.; Cosivi O. (2006) Brucellosis in humans and animals. Geneva: World Health Organization. 189 p.
2. OIE (2009) Bovine Brucellosis. Manual of diagnostic tests and vaccines for terrestrial animals: World Organisation for Animal Health.
3. Bernues A, Manrique E, Maza MT (1997) Economic evaluation of bovine brucellosis and tuberculosis eradication programmes in a mountain area of Spain. Prev Vet Med 30: 137-149.

4. Olsen SC, Stoffregen WS (2005) Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert Rev Vaccines 4: 915-928.
5. McDiarmid A (1957) The Degree and Duration of Immunity in Cattle Resulting from Vaccination with S. 19 Br. abortus Vaccine and its Implication in the Future Control and Eventual Eradication of Brucellosis. Veternary Record 69: 877-879.
6. McDiarmid A (1960) The prophylaxis of brucellosis in cattle. Veternary Record 72: 917-920.
7. Manthei CA. Summary of controlled research with strain 19; 1959. pp. 91-97.
8. Poester FP, Goncalves VS, Paixao TA, Santos RL, Olsen SC, et al. (2006) Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. Vaccine 24: 5327-5334.
9. Redman DR, Deyoe BL, King NB (1967) Resistance of cattle to Brucella abortus following vaccination at two and three months of age. J Am Vet Med Assoc 150: 403-407.
10. Cheville NF, Stevens MG, Jensen AE, Tatum FM, Halling SM (1993) Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of Brucella abortus. Am J Vet Res 54: 1591-1597.
11. Cheville NF, Olsen SC, Jensen AE, Stevens MG, Palmer MV, et al. (1996) Effects of age at vaccination on efficacy of Brucella abortus strain RB51 to protect cattle against brucellosis. Am J Vet Res 57: 1153-1156.
12. Olsen SC (2000) Immune responses and efficacy after administration of a commercial Brucella abortus strain RB51 vaccine to cattle. Vet Ther 1: 183-191.
13. Schurig GG, Roop RM, 2nd, Bagchi T, Boyle S, Buhrman D, et al. (1991) Biological properties of RB51; a stable rough strain of Brucella abortus. Vet Microbiol 28: 171-188.
14. Brasil (2006) Manual Técnico do Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose – PNCEBT. Brasília: Ministerio da Agricultura Pecuária e Abastecimento.
15. Zhan Y, Cheers C (1995) Differential induction of macrophage-derived cytokines by live and dead intracellular bacteria in vitro. Infect Immun 63: 720-723.
16. Pasquali P, Adone R, Gasbarre LC, Pistoia C, Ciuchini F (2001) Mouse cytokine profiles associated with Brucella abortus RB51 vaccination or B. abortus 2308 infection. Infect Immun 69: 6541-6544.
17. He Y, Vemulapalli R, Zeytun A, Schurig GG (2001) Induction of Specific Cytotoxic Lymphocytes in Mice Vaccinated with Brucella abortus RB51. Infection and Immunity 69: 5502-5508.
18. Clapp B, Skyberg JA, Yang X, Thornburg T, Walters N, et al. (2011) Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infect Immun 79: 4165-4174.
19. Ramamoorthy S, Sanakkayala N, Vemulapalli R, Duncan RB, Lindsay DS, et al. (2007) Prevention of lethal experimental infection of C57BL/6 mice by vaccination with Brucella abortus strain RB51 expressing Neospora caninum antigens. Int J Parasitol 37: 1521-1529.
20. Ramamoorthy S, Sanakkayala N, Vemulapalli R, Jain N, Lindsay DS, et al. (2007) Prevention of vertical transmission of Neospora caninum in C57BL/6 mice vaccinated with Brucella abortus strain RB51 expressing N. caninum protective antigens. Int J Parasitol 37: 1531-1538.
21. Vemulapalli R, Cravero S, Calvert CL, Toth TE, Sriranganathan N, et al. (2000) Characterization of Specific Immune Responses of Mice Inoculated with Recombinant Vaccinia Virus Expressing an 18-Kilodalton Outer Membrane Protein of Brucella abortus. Clinical and Vaccine Immunology 7: 114-118.
22. Vemulapalli R, He Y, Boyle SM, Sriranganathan N, Schurig GG (2000) Brucella abortus strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infect Immun 68: 3290-3296.
23. Vemulapalli R, He Y, Sriranganathan N, Boyle SM, Schurig GG (2002) Brucella abortus RB51: enhancing vaccine efficacy and developing multivalent vaccines. Vet Microbiol 90: 521-532.
24. Luo D, Ni B, Li P, Shi W, Zhang S, et al. (2006) Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of Brucella abortus in BALB/c mice. Infect Immun 74: 2734-2741.
25. Fu S, Xu J, Li X, Xie Y, Qiu Y, et al. (2012) Immunization of mice with recombinant protein CobB or AsnC confers protection against Brucella abortus infection. PLoS One 7: e29552.
26. Yu DH, Hu XD, Cai H (2007) A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against Brucella abortus 2308 by inducing specific CTL responses. DNA Cell Biol 26: 435-443.
27. Yu DH, Li M, Hu XD, Cai H (2007) A combined DNA vaccine enhances protective immunity against Mycobacterium tuberculosis and Brucella abortus in the presence of an IL-12 expression vector. Vaccine 25: 6744-6754.

