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Abstract: *Brucella canis* is a small intracellular Gram-negative bacterium that frequently leads to chronic infections highly resistant to antibiotic therapy in dogs. Also, it causes mild human brucellosis compared to other zoonotic *Brucella* spp. Herein we characterize the cellular immune response elicited by *B. canis* by analysing human and canine CD4+ T cells after stimulation with autologous monocyte-derived dendritic cells (MoDCs). Human and canine *B. canis*-primed MoDCs stimulated autologous CD4+ T cells; however, a Th1 response was triggered by human MoDCs, whereas canine MoDCs induced Th1/Th17 responses, with increased CD4+ T cells producing IFN- γ and IL-17A simultaneously. Each pattern of cellular response may contribute to host susceptibility, helping to understand the differences in *B. canis* virulence between these two hosts. In addition, other aspects of canine immunology are unveiled by highlighting the participation of IL-17A- producing canine MoDCs and CD4+ T cells producing IFN- γ and IL-17A.

Professor

Dr. James Gilkerson

Editor-in-Chief

Comparative Immunology, Microbiology & Infectious Diseases

University of Melbourne

Melbourne, Victoria

Australia

August 10th, 2018

Dear Professor Gilkerson:

Please, find enclosed our work entitled "*Brucella canis* induces canine CD4⁺ T cells multi-cytokine Th1/Th17 production via dendritic cell activation" for your consideration to be published in **Comparative Immunology, Microbiology & Infectious Diseases**.

We certificate that our manuscript is original, has not been published elsewhere, either completely, in part, or in another form and it is not presently under consideration for another journal. Furthermore, it is free of conflict of interest, has been conducted by the highest principles of human and animal welfare and read and approved by all of the authors.

Yours sincerely,

Rolando Vernal
Corresponding Author

Highlights:

- Canine monocyte-derived DCs produced IL-12, IFN- γ , TNF- α and IL-17A against *B. canis*.
- Human *B. canis*-primed DCs induced Th1 cytokine production on autologous CD4⁺ T cells.
- Canine *B. canis*-primed DCs induced Th1 and Th17 cytokine production in autologous CD4⁺ T cells.
- Canine CD4⁺ T cells producing IFN- γ and IL-17A simultaneously were induced after stimulation with autologous *B. canis*-primed DCs.

***Brucella canis* induces canine CD4⁺ T cells multi-cytokine Th1/Th17 production via dendritic cell activation**

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Short Title: *B. canis*-induced canine DCs produce Th1/Th17 cytokines

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1 ***Brucella canis* induces canine CD4⁺ T cells multi-cytokine Th1/Th17**
2 **production via dendritic cell activation**

3 **ABSTRACT**

4 *Brucella canis* is a small intracellular Gram-negative bacterium that frequently leads to
5 chronic infections highly resistant to antibiotic therapy in dogs. Also, it causes mild human
6 brucellosis compared to other zoonotic *Brucella spp.* Herein we characterize the cellular
7 immune response elicited by *B. canis* by analysing human and canine CD4⁺ T cells after
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11 increased CD4⁺ T cells producing IFN- γ and IL-17A simultaneously. Each pattern of
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16 **Keywords:** *Brucella canis*; CD4⁺ T cells; dendritic cells; host susceptibility; cytokines.

1. Introduction

B. canis is the main cause of canine brucellosis, a worldwide disseminated disease and highly resistant to antibiotic therapy mainly due to its intracellular location [1]. In the dog (*Canis lupus familiaris*), the primary host for *B. canis*, this infection causes granulomatous lesions in several organs, it is the main cause of reproductive disorders and usually leads to osteodegenerative consequences [2]. Although it is a zoonosis, there are only occasional human case reports, which are characterized by mild and nonspecific symptoms that can be self-limiting or effectively controlled using antibiotic therapy [3].

