



Immunological mechanisms involved in the protection against development of pulmonary tuberculosis in naturally infected goats

Irene Agulló-Ros^a, Rita Vaz-Rodrigues^b, Mercedes Domínguez^c, Álvaro Roy^d, Javier Ortega^d, Inmaculada Moreno^c, Javier Bezos^{d,e}, Lucas Domínguez^e, Isabel G. Fernández de Mera^{b,*}, María A. Risalde^{a,f}

^a Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología, Grupo de Investigación GISAZ, UIC Zoonosis y Enfermedades Emergentes ENZOEM, Universidad de Córdoba, Córdoba, Spain

^b SaBio, Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ronda de Toledo s/n, Ciudad Real, Spain

^c Unidad de Inmunología Microbiana, Centro Nacional de Microbiología, Instituto de Investigación Carlos III, Majadahonda, Madrid, Spain

^d Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Madrid 28040, Spain

^e VISAVET Health Surveillance Centre, Complutense University of Madrid, Madrid, Spain

^f CIBERINFEC, ISCIII - CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain

ARTICLE INFO

Keywords:
Cytokine
Goats
Granuloma
Lung
Immune response
Tuberculosis

ABSTRACT

Tuberculosis (TB) is a notifiable zoonotic disease caused by bacteria of the *Mycobacterium tuberculosis* complex (MTBC) that affects a multitude of domestic and wild species. The main lesions caused by these mycobacteria are tuberculous granulomas, which determine the organism's immune response to the disease. Although TB pathogenesis in cattle has been extensively studied, information regarding its progression in other species of interest for the maintenance and transmission of TB such as goats remains limited. This study aimed to characterise the immune response developed in the lungs of goats naturally infected with mycobacteria of MTBC by assessing key cell populations and immunomodulatory molecules involved in defending against TB. Hence, twelve 6–12-month-old Guadarrama kid goats, initially TB-free, were selected and exposed to *M. bovis* through close contact with other infected goats. Only animals that tested positive by any of the TB diagnostic methods at the end of the experiment were included in the final analysis (n = 9). Gross and microscopic lesions compatible with TB (TBL) in different organs, as well as local response to TB in lungs were evaluated. Our results revealed that after five months of exposure, 44.4 % (4/9) of the *M. bovis*-infected animals exhibited TBL in the lungs (TBLL+), characterized by a predominance of non-cavitary necrotic granulomas. TBLL+ animals showed significantly higher presence of neutrophils, macrophages (MΦs) and lymphocytes along with greater expression of interferon (IFN)- γ . Conversely, the remaining animals did not present macroscopic or microscopic TBL in the lungs (TBLL-) (5/9). However, these goats displayed elevated expression of toll-like receptors (TLR)2 and TLR4 alongside heightened expression of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and interleukin (IL)-10. These results suggest the potential development of an effective immune response that may suppress or delay of TBL in infected animals. Further research is needed to elucidate how these molecules, which are involved in the defence against MTBC, confer protection and modulate their expression during infection for TB control.

1. Introduction

Animal tuberculosis (TB) is a global zoonosis with severe public health challenges which is reemerging in high-income countries (Huang and Zhao, 2022). This chronic inflammatory disease triggered by bacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) can affect humans and a broad range of domestic and wild animals (Thomas

et al., 2021; Balseiro et al., 2020c). Several studies have underscored the high susceptibility of goats to infection by MTBC species, as *Mycobacterium bovis* (*M. bovis*) and *M. caprae* (Thomas et al., 2021), revealing a wide variety of spoligotypes shared with numerous other species, including cattle, sheep, pigs, red deer, wild boar and even humans (Rodríguez et al., 2011; Varela Castro et al., 2021). This observation elucidates potential cross-species transmission and emphasizes the

* Corresponding author.

E-mail address: Marialabel.Garcia@uclm.es (I.G. Fernández de Mera).

<https://doi.org/10.1016/j.vetmic.2024.110320>

Received 30 July 2024; Received in revised form 9 October 2024; Accepted 28 November 2024

Available online 29 November 2024

0378-1135/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

pivotal role of goats as reservoirs for TB (Jiménez-Martín et al., 2024).

Tuberculous compatible lesions (TBL) are compact and organized structures formed by the aggregation of inflammatory cells such as macrophages, epithelioid cells, multinucleated giant cells, lymphocytes, and neutrophils, often surrounding a caseous necrotic core (Thomas et al., 2021; Sholeye et al., 2022). The pulmonary manifestation represents the most prevalent form of TB, with the lungs serving as the primary site for TBL development (Dergal et al., 2023). The high TB transmission rates observed in goats can be attributed to several factors, including the rapid progression of the disease to a stage of generalization of TB granulomas and the formation of cavitory structures in the lung (Sanchez et al., 2011). These characteristic type of TBL are associated to an increased mycobacterial burden and the development of open TB, allowing access to the bronchial tree and an eventual mycobacterial dissemination into the environment (Sanchez et al., 2011).

Individual TB granulomas are dynamic and heterogeneous structures where the local host response plays a critical role in determining the outcome of the infection, often exerting a greater influence of systemic immune responses (Palmer et al., 2019). In this context, the host's defence mechanism against TB entails a complex orchestration among various immune cell types and immunological mediators, where neutrophils and macrophages (MΦs) assume a pivotal role as the first-line of defence (Palmer et al., 2022). MTBC members are phagocytized by MΦs via recognition by toll-like receptors (TLRs), ultimately favouring an intracellular infection (Romero-Adrian, 2015). Infected MΦs subsequently secrete tumor necrosis factor (TNF)- α , facilitating the recruitment of T lymphocytes to the infection site (Sholeye et al., 2022). These T cells execute effector functions and release cytokines such as interferon (IFN)- γ and interleukin (IL)-2, promoting M1 MΦs activation (Romero-Adrian, 2015). This polarization induces the expression of bactericidal enzymes, like inducible nitric oxide synthase (iNOS), thereby enhancing their capacity to eliminate mycobacteria (Palmer et al., 2015; Sholeye et al., 2022). Conversely, the release of IL-10 and transforming growth factor (TGF)- β cytokines by T lymphocytes drives the polarization of M2 MΦs, which exhibit anti-inflammatory properties and engage in tissue remodelling activities (Palmer et al., 2022). Several studies underscore that the balance between pro-inflammatory and anti-inflammatory activities are critical for determining TB disease progression and outcome (Palmer et al., 2022). While numerous studies in cattle have been conducted (Palmer et al., 2015, 2022; Salguero, 2018), information on TB immunopathology in goats remains limited. Thus, the aim of this study was to characterise the immune response developed in the lungs of goats naturally infected with MTBC by assessing the main cell populations and immunomodulatory molecules involved in the defence against TB during recent stages of the disease.

2. Materials and methods

2.1. Study design

Twelve goats of Guadarrama-breed (6–12 months old) were selected from a TB-free herd located in central Spain, where strict biosecurity measures and artificial rearing were implemented. The TB-free status of the herd was confirmed prior to the study through negative results from the single intradermal tuberculin (SIT) test, comparative intradermal tuberculin (CIT) test, a commercial interferon-gamma (IFN- γ) release assay (IGRA), and an in-house indirect ELISA. Subsequently, the selected animals were moved to an experimental farm, where they cohabited in the same enclosure, sharing food and water, for five months in the farm facilities with 30 donor goats from a high prevalence herd ($n = 149$), as determined by the results of SIT test (74.49 % of reactors), IGRA (48.99 % of reactors) and P22 ELISA (79.86 % of reactors). The infection in the herd and donor goats was confirmed by post-mortem bacteriological culture, identifying *M. bovis* spoligotype SB0121. The environmental circulation of mycobacteria within in the experimental farm was confirmed through environmental samples collected from feeders,

drinkers, salt blocks and milking parlours as previously described (Roy et al., 2019). The experimental farm was isolated, with no neighbouring farms in the surrounding area and the facility adhered to Biosafety Level 2 (BSL-2 +) standards. Personnel strictly followed biosecurity measures in accordance with BSL2 + guidelines, including the use of disposable coveralls, gloves, masks, and head coverings, to prevent infection. Cellular and humoral responses to natural exposure were evaluated in the selected animals throughout the study period (Fig. 1).

At 5 months after exposure (T5), all animals were sedated by means of an intravenous injection of xylazine at 0.75 mL/50 kg (2 % Xilagesic, Calier SA, Barcelona, Spain), and subsequently euthanized with an intravenous injection of T-61 at 5 mL/50 kg (Merck Sharp & Dohme Animal Health, S.L., Salamanca, Spain). The goats were then subjected to a systematic necropsy to evaluate the presence and extension of TBL in the following tissues: lung and associated lymph nodes (LNs) (tracheobronchial and mediastinal), tonsils, mandibular and retropharyngeal LNs, mesenteric and ileocecal LNs, spleen, liver, kidneys, ileocecal valve and jejunum. Moreover, other organs were evaluated when any suspicious TBL was observed. Tissue samples from pulmonary LNs and lung lobes were embedded in RNAlater and frozen at -80°C for PCR analysis. Samples of lung lobes for histopathology and immunohistochemical studies were fixed in 10 % neutral buffered formalin for 24–48 hours (h) and subsequently dehydrated and embedded in paraffin using an automatic processor.