28. Andrews E, Salgado P, Folch H, Onate A (2006) Vaccination with live Escherichia coli expressing Brucella abortus Cu/Zn superoxide-dismutase: II. Induction of specific CD8+ cytotoxic lymphocytes and sensitized CD4+ IFN-gamma-producing cell. Microbiol Immunol 50: 389-393.
29. Rosinha GM, Myioshi A, Azevedo V, Splitter GA, Oliveira SC (2002) Molecular and immunological characterisation of recombinant Brucella abortus glyceraldehyde-3-phosphate-dehydrogenase, a T- and B-cell reactive protein that induces partial protection when co-administered with an interleukin-12-expressing plasmid in a DNA vaccine formulation. J Med Microbiol 51: 661-671.
30. Confer AW, Hall SM, Faulkner CB, Espe BH, Deyoe BL, et al. (1985) Effects of challenge dose on the clinical and immune responses of cattle vaccinated with reduced doses of Brucella abortus strain 19. Vet Microbiol 10: 561-575.
31. Smith R, 3rd, Adams LG, Ficht TA, Sowa BA, Wu AM (1990) Immunogenicity of subcellular fractions of Brucella abortus: measurement by in vitro lymphocyte proliferative responses. Vet Immunol Immunopathol 25: 83-97.
32. Wyckoff JH, 3rd, Howland JL, Confer AW (1993) Comparison of Brucella abortus antigen preparations for in vitro stimulation of immune bovine T-lymphocyte cell lines. Vet Immunol Immunopathol 36: 45-64.
33. Palmer MV, Olsen SC, Cheville NF (1997) Safety and immunogenicity of Brucella abortus strain RB51 vaccine in pregnant cattle. Am J Vet Res 58: 472-477.
34. Stevens MG, Olsen SC, Cheville NF (1994) Lymphocyte proliferation in response to immunodominant antigens of Brucella abortus 2308 and RB51 in strain 2308-infected cattle. Infect Immun 62: 4646-4649.
35. Stevens MG, Olsen SC, Cheville NF (1995) Comparative analysis of immune responses in cattle vaccinated with Brucella abortus strain 19 or strain RB51. Vet Immunol Immunopathol 44: 223-235.
36. Banks HT, Sutton KL, Thompson WC, Bocharov G, Roose D, et al. (2011) Estimation of cell proliferation dynamics using CFSE data. Bull Math Biol 73: 116-150.
37. Polci A, Leone A, Di Provvido A (2006) Study of the gene expression of Th1 and Th2 cytokines in the immune response of cows vaccinated with Brucella abortus RB51. Vet Ital 42: 147-155.
38. Singh R, Basera SS, Tewari K, Yadav S, Joshi S, et al. (2012) Safety and immunogenicity of Brucella abortus strain RB51 vaccine in cross bred cattle calves in India. Indian J Exp Biol 50: 239-242.