Numerous immune-mediated mechanisms have been described for other zoonotic *Brucella* spp., including: 1) capacity to evade recognition by receptors expressed in innate immune cellular system, suppressing activation of phagocytic mechanisms [3], 2) persistent bacterial survival in phagocytes by blocking the complete endosome and lysosome fusion, promoting formation of vesicles that allow replication and establishment of bacterial cellular niches [4] and 3) interaction with antigen-presenting cells such as macrophages and DCs thus interfering with cellular activation and antigenic presentation to CD4⁺ T cells, inhibiting the production of Th1 polarizing cytokines and the development of an effective cellular immune response [5]. As a consequence, they produce chronic infections in their primary hosts and humans [6]. These processes have been described for smooth *Brucella* spp. such as *B. melitensis*, *B. abortus*, and *B. suis*; however, many immune aspects related with naturally rough species, such as *B. canis*, are unknown [7, 8].

We previously proposed that although both human and canine MoDCs are activated after stimulation with *B. canis* or its purified LPS, their profiles of cytokine production are

different. Th1 polarizing cytokines are produced by human MoDCs, while in canine cells Th1 and Th17-type cytokines are produced [9]. However, the nature of the responses that those stimulated MoDCs trigger remains unexplained for both species. Therefore, the work presented here naturally follows our previous results, investigating CD4⁺ T cell responses after encountering *B. canis*-primed MoDCs using an *in vitro* model. These results contribute to the understanding of the immune-pathogenesis of *B. canis* since they provide relevant information for therapeutic or preventive alternatives for the efficient control of brucellosis.

2. Material and Methods

2.1. B. canis culture and LPS purification

The *B. canis* strain SCL (GenBank accession number: LGAQ000000000.1) was cultured on 5% bovine serum tryptic soy broth (TSB) (Oxoid Ltd, Hampshire, England) at 37 °C under aerobic conditions. For MoDCs stimulation, *B. canis* cultures, at exponential growth phase, were used. Bacterial concentration was estimated by comparing the OD at 560 nm with a standard curve and verified by plating on 5% bovine serum tryptic soy agar (TSA). LPS was isolated by TRIzol reagent (TRIzol[®] Plus; Invitrogen Corp., Barcelona, Spain) as previously described [9]. The preparation was treated with enzymes (proteinase K, RNase and DNase) and subjected to a purification protocol using alcohols and Folch reagent. *B. canis* manipulation was carried out inside biosafety cabinet and the experimental protocol was approved by the Biosafety Committee, North Campus, Universidad de Chile.

2.2. Human blood samples

Buffy coats from 10 healthy blood donors were obtained from the Clinical Hospital José Joaquín Aguirre, University of Chile. All participants provided signed informed consent. Exclusion criteria considered risk factors for brucellosis and other infectious diseases. The study was approved by the Ethics Committee on Human Research, Universidad de Chile.

2.3. Canine blood samples

Blood samples from 10 healthy dogs of different breeds, were obtained by venipuncture into heparin tubes (Vacutainer®; Becton Dickinson and Company, New Jersey, NJ, USA) after clinical, hematological and biochemical evaluation, together with serological tests for exclusion of distemper, parvovirus and *Erlichia canis*. All dogs had no history of infectious diseases and counterimmuno-electrophoresis serological analyses, using *B. ovis* LPS antigen [10] were performed in order to discard the presence of *B. canis*. Female and male canine donors, 1-7 years old, from private owners, regularly dewormed and vaccinated against distemper, leptospirosis, parvovirus, parainfluenza, canine adenovirus type 2, canine hepatitis, and rabies, were used in these studies. Dog owners provided written informed consent approved by the Committee for Animal Care and Use, Universidad de Chile, and all procedures were conducted according to Institutional Ethical Guidelines.