All handling, testing and sampling procedures were conducted by qualified veterinarians in accordance with European (Directive 2010/63/UE) and Spanish (RD53/2013) legislation. This study was authorized by an institutional ethical committee and approved by the local authorities (PROEX 245.8/20, Comunidad de Madrid).

2.2. Intradermal tuberculin test

The SIT and CIT tests were conducted on each animal included in the study in the origin farm at T-1 (where T# indicates different time points in months before or after the animals were moved to an experimental farm), T0 (exposure), T3 and T5 (end point), using a Dermojet syringe (Akra Dermojet, Pau, France), as described previously (Roy et al., 2019) (Fig. 1). Briefly, bovine purified protein derivative (bPPD) and avian purified protein derivative (aPPD) (0.1 mL; CZ Vaccines, O Porriño, Spain) were inoculated on the left-medial and right-medial side of the neck, respectively, and the reactions were interpreted 72 h later by the same veterinarian. All tests were performed according to the protocol published by the European Union Reference Laboratory (EU-RL) for bovine TB (EURL, 2021). Animals were considered positive to SIT test when their skin fold thickness increased by 4 mm or more, or if clinical signs such as pain, exudation, oedema or necrosis were detected. Animals were considered positive to CIT test, when the bPPD reaction was larger than the aPPD reaction by more than 4 mm or clinical signs were observed on the bPPD inoculation site.

2.3. IGRA test

Blood samples were collected using evacuated tubes (BD Vacutainer Becton, Dickinson and Company, Franklin Lakes, USA) with lithium heparin from the jugular vein at T-1, T0 (exposure), T1, T3 and T5 (end point) (Fig. 1). These samples were then stimulated with bPPD and aPPD at a final concentration of 20 $\mu\text{g}/\text{mL}$ (CZ Vaccines, Porriño, Spain) as described previously (Bezós et al., 2011). Analysis of IFN- γ production in plasma was performed using a commercial IGRA (Bovigam TB kit, Thermo Fisher Scientific, Waltham, USA), following the manufacturer's instructions, and the results were interpreted as described in previous studies (Roy et al., 2019).

2.3.1. Serology

Blood samples at T-1, T0 (exposure), T1, T3 and T5 (end point) were centrifuged (1500 g for 10 min) and sera stored at -20°C until the

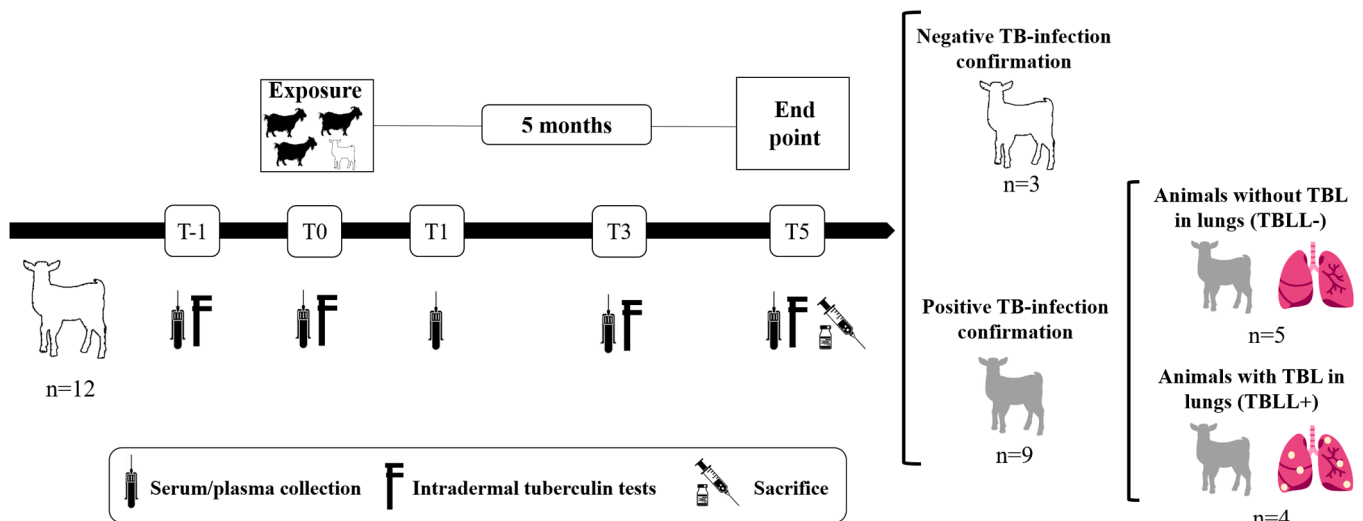


Fig. 1. Summary of the experimental design. White kid goat silhouettes represent the receptor animals, black goat silhouettes represent the donor goats infected with *M. bovis*, and grey goat silhouettes represent the kid goats infected with *M. bovis*. T: months after exposure.

assay to detect antibodies against MTBC (Fig. 1). The in-house indirect ELISA using a purified protein complex from bovine PPD (CZ Vaccines, Porriño, Spain), named P22, was performed as described previously (Roy et al., 2019). The results were expressed as an ELISA percentage (E %), calculated with the following formula: $[\text{sample E\%} = (\text{mean sample optical density (OD)}/2 \times \text{mean of negative control OD}) \times 100]$. Serum samples with E% values greater than 150 were considered positive, and those with E% values between 80 and 150 were considered inconclusive.

2.4. Bacteriology

A pool of lung and tracheobronchial and mediastinal LNs for each animal was placed in a sterile recipient at T5 and stored at -80°C until its bacteriological culture. The culture of samples was performed as previously described Roy et al. (2019). Briefly, the samples were decontaminated with 0.37 % hexadecylpyridinium chloride and then cultured on Coletsos and 0.2 % (w/v) pyruvate-enriched Löwenstein-Jensen media (BioMérieux, Madrid, Spain). Isolates were identified as MTBC using conventional PCR and/or DVR-spoligotyping (Kamerbeek et al., 1997).

2.5. Gross lesions and histopathology

All goats underwent systematic necropsy to assess the presence of TBLs. The gross TBL scoring system was applied in lungs based on the percentage of each lung lobe affected: 0 (no TBL visible), 1 (under 25 % of the lobe affected), 2 (25–50 %), 3 (50–75 %), and 4 (> 75 %). An additional point was assigned to animals with pleural adhesions. The total lung score was calculated by summing the scores of the individual lobes (left apical, left diaphragmatic, right apical, right cardiac, right accessory and right diaphragmatic). In the remaining organs, only was considered the presence or absence of TBLs. Animals presenting gross TB lesions in any tissue were classified as TBL+.

For histopathological analysis, sections were cut at $3\ \mu\text{m}$ and stained with haematoxylin-eosin (HE), using standard procedures. Histopathological TBL in each lung lobe were identified following the criteria described by Wangoo et al., (2005). TB granulomas were divided in four stages (I-IV) based on its size and composition: stage I (initial); stage II (solid); stage III (minimal necrosis); and stage IV (necrosis and mineralisation). Then, the granulomas were grouped into two categories: solid granulomas—comprising the early stages I and II— and necrotic granulomas—comprising the advanced stages III and IV—.

2.6. Immunohistochemistry (IHC)

For the immunohistochemical study, the right caudal lung lobe was selected due to the higher frequency and increased severity of TBL reported in affected animals. Polymorphonuclear neutrophils, activated MΦs and lymphocytes were evaluated and quantified using the avidin-biotin-peroxidase complex (ABC) method, as previously described (Agulló-Ros et al., 2022). Briefly, tissue Section ($3\ \mu\text{m}$) were dewaxed and rehydrated. Endogenous peroxidase activity was exhausted by incubation with 0.3 % hydrogen peroxide in methanol for 30 min at room temperature (RT). Samples were subjected to different pre-treatments for antigen retrieval, depending on the primary antibodies used (Table 1). Subsequently, sections were rinsed three times in PBS (pH 7.2) for 10 min, covered with 1 % normal horse serum (Pierce-Endogen, Woburn, USA) in 0.05 M Tris buffered saline (TBS) (pH 7.6) or 20 % normal goat serum (Thermo Fisher Scientific, Massachusetts, USA) for secondary mouse and rabbit antibodies, respectively, for 30 min at RT. Then slides were incubated with the primary antibodies used (Table 1) overnight at 4°C . After primary incubation, slides were washed in PBS (three times, 5 min each), then incubated with the secondary antibodies for 30 min at RT. Biotinylated horse anti-mouse IgG secondary antibody (Pierce-Endogen, Woburn, USA) diluted 1:200 in TBS containing 1 %

Table 1

Details of the primary antibodies used in the immunohistochemical study.