39. Dorneles EM, Teixeira-Carvalho A, Araujo MS, Lima GK, Martins-Filho OA, et al. (2014) T lymphocytes subsets and cytokine pattern induced by vaccination against bovine brucellosis employing S19 calfhood vaccination and adult RB51 revaccination. Vaccine 32: 6034-6038.
40. Hu XD, Yu DH, Chen ST, Li SX, Cai H (2009) A combined DNA vaccine provides protective immunity against Mycobacterium bovis and Brucella abortus in cattle. DNA Cell Biol 28: 191-199.
41. Sanakkayala N, Sokolovska A, Gulani J, Hogenesch H, Sriranganathan N, et al. (2005) Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant Brucella abortus RB51. Clin Diagn Lab Immunol 12: 1429-1436.
42. Vemulapalli R, Sanakkayala N, Gulani J, Schurig GG, Boyle SM, et al. (2007) Reduced cerebral infection of Neospora caninum in BALB/c mice vaccinated with recombinant Brucella abortus RB51 strains expressing N. caninum SRS2 and GRA7 proteins. Vet Parasitol 148: 219-230.
43. Sabio y Garcia JV, Bigi F, Rossetti O, Campos E (2010) Expression of MPB83 from Mycobacterium bovis in Brucella abortus S19 induces specific cellular immune response against the recombinant antigen in BALB/c mice. Microbes Infect 12: 1236-1243.
44. Sabio y Garcia JV, Farber M, Carrica M, Cravero S, Macedo GC, et al. (2008) Expression of Babesia bovis rhoptry-associated protein 1 (RAP1) in Brucella abortus S19. Microbes Infect 10: 635-641.
45. Alton GG, Jones LM, Angus RD, Verger JM (1988) Techniques for the Brucellosis Laboratory. Paris: Institut National de La Recherche Agronomique.
46. Brasil (2004) Regulamento Técnico do Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal. In: Secretaria de Defesa Agropecuária MdA, Pecuária e Abastecimento, editor. Brasilia: Diário Oficial da União. pp. 6-10.
47. Brasil (2007) Estabelece condições para vacinação de fêmeas bovinas contra brucelose, utilizando vacina não indutora da formação de anticorpos aglutinantes, amostra RB51. In: Secretaria de Defesa Agropecuária MdA, Pecuária e Abastecimento, editor. Brasilia: Diário Ofical da União. pp. 6.
48. Miles AA, Misra SS, Irwin JO (1938) The estimation of the bactericidal power of the blood. J Hyg (Lond) 38: 732-749.
49. Dorneles EM, Araújo MSS, Teixeira-Carvalho A, Martins-Filho OA, Lage AP (2014) Cross-reactivity of anti-human cytokine monoclonal antibodies as a tool to identify novel immunological biomarkers in domestic ruminant. Genetics and Molecular Research.