2.4. Dendritic cell generation and stimulation

Human and canine MoDCs were generated as previously described [9]. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) using an anti-human CD14 monoclonal antibody (clone TÜK4) conjugated to magnetic beads (MACS®;

Miltenyi Biotec, Bergisch Gladbach, Germany), previously shown to cross-react with canine CD14 [11]. The CD14⁺ monocytes were seeded at 10⁶ cells/ mL in RPMI-1640 (Life Technologies, Gaithersburg, MD, USA), containing 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (Gibco Invitrogen Corp., Grand Island, NY, USA) and 100 U/mL polymixin B (Sigma-Aldrich, St. Louis, MO, USA). Canine cell cultures were supplemented with 40 ng/mL recombinant human (rh) GM-CSF and 30 ng/mL recombinant canine (rc) IL-4 (R&D Systems Inc., Minneapolis, MN, USA), while human cells cultures were supplemented with 20 ng/mL rhGM-CSF and rhIL-4 (R&D Systems Inc.) every 2 days. Differentiated MoDCs were then primed with live *B. canis* (MOI=200), 1 µg/mL *B. canis* purified LPS or 1 µg/mL LPS from *Escherichia coli* strain 0128:B12 (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. Non-stimulated MoDCs were used as negative control. For each individual, experiments were performed separately.

2.5. CD4⁺ T cell isolation and stimulation

Human and canine CD4⁺ T cells were negatively selected by MACS from the peripheral blood CD14⁻ fraction. The naïve CD4⁺ T Cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) was used for human samples. Canine cells were incubated with anti-canine CD11b (clone CA16.3E10) and anti-human CD21 (clone LT21) previously shown to react with canine CD21 [12] (Invitrogen, Carlsbad, CA, USA), followed by Anti-Mouse IgG MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The purified lymphocytes were kept in culture during the 7 days necessary for the differentiation and activation of MoDCs, replacing 50% of the medium every 48 hours. For CD4⁺ T cell stimulation assays, 10⁶ cells/mL were cultured for 5 days with autologous

primed or non-stimulated MoDCs (ratio 5: 1) previously washed and treated with antibiotics.

2.6. Extracellular staining and proliferation assay

The purity and activation of canine and human CD4⁺ T cells were determined by detection of surface markers and cell proliferation assay using flow cytometry. Prior stimulation, isolated lymphocytes were incubated with 10 µg/mL CellTrace Violet Proliferation kit (Invitrogen, Carlsbad, CA, USA) at 37 °C for 15 minutes and washed twice with PBS/5% FBS. After stimulation with autologous MoDCs, T cells were stained with Zombie aqua viability dye (Biolegend, San Diego, CA, USA). Following two washes and a blocking step with 5% bovine serum, canine or human anti-CD4 (clones YKIX302.9 or OKT4) and anti-CD25 (clones P4A10 or 2A3) were used for detection of surface markers (BD Biosciences Pharmingen, San Jose, CA, USA). The monoclonal antibodies were titrated and diluted in PBS/0.5% BSA. For each experiment, a minimum of 50,000 live/single events were recorded using a LSR FORTESSA X-20 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A total of 3 canine and 3 human samples were analyzed separately, using Fluorescence minus one (FMO) controls and data were expressed as mean ± SD percentage of CD-positive cells and mean fluorescence intensity (MFI) for each surface marker.

2.7. Intracellular staining

Canine MoDCs or CD4⁺ T cells were treated with 5 µg/mL Brefeldin-A at 37 °C for the last 6 hours of culture. Cells were treated with Flow Cytometry Fixation and Permeabilization Buffers (R&D Systems, Minneapolis, MN, USA) according to

manufacturer's instructions. Monoclonal antibodies for canine cytokines: IL-12, IL-17A, IFN- γ and TNF- α (Novus Biologicals, Littleton, CO, USA) were conjugated to different fluorochromes, using the Lightning-Link kits (Innova Biosciences, Cambridge, UK), following manufacturer's instructions. Intracellular labelling was performed by incubating the cells for 30 minutes at 4 °C with antibodies diluted in the permeabilization solution and washed twice with this solution, containing BSA 0.5%. Cells were gated according their forward (FSC) and side (SSC) scatter characteristics, viability staining and the CD11c (clone N418) and CD4 extracellular markers using specific monoclonal antibodies (Invitrogen, Carlsbad, CA, USA). PMA/Ionomycin-treated CD4⁺ T cells were used as positive control (BD Biosciences Pharmingen, San José, CA, USA). Fluorescence minus one (FMO) together with isotype-matched control antibodies were used to determine the negative cell populations and data were expressed as mean $\pm$ SD percentage of positive cells for each intracellular marker. A total of 3 canine MoDC or 3 canine CD4⁺ T cell samples were analyzed separately.