Specificity	Antigen or cell detected	mAb/pAb	Dilution	Pre-treatment	Source
Anti-human Lysozyme	Neutrophils	pAb	1:200	Proteinase K ^a	Dako/Agilent
Anti-rabbit iNOS	M1 macrophages	pAb	1:100	CB-autoclave ^b	Merck/Millipore
Anti-human CD163	M2 macrophages	mAb	1:200	CB-autoclave ^b	Bio-Rad/AbD
Anti-human CD3	T lymphocytes	pAb	1:100	CB-microwave ^c	Serotec/Dako/Agilent
Anti-human CD79α	B lymphocytes	mAb	1:25	CB-microwave ^c	Dako/Agilent

mAb: monoclonal antibody; pAb: polyclonal antibody; CB: citrate buffer

^a Incubation with 0.2 % proteinase K (Sigma-Aldrich Chemie, Buchs, Switzerland) in Tris buffer for 8 min at 37°C in oven.

^b Incubation with 0.1 M tri-sodium citrate dihydrate (pH6), autoclaved for 10 min at 121°C and 1 atm.

^c Incubation with 0.1 M tri-sodium citrate dihydrate (pH 6), microwave for 6 min at sub-boiling temperature.

normal horse serum was used for primary mouse antibodies, and biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, CA, USA) diluted 1:200 in PBS containing 10 % normal goat serum for primary rabbit antibodies. After three further 5 min washes in PBS, the samples were incubated with ABC complex (Vectastain® ABC Elite Kit, Vector Laboratories, CA, USA) for 1 h at RT. All tissue sections were rinsed in TBS and incubated with chromogen solution (NovaRED® Substrate Kit, Vector Laboratories). Finally, the slides were counterstained with Harris' haematoxylin. Details of primary monoclonal and polyclonal antibodies previously tested on goats can be found in Table 1. Slides with lungs from goats experimentally infected with *M. caprae* were used as positive controls. Non-immune mouse or rabbit sera (Dako/Agilent, Glostrup, Denmark) were used instead of specific primary antibodies as additional negative controls.

To assess the number of immunolabeled cells and to compare results obtained using different antibodies, one paraffin-wax block from the right caudal lung lobe of each animal was selected. Cell counts were conducted by two experienced observers in 25 randomly fields of 0.2 mm² (IAR and MAR), and they were blinded to which group was being analysed. Cellular identification was based on morphological features, location and size of cells. The results obtained were presented as number of positive cells per 0.2 mm².

2.7. Immunomodulatory gene expression

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to evaluate the expression levels of genes encoding toll-like receptors (TLR2 and TLR4), enzymes (iNOS) and cytokines (TNF- α , INF- γ , IL-2, IL-10 and TGF- β) in lung tissue. Tissue samples measuring 0.5 \times 0.5 cm were pooled from each animal, including both the left (cranial and caudal lobes) and right (cranial, middle, accessory, and caudal lobes) lungs, and preserved in RNAlater (Sigma, Madrid, Spain). In animals with macroscopic lesions, samples were collected from both unaffected lung areas and TBL-affected regions. Tissue samples from all lung lobes were combined and homogenized to ensure uniform consistency. From this homogenized mixture, 20 mg was used for mRNA extraction using the RNeasy Plus kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The mRNA concentration (ng/ μ L) was quantified by measuring the absorbance at 260 nm (OD₂₆₀) with a Nanodrop One spectrophotometer (Thermo Scientific, Waltham, USA), and purity was assessed by determining the OD₂₆₀/OD₂₈₀ ratio, which ranged between 1.9 and 2. Subsequently, mRNA concentrations were standardized to 20 ng/ μ L. All samples were then stored at -80 °C until further analysis.

For real time RT-PCR, it was used the SYBR Green iTaq Universal One-Step RT-qPCR kit (Bio-Rad, Hercules, CA, USA), and the final volume was adjusted to 10 μ L containing: 5 μ L of iTaq universal SYBR Green reaction mix (2x), 0.125 μ L of iScript reverse-transcriptase, 1 μ L of primer forward, 1 μ L of primer reverse, 2 μ L of RNA sample and 0.875 μ L of nuclease-free water. Each PCR reaction included two technical replicates and nuclease-free water was used as negative control. Amplification conditions were identical for all reactions and consisted of 15 min at 50 °C, 1 min at 95 °C, and 40 cycles of 10 sec at 95 °C and

30 sec at 60 °C. Melting curve analysis was performed by heating the samples to 95 °C at 0.5 °C increments every 6 sec. The sequences of the forward and reverse primers utilized are summarized in Table 2. GAPDH was the housekeeping gene used to normalize the expression of the analysed genes (Ravazzolo et al., 2006), applying the 2^{- $\Delta\Delta$ Ct} method (relative quantification), as previously described by Livak and Schmittgen (2001).

2.8. Statistical analysis

Graphical data and statistical analysis were conducted using R software, version 4.2.2 (R Core Team, 2022). A principal component analysis (PCA) was performed using the *prcomp* function from the *stats* package and included a data matrix with 13 scaled variables from immunomodulatory molecules (TLR2, TLR4, iNOS, TNF- α , INF- γ , IL-2, IL-10 and TGF- β) and immunostained cell populations (neutrophils, M1 M Φ s, M2 M Φ s, T lymphocytes and B lymphocytes). With PCA reducing dataset dimensionality, a biplot was created with the *fviz_pca_biplot* function from the *factoextra* package (Kassambara and Mundt, 2020) to compare clustering patterns of TBL+ and TBL- groups (Supplementary Fig. 1).

The Kruskal-Wallis test was employed to examine changes among the immunomodulatory molecules and the immunostained cell populations per group. Subsequently, the Wilcoxon test was performed to pinpoint molecules or cell populations that exhibited significant differences between groups. Furthermore, a correlation matrix was generated by utilizing the Pearson method with the *cor* function from the *stats* package in order to compare within TBL+ and TBL- groups the variables previously mentioned. The p-values for each pair of input features were generated by the *cor.mtest* function with a coefficient interval of 95 %. Significant correlations with a p-value lower than 0.01 were showcased in a correlation plot utilizing the *corrplot* function and package. Afterwards, the non-parametric Mann-Whitney *U* test was used to compare data from immunomodulatory molecules and immunostained cell populations between TBL+ and TBL- groups.

To identify the most determinant immunological factors associated with the presence or absence of granulomas, we fitted a generalized linear model (GLM) with a binomial regression and logit link function. TBL+ /TBL- was set as the binomial response variable using the *glm* function from the *stats* package (R Core Team, 2022). Fixed factors that presented strong significant linear relationships were excluded from the model in order to guarantee that only independent predictors were employed. To eliminate multicollinearity between potential predictor variables, covariables that presented a variance inflation factor (VIF) over 5 were orderly excluded from the model. For final predictors assortment, we carried out a stepwise variable selection with iterations between the forward and backwards steps, focusing on models with the lowest corrected Akaike information criterion (AIC) scores to minimize the chances of over-fitting. Final model fit was performed based on predictive capacity, presence of normality and absence of residual patterns in data variation. All non-mentioned plots were produced using the *ggplot2* package (Wickham, 2016).

Table 2

Primer sets used in this study for quantify immunomodulatory molecules.

Target	Forward primer	Reverse primer	Ref.
TLR-2	5'-CTGTGTGCGTCTTCCTCAGA-3'	5'-TCAGGGAGCAGAGTAACCAGA-3'	E et al., 2016
TLR-4	5'-GGTTTCCACAAAAGCCGTAA-3'	5'-AGGACGATGAAGATGATGCC-3'	E et al., 2016
iNOS	5'-CCAGCCCAAGGTCTATGTTTC-3'	5'-TAGTCTCCACCTGCTCCTC-3'	Yadav et al., 2016
TNF α	5'-AGAAGGGAGATCGCCTCAGT-3'	5'-AGAAGGGGATGAGGAGGGTC-3'	Jarczak et al., 2019
INF γ	5'-TAGCTAAGGGTGGGCTCTTTTCTCA-3'	5'-TGAGGAGGAGGAGAACATTACATTGA-3'	Jarczak et al., 2019
IL-2	5'-GCAAACGGTGCAACTACTTCAA-3'	5'-TGAGTTTCTCGGGATTTTAACTTT-3'	Ravazzolo et al., 2006
IL-10	5'-CGGCGCTGTCATCGTTTT-3'	5'-TCTTGAGCATATTGAAGACTCTCTTC-3'	Ravazzolo et al. 2006
TGF β	5'-CCTGCTGAGGCTCAAGTTAAAAGT-3'	5'-CTGAGGTAGGCCAGGAATT-3'	Ravazzolo et al. 2006
GADPH	5'-GGCAAGTTCATGGCACAGT 3'	5-ACGTACTCAGCACCAGCATCAC 3'	Ravazzolo et al. 2006

3. Results

3.1. Animal tuberculous status

Three animals were excluded from the final analysis as *M. bovis* infection could not be confirmed by any diagnostic technique used throughout the study. The present study included animals that tested positive using at least one TB diagnostic technique at the end of the experiment (n = 9). No clinical signs were reported in the selected goats.