50. Kwong LS, Hope JC, Thom ML, Sopp P, Duggan S, et al. (2002) Development of an ELISA for bovine IL-10. Vet Immunol Immunopathol 85: 213-223.
51. Colby LA, Schurig GG, Elzer PH (2002) An indirect ELISA to detect the serologic response of elk (Cervus elaphus nelsoni) inoculated with Brucella abortus strain RB51. J Wildl Dis 38: 752-759.
52. Myers DM, Jones LM, Varela-Diaz VM (1972) Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by Brucella ovis and other Brucellae. Applied Microbiology 23: 894-902.
53. Chongsuvivatwong V (2012) Epicalc: Epidemiological calculator. 2.15.1.0. ed: R package
54. R RCT (2013) A language and environment for statistical computing. Viena, Austria: R Foundation for Statistical Computing.
55. Siegel S, Castellan NJ (1988) Nonparametric Statistics for the Behavioral Sciences: McGraw-Hill.
56. Srisuradetchai P, Borkowski JJ (2013) Skillings.Mack: The Skillings-Mack test Statistic for block designs with missing observations. 1.0 ed: R package.
57. Sampaio IBM (2002) Estatística aplicada à experimentação animal. Belo Horizonte: Fundação de Ensino e Pesquisa em Medicina Veterinária e Zootecnia.
58. Zhan Y, Yang J, Cheers C (1993) Cytokine response of T-cell subsets from Brucella abortus-infected mice to soluble Brucella proteins. Infect Immun 61: 2841-2847.
59. Araya LN, Elzer PH, Rowe GE, Enright FM, Winter AJ (1989) Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with Brucella abortus. J Immunol 143: 3330-3337.
60. Araya LN, Winter AJ (1990) Comparative protection of mice against virulent and attenuated strains of Brucella abortus by passive transfer of immune T cells or serum. Infect Immun 58: 254-256.
61. Splitter G, Oliveira S, Carey M, Miller C, Ko J, et al. (1996) T lymphocyte mediated protection against facultative intracellular bacteria. Vet Immunol Immunopathol 54: 309-319.
62. Trapani JA, Smyth MJ (2002) Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2: 735-747.
63. Hersperger AR, Makedonas G, Betts MR (2008) Flow cytometric detection of perforin upregulation in human CD8 T cells. Cytometry A 73: 1050-1057.
64. Oliveira SC, Splitter GA (1995) CD8+ type 1 CD44hi CD45 RBlo T lymphocytes control intracellular Brucella abortus infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice. Eur J Immunol 25: 2551-2557.
65. Hirota K, Martin B, Veldhoen M (2010) Development, regulation and functional capacities of Th17 cells. Semin Immunopathol 32: 3-16.
66. Stevens MG, Olsen SC, Pugh GW, Jr., Palmer MV (1994) Immune and pathologic responses in mice infected with Brucella abortus 19, RB51, or 2308. Infect Immun 62: 3206-3212.
67. Tobias L, Schurig GG, Cordes DO (1992) Comparative behaviour of Brucella abortus strains 19 and RB51 in the pregnant mouse. Res Vet Sci 53: 179-183.
68. LaSalle JM, Ota K, Hafler DA (1991) Presentation of autoantigen by human T cells. J Immunol 147: 774-780.
69. Murphy EA, Parent M, Sathiyaseelan J, Jiang X, Baldwin CL (2001) Immune control of Brucella abortus 2308 infections in BALB/c mice. FEMS Immunol Med Microbiol 32: 85-88.
70. Brandao AP, Oliveira FS, Carvalho NB, Vieira LQ, Azevedo V, et al. (2012) Host susceptibility to Brucella abortus infection is more pronounced in IFN-gamma knockout than IL-12/beta2-microglobulin double-deficient mice. Clin Dev Immunol 2012: 589494.
71. Pasquevich KA, Ibanez AE, Coria LM, Garcia Samartino C, Estein SM, et al. (2011) An oral vaccine based on U-Omp19 induces protection against B. abortus mucosal challenge by inducing an adaptive IL-17 immune response in mice. PLoS One 6: e16203.
72. Siegrist C-A (2013) 2 - Vaccine immunology. In: Offit SAPAOA, editor. Vaccines (Sixth Edition). London: W.B. Saunders. pp. 14-32.
73. Diaz R, Jones LM, Leong D, Wilson JB (1968) Surface antigens of smooth brucellae. J Bacteriol 96: 893-901.
74. Estes DM, Brown WC (2002) Type 1 and type 2 responses in regulation of Ig isotype expression in cattle. Vet Immunol Immunopathol 90: 1-10.
75. Xavier MN, Winter MG, Spees AM, Nguyen K, Atluri VL, et al. (2013) CD4(+) T Cell-derived IL-10 Promotes Brucella abortus Persistence via Modulation of Macrophage Function. Plos Pathogens 9.
76. Hoek A, Rutten VP, Kool J, Arkesteijn GJ, Bouwstra RJ, et al. (2009) Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. Vet Res 40: 6.