2.8. Expression of cytokines by RT-qPCR

Total cytoplasmic RNA was isolated from stimulated cells using 400 μ L of ice-cold lysis buffer containing 0.5% Igepal[®] CA-630 (Sigma-Aldrich, Saint Louis, MO, USA) as previously described [9]. First-strand cDNA strand was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesis kit for RT-qPCR[®]; Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol. The mRNA expression of IL-1 β , IL-4, IL-10, IL-12, IL-13, IL-17A, IFN- γ , TNF- α and TGF- β 1 was determined using KAPA[™] SYBR[®] Fast qPCR reagent (KAPA Biosystems, Woburn, MA, USA) in a StepOnePlus[®] RT-qPCR equipment (Applied Biosystems, Singapore) [9]. 18S

ribosomal RNA (18S rRNA) expression levels were used as endogenous control. A total of 10 canine and 10 human independent experiments of CD4⁺ T cells were analyzed in duplicate.

2.9. Production of cytokines by ELISA

The supernatants from canine and human CD4⁺ T cells stimulated with MoDCs were collected and the secreted levels of IL-17A, IFN- γ and TNF- α were determined by ELISA assays (R&D Systems, Minneapolis, MN, USA), using an automatic microplate spectrophotometer (Synergy HT; Bio- Tek Instrument Inc., Winooski, VT, USA), following the manufacturer's instructions. Data from 8 independent experiments are shown as mean $\pm$ SD.

2.10. Statistical analysis

The flow cytometry data were analyzed using the FlowJo software v9.6.4 (TreeStar, Ashland, ORE, USA), represented as histograms, and expressed as the percentage of positive cells over the total $\pm$ standard deviation. The RT-qPCR data were analyzed using the StepOne Software v2.2.2 (Applied Biosystems) and the relative quantification was obtained by normalizing the cytokine expression to endogenous control expression using the $2^{-\Delta\Delta C_t}$ method. The ELISA data were calculated using a four-parameter logistic equation. Data were expressed as mean $\pm$ standard deviation and statistically analyzed using the GraphPad Prism software v5.0 (GraphPad software Inc., La Jolla, CA, USA). The normality of data distribution was determined using the Kolmogorov-Smirnov test. The differences between groups were determined using the one-way ANOVA test followed by Tukey or Bonferroni post-hoc test. Statistical significance was assumed when P -value < 0.05.

3. Results

3.1. Increased proportion of canine MoDCs producing IL-12, IFN- γ , TNF- α and IL-17A against *B. canis*.

Previously, our data showed that canine monocyte-derived MoDC cultures expressed and secreted increased amounts of Th1 and Th17-type cytokines, as compared with non-stimulated MoDC cultures [9]. About 90% of the obtained cells expressed superficial markers compatible with MoDC immunophenotype (90% purity). Following up on those aforementioned studies, the production of these cytokines was confirmed in MoDCs using flow cytometry, on a cell per cell basis. Increased proportion of canine MoDCs produced Th1-type cytokines: IL-12, IFN- γ and TNF- α , against live *B. canis*. Also, MoDCs stimulated with live *B. canis* or its purified LPS increased the proportion of MoDCs producing the Th17-type cytokine: IL-17A (Fig. 1).

3.2. Canine and human CD4⁺ T cells are activated by *B. canis* stimulated MoDCs

Once confirmed that canine *B. canis*-primed MoDCs produced Th1 and Th17-type cytokines, and human MoDCs were polarized for Th1-type cytokine production, we asked whether these cells could induce the veering of autologous CD4⁺ T cells towards each respective subset. Canine T cell isolation was confirmed and the purity of cell cultures was compared with that of human cultures by flow cytometry analysis (Fig 2A-B). Nearly 95% of the canine and human live cells corresponded to lymphocytes. Furthermore, after culturing those cells for 7 days in RPMI media, about 99% expressed CD4.