Results of different diagnostic tests (SIT, CIT, IGRA, ELISA, culture and macroscopic lesions) at the study's end point are summarised in [Supplementary Table 1](#). When the animals were euthanized at the end of the study (T5), all the selected animals (n = 9) were positive reactors to SIT. Regarding bacteriological culture, *M. bovis* SB0121 was isolated from the respiratory pool of 66.7 % (6/9) of the goats. Gross TBLs were also detected in 66.7 % (6/9) of the animals and were characterized by local to multifocal, well-circumscribed, rounded nodules with a yellowish appearance and caseous or purulent consistency, sometimes exhibiting

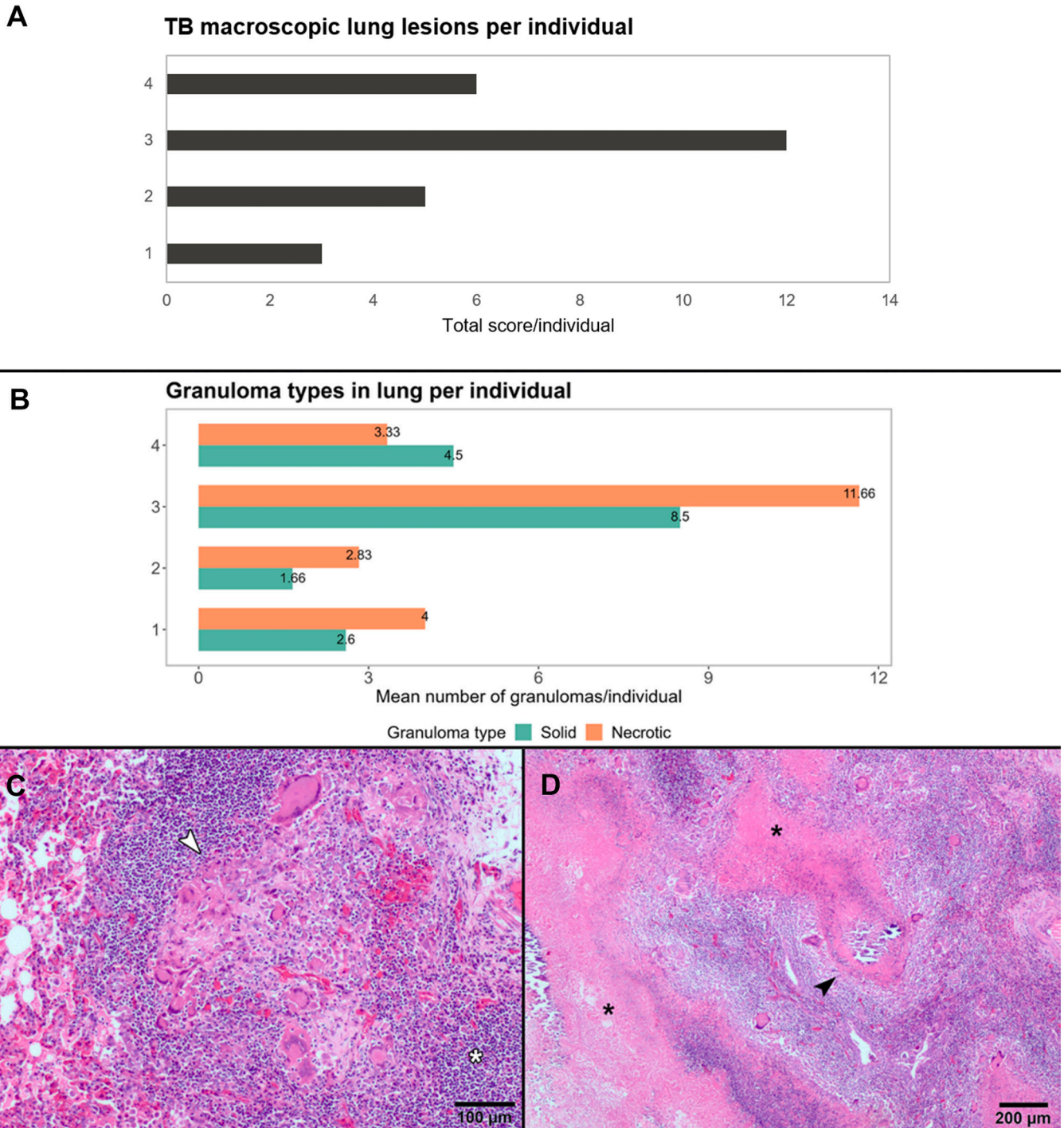


Fig. 2. A) Graphical representation of the total gross TBL score (sum) in the lungs for individual. B) Graphical representation depicting the average number of solid and necrotic granulomas observed across all lung lobes per individual (n = 4). C) Lung parenchyma of a goat showing an immature granuloma (white arrowhead) composed mainly of epithelioid macrophages and Langhans giant cells, surrounded by a mononuclear inflammatory infiltrate (white asterisk). D) Multifocal mature granuloma (black arrowhead) in the lung of a goat, with several calcification foci and extensive areas of necrosis (black asterisks). Staining: haematoxylin-eosin.

calcification. Occasionally, they were surrounded by fibrous tissue, ranging from small to coalescent lesions involving the greater part of an organ. Animals with TBL tested positive in cellular and humoral assays, as well as in culture. In contrast, animals without TBL tested positive only in SIT and CIT assays and were negative in *in vitro* tests. TBL were detected in the lungs (TBLL) of 66.7 % (4/6) of the goats, with no cavitory lesions reported (Fig. 2A). In addition, these animals showed lesions in the liver, tracheobronchial and mediastinal LNs. Conversely, the remaining animals (2/6) only presented TBL in tracheobronchial and mediastinal LNs.

3.2. Microscopic assessment of TBLL

Microscopic TBLL were reported in 44.4 % of the goats analysed (4/9). These lesions were characterized by the presence of granulomas in different stages of development across all lung lobes. Both solid (stage I and II) and necrotic granulomas (stage III and IV) were observed in all

TBLL+ animals (n = 4) (Fig. 2B). Solid granulomas, primarily consisted of clusters of epithelioid MΦs, with interspersed lymphocytes, neutrophils, and Langhans giant cells (LGCs) (Fig. 2C), accounted for 44.3 % of the total granulomas detected (Fig. 2B). Necrotic granulomas, characterized by focal and multifocal granulomas with extensive necrotic areas and mineralization, accompanied by MΦs, lymphocytes, neutrophils, and LGCs in the outer periphery (Fig. 2D), accounted for 55.7 % of the total granulomas observed (Fig. 2B). In most cases, these granulomas were surrounded by an incomplete connective tissue capsule.

3.3. Local immune cell response to *M. bovis* infection

Polymorphonuclear neutrophils were identified by the lysozyme marker, exhibiting a cytoplasmic staining pattern. These cells were observed diffusely in the lung parenchyma (Fig. 3A) in TBLL- (n = 5) and TBLL+ (n = 4) groups, as well as in all stages of granulomas in TBLL+ animals, mainly found in necrotic centres and the outer