77. Travis MA, Sheppard D (2014) TGF-beta activation and function in immunity. Annu Rev Immunol 32: 51-82.
78. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011) The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1813: 878-888.

SUPPLEMENTARY FIGURES

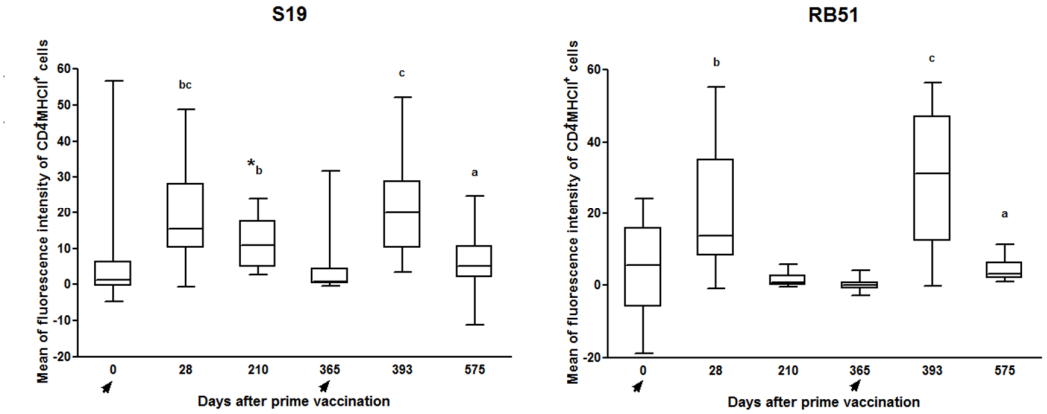

Figure S1. Mean of fluorescence intensity of MHC class II on $CD4^+$ T-cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with ɣ-irradiated *B. abortus* 2308. The results are expressed box plot chart. The day when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