B. canis-primed autologous MoDCs activated human (Fig. 2C) and canine (Fig. 2D) CD4⁺ T cells, shown by an increased proportion of CD25⁺ proliferating cells within the total population of CD4⁺ T cells. By independently analyzing the expression of CD25 (Fig. 2E-H, M-P) or the percentage of proliferating cells within the total CD4⁺CD25⁺ T cells (Fig. 2I-L, Q-T), no significant differences were found between stimuli or between cells of both species. Nevertheless, cells proliferating in response to *B. canis*-primed canine MoDCs reached approximately 30% of the CD4⁺CD25⁺ T cells.

3.3. B. canis primed MoDCs induce Th1 and Th1/Th17 cytokine expression in human or canine CD4⁺ T cells, respectively

Cultures of human CD4⁺ T cells stimulated with *B. canis*-primed MoDCs expressed Th1 cytokines: IL-1 β , IFN- γ and TNF- α (Fig. 3A), whereas canine CD4⁺ T cells were biased towards a Th1/Th17 cytokine profile: IL-17A, IFN- γ and TNF- α (Fig. 3B). No increase in cytokines was detected in human or canine lymphocytes when stimulated with *B. canis* or *E. coli* LPS (data not shown).

The described changes in the cytokine mRNA levels were consistent with the changes in protein levels evaluated by ELISA (Fig. 4). Human CD4⁺ T cells secreted increased level of the Th1 cytokine IFN- γ (Fig. 4A,C,E) whereas canine CD4⁺ T cells secreted increased Th1/Th17 cytokine levels with IFN- γ , TNF- α and IL17A (Fig. 4B,D,F).

3.4. B. canis primed MoDCs induce canine CD4⁺ T cells producing IFN- γ and IL-17A simultaneously.

To elucidate whether the increased levels of IFN- γ and IL-17A were secreted by the same cell or not, the proportion of IFN- γ , IL-17A or IFN- γ /IL-17A producing cells was evaluated within the total of previously stimulated canine CD4⁺ T cells by flow cytometry. A significant increase in IFN- γ IL-17A⁺ and IFN- γ ⁺IL-17A⁺ cells was detected when live *B. canis*-primed autologous MoDCs were used for lymphocyte stimulation. None of the 3 stimuli increased the proportion of IFN- γ ⁺IL-17A⁻ cells (Fig. 5).

4. Discussion

Canine brucellosis is a highly disseminated disease worldwide, is the main cause of canine reproductive alterations and produces severe multisystemic inflammatory lesions [2]. It requires combined antibiotic therapy that may last several months and relapses are frequent [1]. Conversely, the number of human brucellosis cases due to *B. canis* is low, considering the close proximity between both species. It frequently produces mild brucellosis compared to other zoonotic *Brucella spp.*, and antibiotic therapy is effective [3, 2]. A Th1 response is critical for the efficient elimination of *Brucella spp.* [13]. Based on these observations, we hypothesized that humans and dogs may trigger different cellular immune response, explaining at least partly, the different virulence of *B. canis* in both species.

We recently described the expression and secretion of Th1 and Th17-type cytokines, including IL-17A in canine MoDCs stimulated with *B. canis* or its purified LPS [9]. Although some innate immune cells, including $\gamma\delta$ T lymphocytes, iNKT and NK cells produce IL-17A [14], there were no records of IL-17A production in MoDCs from canines or other species prior to this work. Here we confirmed by intracellular staining, the

increment of IL-17A-producing MoDCs upon challenge with *B. canis* or its LPS, in addition to the increase of MoDCs producing: IL-12, IFN- γ and TNF- α against *B. canis* (Fig. 1).