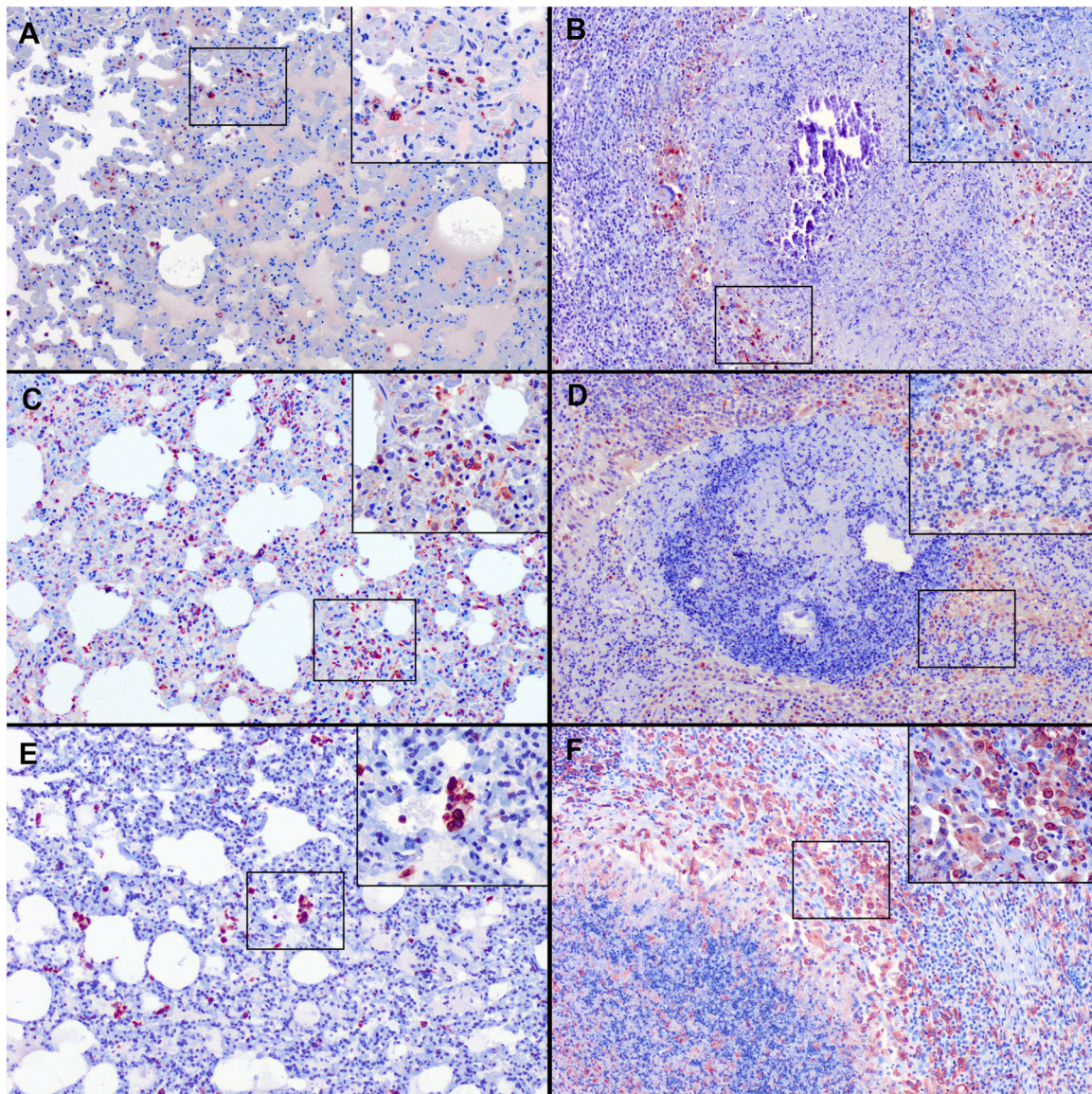


Fig. 3. Lysozyme expression in the lung parenchyma of a goat without tuberculous-compatible lung lesions (TBLL)- (A). Lysozyme expression in the necrotic core and outer periphery of advanced granuloma in a TBLL+ animal (B). Insets show close-up images at higher magnification displaying Lysozyme⁺ neutrophils. Immunolabeling of M1 MΦs with iNOS antibody in the lung parenchyma of TBLL- goat (C) and M1 MΦs surrounding the necrotic core of advanced lung granuloma in TBLL+ animal (D). Insets show close-up images at higher magnification displaying iNOS⁺ MΦs. CD163⁺ immunohistochemical detection of M2 MΦs in the lung parenchyma of TBLL- goat (E) and M2 MΦs encircling the necrotic area of an advanced lung granuloma in TBLL+ animal (F). Insets show close-up images at higher magnification displaying CD163⁺ MΦs. Scale bars = 100 μm.

periphery of advanced lesions (stages III and IV) (Fig. 3B). They constituted the least numerous cellular populations in the TB granulomas, with expression significantly higher in TBLL+ goats ($p = 0.0365$) (Fig. 4). iNOS, serving as a M1 MΦs marker, was detected in both groups showing cytoplasmic granular staining in diffuse inflammatory infiltrates of epithelioid cells of pulmonary parenchyma in TBLL+ and TBLL- animals (Fig. 3C). M1 MΦs expression was significantly higher ($p = 0.0317$) in the TBLL+ group (Fig. 4), predominantly concentrated surrounding necrotic and/or mineralized areas in advanced necrotic granulomas (Fig. 3D) or with a diffuse distribution in non-necrotic granulomas. Similarly, M2 MΦs, identified by the CD163 marker, exhibited a granular cytoplasmic staining diffusely distributed in the lung parenchyma in TBLL- goats (Fig. 3E). In TBLL+ animals, this staining was mainly concentrated around necrotic/mineralized areas of mature granulomas (Fig. 3F). Additionally, M2 MΦs expression was significantly higher ($p = 0.0195$) in the TBLL+ group, as previously reported for M1 MΦs (Fig. 4). T lymphocytes, identified by CD3 marker through a membrane-associated staining pattern, and B lymphocytes, identified by CD79αcy+ marker exhibiting a cytoplasmic staining pattern, were less abundant in TBLL- animals, displaying a random distribution within the lung parenchyma. In TBLL+ goats, these cells were detected in solid granulomas and at the periphery of necrotic granulomas. No significant differences were observed in the mean number of total B and T immunolabeled cells between the TBLL+ and TBLL- groups (Fig. 4).

3.4. Immunomodulatory molecules gene expression

The differences of level expression of immunomodulatory molecules were evaluated in the lungs from TBLL- ($n = 5$) and TBLL+ ($n = 4$) animals. Significantly higher gene expression levels of TLR4 ($p = 0.0317$) and IL-10 ($p = 0.0159$) were observed in TBLL- goats compared to TBLL+ animals, with a 4.87 and 6.72-fold increase reported, respectively (Fig. 5). Although not statistically significant, TBLL- animals showed a tendency towards increased expression of TLR2 and iNOS (Fig. 5). In contrast, IFN- γ expression levels were similar between both groups, with a higher value observed in only a single TBLL+ goat.

3.5. Correlation between TBLL development, cell immune populations and immunomodulatory expression

To evaluate the predominant immunological factors influencing the presence or absence of pulmonary granulomas, a generalized linear

model (GLM) was conducted in the selected animals ($n = 9$), employing a binomial regression with a logit link function. The stepwise variable selection showed that the most parsimonious regression model was the univariable model constituted by the fixed effect factor of M1 MΦs. Therefore, a logistic regression was performed to assess the effects of M1 MΦs on the likelihood of having granulomas. This model was statistically significant, $\chi^2(1) = 7.50$, $p < 0.05$ and explained 76 % (Cragg-Uhler pseudo- R^2) of the variance in granuloma presence.

All statistically significant correlations between cell populations and immunomodulatory molecules analysed in the present study are depicted in Fig. 6. However, only correlations with a moderate to strong coefficient ($\pm 0.6-1$) were considered to highlight the most significant associations. Notably, in the TBLL+ group, a high positive correlation (>0.87) was reported between M1 and M2 MΦs, as well as T lymphocytes. Moreover, in the presence of TBLL, iNOS, IFN- γ , IL-10 and TGF- β exhibited a high positive correlation (>0.85) with activated MΦs and T lymphocytes, while these cells and immunological mediators displayed a strong negative correlation with TLR2 and TLR4 (ranging from -0.65 to -0.91). Conversely, TBLL- goats showed a positive correlation between TLR4 with M1 MΦs and B lymphocytes (>0.82), and negative between iNOS, TNF- α and IFN- γ with TLR2 and TLR4 (<-0.60). However, in the absence of TBLL, a high positive correlation (>0.88) was observed between iNOS, TNF- α , IFN- γ and IL-2.

4. Discussion

Research on TB immunopathogenesis in goats, a species with pathological findings similar to those observed in humans, offers valuable opportunities to enhance the understanding of this complex and not fully elucidated area. The present study evaluates the immune response developed in caprine pulmonary TB, through key cells and immunomodulatory molecules involved in the defence against mycobacteria about formation and development of TBL in kid goats naturally infected with *M. bovis*. Thus, while cytokine gene expression patterns have been previously documented in TB in cattle, mice, rabbits, non-human primates, humans (Domingo-Gonzalez et al., 2016; Palmer et al., 2015, 2019; Salguero et al., 2017), and to a lesser extent in goats (Sanchez et al., 2011), the assessment of differences in the early immune response to TB in infected animals that develop lesions compared to those that do not, has not been analysed to date.

In this study, 75 % of the animals were included based on positive results from any TB-diagnostic test at the end of the study (9/12). Notably, two-thirds of goats tested positive in all cellular (SIT, CIT,

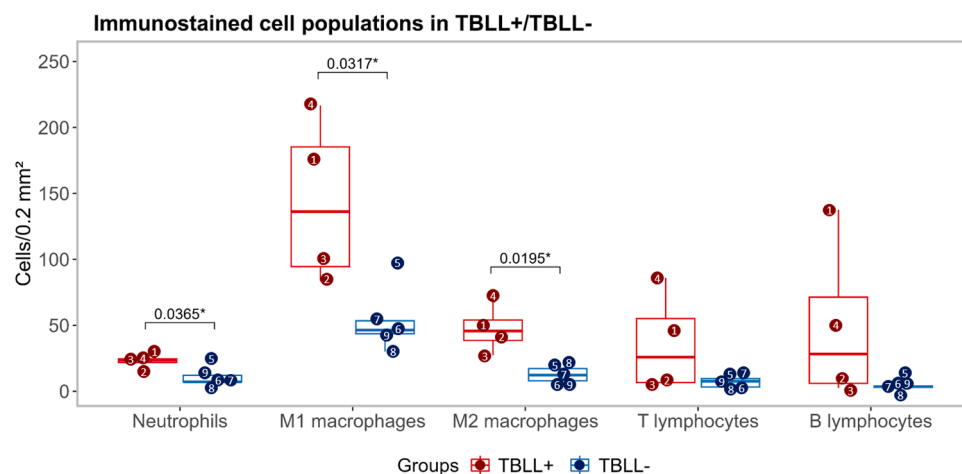


Fig. 4. Quantitative assessment (median, IQR) of immunopositive M1 MΦs (iNOS+), M2 MΦs (CD163 +), neutrophils (lysozyme), T lymphocytes (CD3 +) and B lymphocytes (CD79 +) in the right caudal lung lobe of goats naturally infected with *M. bovis*, categorized as having tuberculous-compatible lung lesions (TBLL+) and those without lung lesions (TBLL-). The numbers within the circles correspond to the individual animals included in the study (1–9). * $P < 0.05$: significant differences between groups.