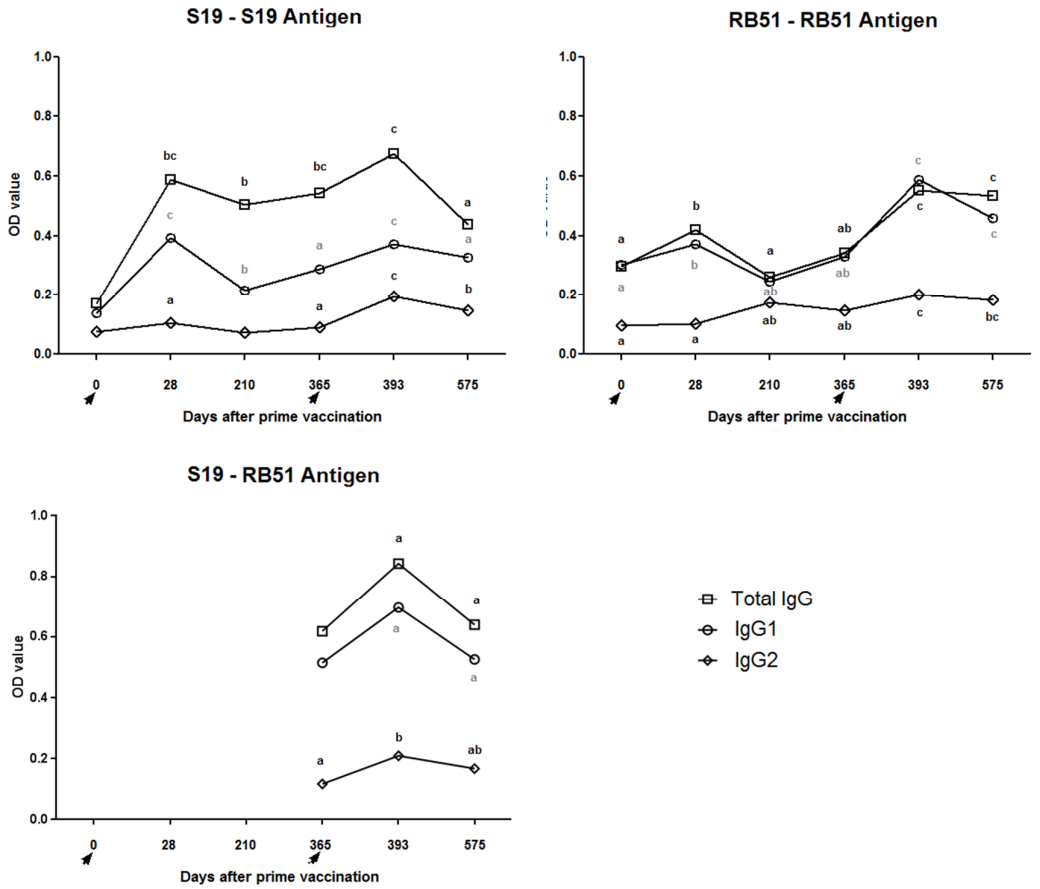

Figure S2. Immunoglobulin profile of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle measured by I-ELISA using S19 and RB51 lysed heat-killed antigens. The results are expressed as mean. Data for total IgG are shown by ( ), for IgG1 by ( ) and for IgG2 by ( ). The day when the vaccinations occurred are highlighted with arrows. Lowercase letters indicate statistical difference between days in same group (ANOVA followed by paired t-test).

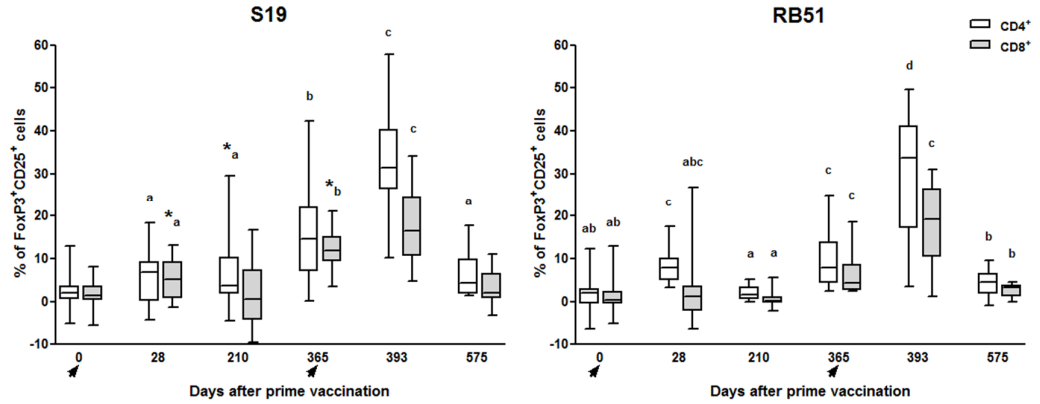

Figure S3. Expression of FoxP3 and CD25 by $CD4^+$ (white box plot) and $CD8^+$ (light gray box plot) T-cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with ɣ-irradiated *B. abortus* 2308. The results are expressed in box plot chart. The day when the vaccinations occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