To carry out this investigation, human naïve CD4⁺ T cells were used. Considering the lack of commercial kits for the specific isolation of canine cells, potential donors with risk factors for brucellosis were discarded. To achieve CD4⁺ T cell enrichment, a negative selection protocol was implemented, excluding cell populations by antibody detection of: the monocyte marker CD14 [15], CD21 expressed in B lymphocytes [12] and the myeloid marker CD11b expressed in granulocytes, NK cells and $\gamma\delta$ lymphocytes [16-18]. After 7 days of incubation and without prior depletion of CD8⁺ cells, highly pure cultures of CD4⁺ T cells were obtained (Fig. 2). The higher susceptibility of CD8⁺ lymphocytes to the lack of IL-2 supplementation, in comparison to CD4⁺ lymphocytes [19] could explain this result.

Human and canine *B. canis*-primed MoDCs activated autologous lymphocytes, increasing CD25 expression and cell proliferation (Fig. 2). Human cultures developed a Th1 response against *B. canis*, with increased expression of IL-1 β , IFN- γ and TNF- α ; while a Th1/Th17 profile was triggered in canine lymphocytes, with the expression of IL-17A, IFN- γ and TNF- α (Fig. 3). These results were consistent with the changes in cytokine secretion detected at protein levels (Fig. 4). Considering IFN- γ -producing Th1 lymphocytes are efficient and protective against intracellular infections, such as *Brucella spp*, the development of this cellular response in human cells could partly explain a higher host resistance to *B. canis* infection. Conversely, a Th17 polarization could contribute to the development of granulomatous lesions during canine brucellosis, as occurs in TBC infections [20]. Furthermore, although IL-17A production contributes to eliminate some

intracellular bacteria, such as *L. monocytogenes* or *S. enterica*, it is mainly by granulocyte recruitment [21], which is ineffective against infection with *Brucella spp.*, due to its high resistance to neutrophil killing [22].

Although the development of a Th17 response induced by oral vaccines confers mucosal protection against *Brucella spp.*, the neutralization of IL-17 does not affect bacterial proliferation in non-immunized mice [23]. Moreover, the development of a Th17 response affects host susceptibility and virulence of *Brucella spp.*, impairing an effective Th1 response. Patients with active brucellosis have increased blood levels of IL-17A, that decrease at the end of antibiotic treatment [24]. On the other hand, vaccination with RB51 confers a protective Th1 response against *B. abortus* in young mice, whereas in older mice induces an inefficient Th17 response [25].

Considering that the Th17 phenotype confers some cell plasticity, which can lead to different cell subsets [26], the production of IL-17A and IFN- γ by canine CD4⁺ T cells was evaluated by intracellular staining. *B. canis* induced an increase in CD4⁺ T cells producing IL-17A and also CD4⁺ T cells that simultaneously produced IFN- γ and IL-17A (Fig. 5). Th17 cells that acquire the ability of IL-17/IFN- γ production promote chronicity in inflammatory disorders [26, 27]. *In vitro* stimulation of leukocytes from tuberculosis patients with extracts of *M. tuberculosis* induced increased IL-17 levels compared to healthy vaccinated donors. The main source were CD4⁺ T cells producing IFN- γ and IL-17A, also their presence was directly related with "low responders" patients, with severe pulmonary lesions and a long clinical evolution [28]. Macrophages infected with *Brucella spp.* biased Th1 lymphocytes producing IFN- γ towards an osteoclastogenic Th17 phenotype, responsible for osteoarticular lesions in brucellosis [29]. In addition, the

282 presence of TNF- α enhances the formation of pathogenic CD4⁺ T cells producing IFN- γ
283 and IL-17A, from Th17 cells and promotes IL-17A dependent osteoclastogenesis.
284 Therefore, neutralization of TNF- α improves the symptomatology in a variety of
285 pathologies [27, 26, 30].

286 In other species, CD4⁺ T cells producing IFN- γ and IL-17A are widely associated
287 with autoimmune pathologies, such as: multiple sclerosis, diabetes mellitus, rheumatoid
288 arthritis and Sjögren's syndrome, among others [31]. Canine brucellosis has an
289 autoimmune component, manifested by antibody mediated destruction of sperm [32, 33],
290 which also occurs in rams infected with *B. ovis* [34]. In addition, IL-17 promotes the
291 formation of autoantibodies in other pathologies [35] which could be plausible in the case
292 of *B. canis* and the results presented here. Further investigations are required to confirm this
293 hypothesis.