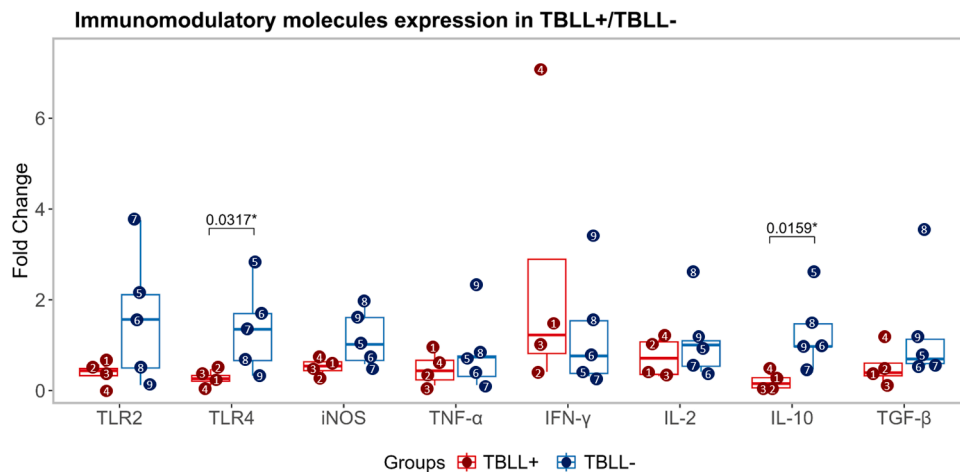


Fig. 5. Immunomodulatory molecules gene expression in lung tissues of goats naturally infected with *M. bovis* with tuberculous-compatible lung lesions (TBLL+) and without lung lesions (TBLL-). The numbers within the circles correspond to the individual animals included in the study (1–9). * $P < 0.05$: significant differences between groups.

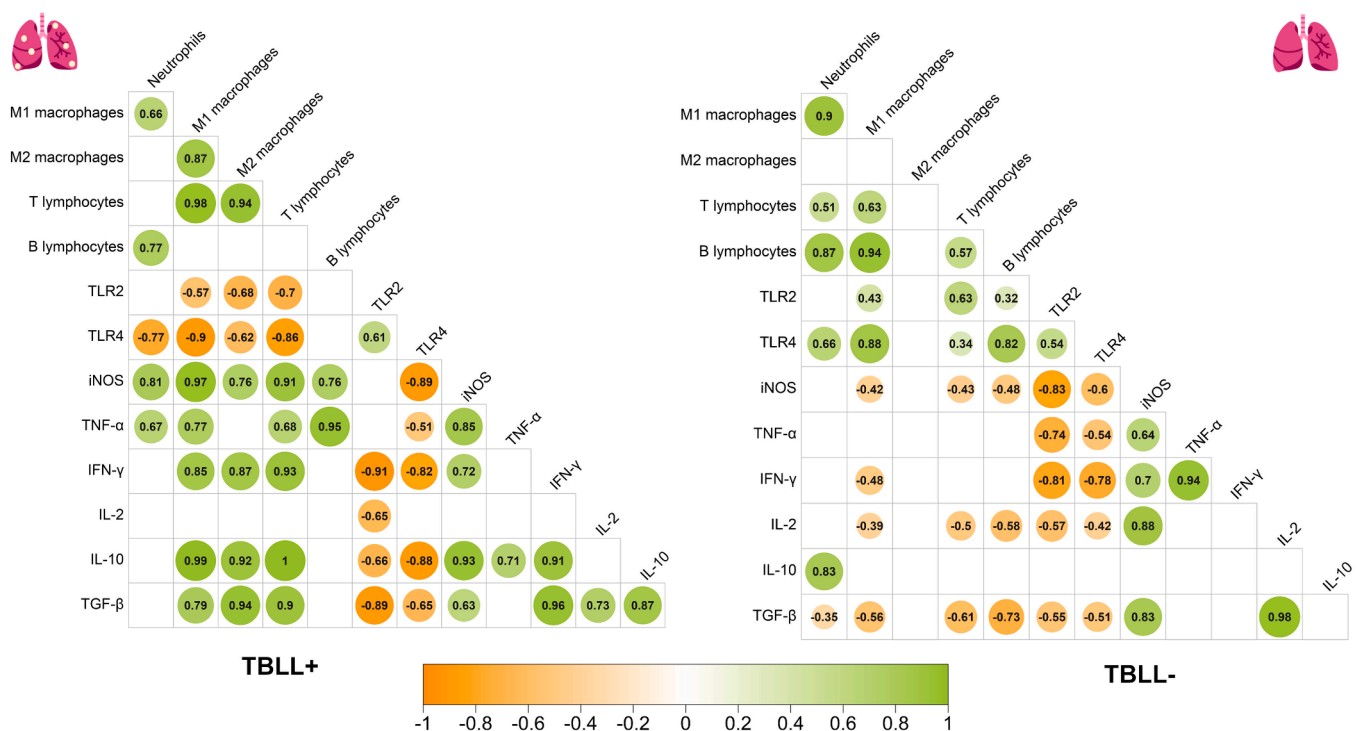


Fig. 6. Correlation matrix between cell populations and immunomodulatory molecules with significant values ($p < 0.05$) in TBLL+ (N = 4) and TBLL- (N = 5) groups using Pearson Correlation.

IGRA) and humoral (ELISA) assays (6/9), while one-third were negative to IGRA and ELISA (3/9). In previous studies on natural exposure to tuberculous mycobacteria in goats, initial positive reactions to SIT (Bezoz et al., 2017) and IGRA tests (Roy et al., 2019) were detected in some animals as early as 3 months' post exposure (Bezoz et al., 2017), while positive ELISA results were observed at 7 months' post-exposure (Roy et al., 2019). Additionally, gross TBL were observed in 66.7 % (6/9) of the animals exposed to *M. bovis* infected goats, affecting the lungs, pulmonary LNs, and occasionally the liver. The pulmonary form is the most common presentation of TB in both animals and humans (Palmer et al., 2022; Dergal et al., 2023). This is consistent with our findings, which showed that 66.7 % (4/6) of the animals exhibited TBL in the lungs. Microscopic TBLL consisted of both solid and necrotic granulomas, with a higher presence of advanced stages III and IV in

75 % of the goats with TBLL (3/4). Clusters of epithelioid macrophages, interspersed with lymphocytes, neutrophils, and LGCs, represented the solid granulomas, while extensive necrotic areas and mineralization, surrounded by an incomplete connective tissue capsule, characterized the necrotic granulomas. These lesions have been previously described in domestic ruminants (Wangoo et al., 2005; Palmer et al., 2019; Wedlich et al., 2022), red deer and wild boar (Zanella et al., 2008). TB granuloma formation is associated with tissue damage and morbidity, attributed to dysregulated inflammation (Wilson et al., 2019). The predominance of necrotic granulomas over solid granulomas in affected animals not only indicates a lack of disease control but also correlates with heightened mycobacterial excretion into the environment and increased transmission rates (Palmer et al., 2022). However, in our study, several MTBC infected goats did not develop TBLL (5/9), thus

necessitating a detailed exploration of the immunological mechanisms potentially implicated in this phenomenon.

The key cellular populations and immunological mediators involved in the response to TB were analysed to evaluate differences between animals exhibiting TBLL and those without TBLL. In TBLL- animals ($n = 5$), no remarkable findings regarding cell populations were observed, with their numbers lower than those noted in animals with TB granulomas, which are inflammatory structures characterized by an increase in immunocompetent cells such as MΦs, epithelioid MΦs, neutrophils, lymphocytes and plasma cells (Sholeye et al., 2022). Contrary, TBLL+ animals ($n = 4$) exhibited significantly higher presence of neutrophils, MΦs, and lymphocytes, which are central to the immune response against mycobacteria and are major constituents of tuberculous granulomas (Palmer et al., 2022). Neutrophils, although the least abundant cell population in TBLL, were consistently found in necrotic areas and at the periphery, aligning with previous findings in necrotic granulomas (Sanchez et al., 2011; Larenas-Muñoz et al., 2023). In contrast, a high presence of these cells were reported in TB infected goats in cavitary lesions (Sanchez et al., 2011), which are associated with a high shedding of mycobacteria into the environment (Gonzalez-Juarrero et al., 2013). However, these lesions were not observed in our study, likely due to shorter exposure periods and disease courses (Alsayed and Gunosewoyo, 2023). On the other hand, T lymphocytes increase in TBLL+ animals were correlated with an upward trend of IFN- γ , as described previously in adult infected goats (Sanchez et al., 2011), being a positive correlation between this cytokine and T cells reported. Similar results were noted in experimentally infected cattle, where IFN- γ expression level was significantly higher within granulomas compared to non-granuloma areas (Palmer et al., 2015). This cytokine plays a key role in protective Th1 responses to mycobacteria, promoting MΦs activation and iNOS induction (Palmer et al., 2022). M1 MΦs, which showed a positive correlation with iNOS, were the most prevalent cell population in TBLL, primarily located in the initial lesion composed of activated MΦs and surrounding the necrotic areas of advanced granulomas. This observation aligns with studies in naturally infected cattle (Palmer et al., 2019), wild boar (García-Jiménez et al., 2013) and guinea pig (Larenas-Muñoz et al., 2023), emphasizing the crucial role of these phagocytes in granuloma formation (Palmer et al., 2022). However, our findings contrast with previous observations where iNOS+ expression level in MΦs (M1) was reduced in cavitary granulomas and, even absent in necrotic granulomas of adult goats (3–5 years) (Sanchez et al., 2011). These differences may be attributed to an exhaustion or dysregulation of the protective Th1 response due to prolonged or intense exposure to mycobacteria, leading to a decreased effectiveness of this immune mediator and, consequently, increased lung damage (Lombardi et al., 2021).