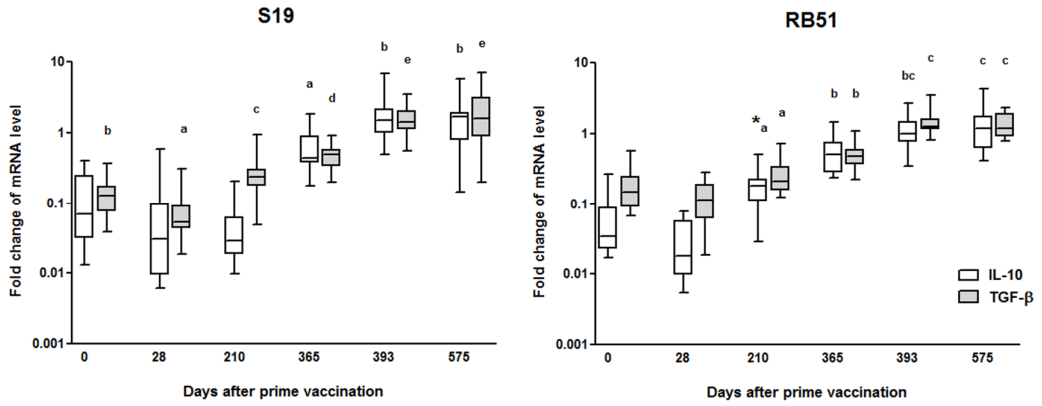

Figure S4. mRNA level of IL-10 and TGF-β in peripheral blood mononuclear cells of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle upon in vitro stimulation with γ-irradiated *B. abortus* 2308. The results are expressed box plot chart. Data of PBMC mRNA level of IL-10 (white box plots) and TGF-β (light gray box plots) are shown. The day when the RB51 revaccination occurred are highlighted with arrows. Significant differences ($P < 0.05$) between vaccination regimens (in same day) are indicated by asterisks (Mann-Whitney-test), and lowercase letters indicate statistical difference between days in same group (Skillings Mack test followed by Wilcoxon signed rank test).

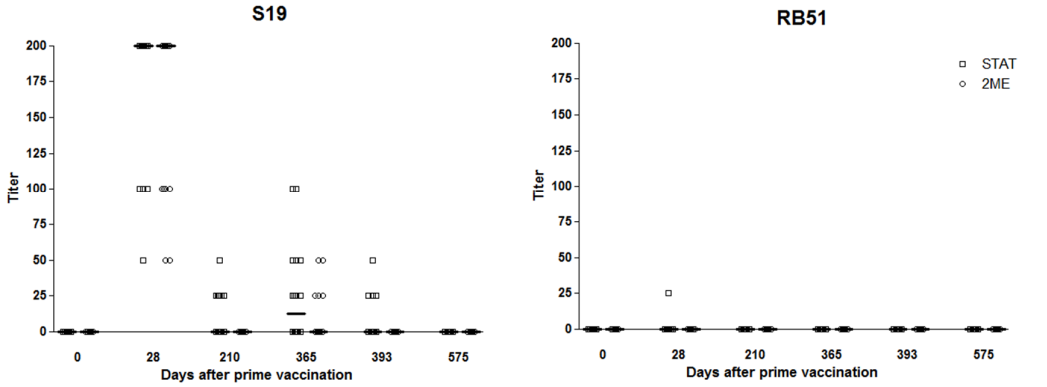

Figure S5. Serologic titer of S19 and RB51 prime vaccinated, and RB51 revaccinated cattle in 2-Mercaptoetanol (2ME) and standard tube agglutination test (STAT).

OVERALL CONCLUSIONS

Overall, the present results showed that in cattle the immune response to S19 or RB51 vaccination is characterized by proliferation of specific $CD4^+$ and $CD8^+$ T-cells; IFN-γ and IL-17A production, mainly by $CD4^+$ T-cells; cytotoxic activity exercised by $CD8^+$ T-cells; IL-6 secretion; induction of $CD4^+$ and $CD8^+$ memory cells; production of immunoglobulin, mainly of IgG1 class; and expression of the phenotypes of activation in T-cells. The main differences in the immune response stimulated by S19 compared to RB51 were the higher persistency of the IFN-γ response and $CD4^+$ memory cells, induction of $CD21^+$ memory cells and higher secretion of IL-6. After RB51 revaccination, the immune response was chiefly characterized by increase in IFN-γ expression, proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells, cytotoxic $CD8^+$ T-cells and decrease in IL-6 production in both groups. However, a different polarization of the immune response, $CD4^+$- or $CD8^+$-dominant, was observed after the booster with RB51, for S19 and RB51 prime-vaccinated animals, respectively. Compared to S19 group after the RB51 booster, RB51 prime -accinated animals exhibited significantly higher proliferation of $CD8^+$ T-cells, cytotoxic phenotype on $CD8^+$ T-cells, expression of IFN-γ by $CD4^+$ and $CD8^+$ T-cells and expression of IL-17A by $CD4^+$ T-cells. Our results indicate that after first vaccination both vaccine strains (S19 and RB51) induce a strong and complex immune response dominated by Th1 profile, although after RB51 revaccination the differences between immune profiles induced by prime-vaccination become more accentuated.