294 In synthesis, our results suggest that different cellular responses in dogs and humans
295 could partially determine the greater susceptibility of domestic canines to *B. canis*
296 infection, with severe symptoms such as osteoarticular and granulomatous lesions, thus
297 promoting chronic infections. A Th1/Th17 response in canines could interfere with the
298 development of an effective Th1 response, also CD4⁺ T cells producing IFN- γ and IL-17A
299 could represent a pathogenic phenotype, which contributes to the inflammatory and
300 autoimmune processes of canine brucellosis, whereas the development of a Th1 response in
301 humans would confer a greater resistance and efficiency to this species against *B. canis*
302 infection.

303 **Competing interests**

304 The authors declare that they have no competing interests.

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Figure legends

Fig. 1. Intracellular detection of Th1 and Th17-type cytokines in *B. canis* stimulated canine MoDCs. Representative dot plots show the proportion of MoDCs producing cytokines was assessed by flow cytometry analysis. Canine MoDCs were identified according to FSC and SSC parameters, together with CD11c expression and the use of a Live/Dead staining reagent. Intracellular cytokine detection was determined in MoDCs stimulated with Live *B. canis* (B.C) or LPS from B.C or *E. coli* (E.C) and compared to FMO (fluorescence minus one) with isotype control.

Fig. 2. Activation of human and canine CD4⁺ T cells by *B. canis* stimulated MoDCs. Flow cytometry analysis of human and canine CD4⁺ T cells after 5 days of incubation with Live *B. canis* (B.C) or LPS (B.C or E.C) stimulated autologous MoDCs. First, the purity of human (A) and canine (B) lymphocyte cultures was confirmed according to FSC and SSC parameters, along with Live/Dead staining and CD4 expression. Human (C) and canine (D) CD4⁺ T cell activation was determined according to CD25 expression and cell proliferation. The single expression of CD25 is shown for human (E-H) and canine (M-P) cells. The percentage of cells proliferating within the total population of CD4⁺ T cells is also presented for human (I-L) and canine (Q-T) cultures.

Fig. 3. Different cytokine expression pattern in human or canine CD4⁺ T cells induced by *B. canis* stimulated MoDCs. RT-qPCR analysis shows cytokine quantification represented as mRNA fold-change in human (A) and canine (B) CD4⁺ T cells after 5 days of incubation with autologous *B. canis* stimulated MoDCs. The cytokine mRNA expression

in CD4⁺ lymphocytes incubated with non-stimulated MoDCs was considered as 1, as a reference for fold-change in expression (N.S.).

Fig. 4. Cytokine secretion detection in human or canine CD4⁺ T cells against *B. canis*. ELISA analysis of supernatants from CD4⁺ T cells incubated with live *B. canis* (B.C) or LPS (B.C or E.C) stimulated MoDCs. Quantification of IL-17A, IFN- γ and TNF- α from human (A) and canine (B) cell cultures is represented as pg/mL. CD4⁺ lymphocytes incubated with non-stimulated MoDCs were considered as negative controls.

Fig. 5. Intracellular staining of IFN- γ and IL-17 in canine CD4⁺ T cells induced by *B. canis* stimulated MoDCs. Representative flow cytometry dot plots, showing the percentage of canine CD4⁺ T cells expressing IFN- γ and IL-17A after 5 days of incubation with *B. canis* (A), *B. canis* LPS (B), *E. coli* LPS (C) stimulated MoDCs or non-stimulated (D) autologous MoDCs. The bar graph shows the differences in the percentage increase of cells expressing IFN- γ , IL-17A and IFN- γ /IL-17A against different stimuli (E). PMA/ionomycin-treated CD4⁺ T cells were used as positive control for intracellular staining of IFN- γ (F) or IL-17A (G).

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