The TBLL- group exhibited higher expression levels of immunomodulatory genes compared to the TBLL+ animals. Specifically, we observed 4.25- and 4.87- fold reductions in the expression of TLR2 and TLR4, respectively, in TBLL+ goats compared to TBLL- animals. These are critical transmembrane receptors in host defence against mycobacteria through direct or indirect processes such as mycobacterial recognition, innate and adaptive immunity orchestration, antimicrobial activities, and cytokine production regulation (Hu and Spaink, 2022). Indeed, in TBLL- animals the expression of TLR4 showed a positive correlation with M1 MΦs and B lymphocytes, suggesting a relationship between the effective activation of the TLR signal pathway and the resistance against TB (Cassidy and Martineau, 2014). Similarly, in the TBLL- animals the pro-inflammatory cytokines TNF- α and IL-2 exhibited a positive correlation with iNOS, essential elements in the protective immune response against mycobacteria (Palmer et al., 2022). Additionally, we observed a 6.72-fold increase in the expression level of IL-10 gene in TBLL- group, consistent with findings in infected cattle, where greater expression of this cytokine has been associated with reduced pathology and bacterial load (Palmer et al., 2015). Therefore, the higher level of expression of several immunomodulatory molecules observed in

TBLL- animals suggests an effective immune response potentially involved in suppressing the formation and progression of tuberculous granulomas in lung.

Nevertheless, the results obtained in our study should be interpreted with caution, since conducting experimental studies with naturally infected animals, where the animals included in the study consistently had direct contact with TB infected goats through exposure to aerosols and by sharing feed and water points, has both limitations and strengths. Inherent limitations include variable disease presentation, lack of control over infection timing and intensity, and high individual variability within the study donor population, complicating the identification of consistent patterns. Moreover, the small sample size analysed in this study introduces further challenges, such as reduced generalizability of the results, diminished statistical power, and limited ability to explore biological heterogeneity. However, the findings emphasized the effectiveness of employing a direct and continuous contact model with infected animals for experimental studies, offering a realistic assessment of TB pathogenesis in natural conditions and insights into disease progression in a controlled environment (Roy et al., 2019), a methodology previously utilized by other authors (Roy et al., 2019). Additionally, the study design maintains ecological validity, considering the natural behaviours and interactions of infected animals, enhancing this approach the translational relevance of findings to TB human. This natural transmission model is one of the main strengths of this study, as it closely mimics the progression of the disease.

5. Conclusions

In conclusion, the present study highlights significant variations in the immune response among naturally TB-infected goats in early stages, manifested through differential tuberculous lesion development, cellular populations, and the gene expression of certain molecules in the lungs. Notably, M1 MΦs emerge as key contributors to granuloma formation. Furthermore, the elevated expression of TLR2 and TLR4 in MTBC infected animals without presence of TBL in lungs appears to be associated with TB resistance. This heightened gene expression in these animals is also observed in pro-inflammatory cytokines, iNOS, and IL-10, indicating the potential development of an effective immune response that may suppress formation of tuberculous lesions. Our findings emphasize the complexity of the immune response in TB-infected goats and underscore the necessity for further research with a larger number of animals to enhance our understanding of immunopathogenesis in natural conditions. This knowledge is necessary to develop and assess novel vaccine candidates and immunodiagnostic tools, contributing to the control and eradication of TB in both animal and human populations.

Funding

This work was supported by grants from the University of Cordoba (REF: UCO-FEDER-1381385-R). Moreover, this research was made possible by funding from ICRAD, an ERA-NET co-funded under the European Union's Horizon 2020 research and innovation program (<https://ec.europa.eu/programmes/horizon2020/en>) under Grant Agreement No. 862605, and the Spanish Ministry of Science, Innovation and Universities (MCIN/AEI/10.13039/501100011033) through the project "Improving the diagnosis of tuberculosis in domestic ruminants through the use of new antigens and test platforms" (reference PCI2023-143368). Moreover, IAR is supported by the FPU grant of the Ministerio de Ciencia, Innovación y Universidades (FPU19/ 03969). RVR was funded by Universidad de Castilla-La Mancha (UCLM), Spain and the European Social Fund (ESF) (grant 2022-PRED-20675). JO was supported by an FPU contract-fellowship (Formación de Profesorado Universitario) from the Ministerio de Ciencia, Innovación y Universidades (FPU18/ 05197).

CRediT authorship contribution statement

Irene Agulló-Ros: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Rita Vaz-Rodrigues:** Writing – review & editing, Methodology, Data curation. **Mercedes Domínguez:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Álvaro Roy:** Writing – review & editing, Methodology, Data curation. **Isabel G. Fernández de Mera:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **María A. Rivalde:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Javier Ortega:** Writing – review & editing, Methodology, Conceptualization. **Inmaculada Moreno:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Javier Bezos:** Writing – review & editing, Supervision, Data curation. **Lucas Domínguez:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetmic.2024.110320](https://doi.org/10.1016/j.vetmic.2024.110320).

References

- Agulló-Ros, I., Andrada Borzollino, M.A., Pérez-Sancho, M., Roy, Á., Bezos, J., Bonnet, T., Moreno, I., Paz Sánchez, Y., Domínguez, M., Gómez-Villamandos, J.C., Domínguez, L., Rivalde, M.A., 2022. Effect of heat-inactivated *Mycobacterium avium* subspecies *paratuberculosis* (MAP) vaccine on the lesions and immunopathology developed in target tissues of naturally MAP-infected goats. *Vet. Microbiol.* 273, 109543. <https://doi.org/10.1016/j.vetmic.2022.109543>.
- Alsayed, S.S.R., Gunosewoyo, H., 2023. Tuberculosis: pathogenesis, current treatment regimens and new drug targets. *Int J Mol Sci.* 24, 5202. <https://doi.org/10.3390/ijms24065202>.
- Balseiro, A., Thomas, J., Gortázar, C., Rivalde, M.A., 2020c. Development and challenges in animal tuberculosis vaccination. *Pathogens* 9, 472. <https://doi.org/10.3390/pathogens9060472>.
- Bezós, J., Alvarez, J., de Juan, L., Romero, B., Rodríguez, S., Fernández-de-Mera, I.G., Hewinson, R.G., Vordermeier, M., Mateos, A., Domínguez, L., Aranaz, A., 2011. Assessment of *in vivo* and *in vitro* tuberculosis diagnostic tests in *Mycobacterium caprae* naturally infected caprine flocks. *Prev. Vet. Med.* 100, 187–192. <https://doi.org/10.1016/j.prevetmed.2011.03.012>.
- Cassidy, J.P., Martineau, A.R., 2014. Innate resistance to tuberculosis in man, cattle and laboratory animal models: nipping disease in the bud? *J. Comp. Pathol.* 151, 291–308. <https://doi.org/10.1016/j.jcpa.2014.08.001>.
- Dergal, N.B., Ghermi, M., Imre, K., Morar, A., Acaroz, U., Arslan-Acaroz, D., Herman, V., Ayad, A., 2023. estimated prevalence of tuberculosis in ruminants from slaughterhouses in constantine province (Northeastern Algeria): a 10-year retrospective survey (2011–2020). *Life* 13, 817. <https://doi.org/10.3390/life13030817>.
- Domingo-Gonzalez, R., Prince, O., Cooper, A., Khader, S., 2016. Cytokines and chemokines in *Mycobacterium tuberculosis* infection. *Microbiol. Spectr.* 4, 10.1128/microbiolspec. <https://doi.org/10.1128/microbiolspec.TBTB2-0018-2016>.
- E, G.X., Zhao, Y.J., Ma, Y.H., Chu, M.X., Hong, Q.H., Lv, S.J., Zhu, L., Li, F.K., Han, Y.G., Wu, X., Huang, Y.F., 2016. Differential expression of Toll-like receptors in goat dominant and nondominant follicles. *Genet. Mol. Res.* 15. <https://doi.org/10.4238/gmr15049157>.
- García-Jiménez, W.L., Salguero, F.J., Fernández-Llario, P., Martínez, R., Risco, D., Gough, J., Ortiz-Peláez, A., Hermoso-de-Mendoza, J., Gómez, L., 2013. Immunopathology of granulomas produced by *Mycobacterium bovis* in naturally infected wild boar. *Vet. Immunol. Immunopathol.* 156, 54–63. <https://doi.org/10.1016/j.vetimm.2013.09.008>.
- Gonzalez-Juarrero, M., Bosco-Lauth, A., Podell, B., Soffler, C., Brooks, E., Izzo, A., Sanchez-Campillo, J., Bowen, R., 2013. Experimental aerosol *Mycobacterium bovis* model of infection in goats. *Tuberculosis* 93, 558–564. <https://doi.org/10.1016/j.tube.2013.05.006>.
- Hu, W., Spink, H.P., 2022. The role of TLR2 in infectious diseases caused by mycobacteria: from cell biology to therapeutic target. *Biology* 11, 246. <https://doi.org/10.3390/biology11020246>.
- Huang, F., Zhao, Y., 2022. Global control of tuberculosis: current status and future prospects. *Zoonoses*. 2. <https://doi.org/10.15212/ZOONOSES-2021-0021>.
- Jarczák, J., Stoniewska, D., Kaba, J., Bagnicka, E., 2019. The expression of cytokines in the milk somatic cells, blood leukocytes and serum of goats infected with small ruminant lentivirus. *BMC Vet. Res.* 15, 424. <https://doi.org/10.1186/s12917-019-2182-4>.
- Jiménez-Martín, D., García-Bocanegra, I., Rivalde, M.A., Napp, S., Domínguez, M., Romero, B., Moreno, I., Martínez, R., Cano-Terriza, D., 2024. *Mycobacterium tuberculosis* complex in domestic goats in Southern Spain. *Prev. Vet. Med.* 227, 106204. <https://doi.org/10.1016/j.prevetmed.2024.106204>.
- Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* 35, 907–914. <https://doi.org/10.1128/jcm.35.4.907-914.1997>.
- Kassambara, A., Mundt, F., 2020. Extract and visualize the results of multivariate data analyses [R package factoextra version 1.0.7] [WWW Document]. URL (<https://www.semanticscholar.org/author/A.-Kassambara/6376218>) (accessed 4.16.24).
- Larenas-Muñoz, F., Ruedas-Torres, I., Hunter, L., Bird, A., Agulló-Ros, I., Winsbury, R., Clark, S., Rayner, E., Salguero, F.J., 2023. Characterisation and development of histopathological lesions in a guinea pig model of *Mycobacterium tuberculosis* infection. *Front. Vet. Sci.* 10, 1264200. <https://doi.org/10.3389/fvets.2023.1264200>.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>.
- Lombardi, A., Villa, S., Castelli, V., Bandera, A., Gori, A., 2021. T-cell exhaustion in *Mycobacterium tuberculosis* and nontuberculous mycobacteria infection: pathophysiology and therapeutic perspectives. *Microorganisms* 9, 2460. <https://doi.org/10.3390/microorganisms9122460>.
- Palmer, M.V., Kanipe, C., Boggiatto, P.M., 2022. The bovine tuberculoid granuloma. *Pathogens* 11, 61. <https://doi.org/10.3390/pathogens11010061>.
- Palmer, M.V., Thacker, T.C., Waters, W.R., 2015. Analysis of cytokine gene expression using a novel chromogenic in-situ hybridization method in pulmonary granulomas of cattle infected experimentally by aerosolized *Mycobacterium bovis*. *J. Comp. Pathol.* 153, 150–159. <https://doi.org/10.1016/j.jcpa.2015.06.004>.
- Palmer, M.V., Wiarda, J., Kanipe, C., Thacker, T.C., 2019. Early pulmonary lesions in cattle infected via aerosolized *Mycobacterium bovis*. *Vet. Pathol.* 56, 544–554. <https://doi.org/10.1177/0300985819833454>.
- Ravazzolo, A.P., Nenci, C., Vogt, H.-R., Waldvogel, A., Obexer-Ruff, G., Peterhans, E., Berton, G., 2006. Viral load, organ distribution, histopathological lesions, and cytokine mRNA expression in goats infected with a molecular clone of the caprine arthritis encephalitis virus. *Virology* 350, 116–127. <https://doi.org/10.1016/j.virol.2006.02.014>.
- Rodríguez, S., Bezós, J., Romero, B., de Juan, L., Álvarez, J., Castellanos, E., Moya, N., Lozano, F., Javed, M.T., Sáez-Llorente, J.L., Liébana, E., Mateos, A., Domínguez, L., Aranaz, A., Tuberculosis, M. of A., 2011. *Mycobacterium caprae* Infection in livestock and wildlife. *Spain Emerg. Infect. Dis.* 17, 532–535. <https://doi.org/10.3201/eid1703.100618>.
- Romero-Adrian, T., 2015. Role of cytokines and other factors involved in the *Mycobacterium tuberculosis* infection. *World J. Immunol.* 5. <https://doi.org/10.5411/wji.v5.i1.16>.
- Roy, A., Tomé, I., Romero, B., Lorente-Leal, V., Infantes-Lorenzo, J.A., Domínguez, M., Martín, C., Aguiló, N., Puentes, E., Rodríguez, E., de Juan, L., Rivalde, M.A., Gortázar, C., Domínguez, L., Bezós, J., 2019. Evaluation of the immunogenicity and efficacy of BCG and MTBVAC vaccines using a natural transmission model of tuberculosis. *Vet. Res.* 50, 82. <https://doi.org/10.1186/s13567-019-0702-7>.
- Salguero, F.J., 2018. The pathology and pathogenesis of *Mycobacterium bovis* infection. *Bov. Tuberc.* CABI Books. 122–139. <https://doi.org/10.1079/9781786391520.0122>.
- Salguero, F.J., Gibson, S., Garcia-Jimenez, W., Gough, J., Strickland, T.S., Vordermeier, H.M., Villarreal-Ramos, B., 2017. Differential cell composition and cytokine expression within lymph node granulomas from BCG-vaccinated and non-vaccinated cattle experimentally infected with *Mycobacterium bovis*. *Transbound. Emerg. Dis.* 64, 1734–1749. <https://doi.org/10.1111/tbed.12561>.
- Sanchez, J., Tomás, L., Ortega, N., Buendía, A.J., del Rio, L., Salinas, J., Bezós, J., Caro, M.R., Navarro, J.A., 2011. Microscopical and immunological features of tuberculoid granulomata and cavitary pulmonary tuberculosis in naturally infected goats. *J. Comp. Pathol.* 145, 107–117. <https://doi.org/10.1016/j.jcpa.2010.12.006>.
- Sholeye, A.R., Williams, A.A., Loots, D.T., Tutu van Furth, A.M., van der Kuip, M., Mason, S., 2022. Tuberculous granuloma: emerging insights from proteomics and metabolomics. *Front. Neurol.* 13, 804838. <https://doi.org/10.3389/fneur.2022.804838>.
- Thomas, J., Balseiro, A., Gortázar, C., Rivalde, M.A., 2021. Diagnosis of tuberculosis in wildlife: a systematic review. *Vet. Res.* 52, 31. <https://doi.org/10.1186/s13567-020-00881-y>.
- Varela Castro, L., Gerrikagoitia, X., Alvarez, V., Geijo, M., Barral, M., Sevilla, I., 2021. A long-term survey on *Mycobacterium tuberculosis* complex in wild mammals from a bovine tuberculosis low prevalence area. *Eur. J. Wildl. Res.* 67. <https://doi.org/10.1007/s10344-021-01489-z>.
- Wangoo, A., Johnson, L., Gough, J., Ackbar, R., Inglut, S., Hicks, D., Spencer, Y., Hewinson, G., Vordermeier, M., 2005. Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, gammadelta (WC1+) T cells and CD 68+ cells. *J. Comp. Pathol.* 133, 223–234. <https://doi.org/10.1016/j.jcpa.2005.05.001>.
- Wedlich, N., Figl, J., Liebler-Tenorio, E.M., Köhler, H., von Pückler, K., Rissmann, M., Petow, S., Barth, S.A., Reinhold, P., Ulrich, R., Grode, L., Kaufmann, S.H.E., Menge, C., 2022. Video endoscopy-guided intrabronchial spray inoculation of *Mycobacterium bovis* in goats and comparative assessment of lung lesions with

- various imaging methods. *Front. Vet. Sci.* 9. <https://doi.org/10.3389/fvets.2022.877322>.
- Wickham, H., 2016. *ggplot2: Elegant Graphics for Data Analysis*. Springer International Publishing.
- Wilson, J.L., Mayr, H.K., Weichhart, T., 2019. Metabolic programming of macrophages: Implications in the Pathogenesis of Granulomatous disease. *Front. Immunol.* 10. <https://doi.org/10.3389/fimmu.2019.02265>.
- Yadav, V.P., Dangi, S.S., Chouhan, V.S., Gupta, M., Dangi, S.K., Singh, G., Maurya, V.P., Kumar, P., Sarkar, M., 2016. Expression analysis of NOS family and HSP genes during thermal stress in goat (*Capra hircus*). *Int. J. Biometeorol.* 60, 381–389. <https://doi.org/10.1007/s00484-015-1035-5>.
- Zanella, G., Duvauchelle, A., Hars, J., Moutou, F., Boschiroli, M.L., Durand, B., 2008. Patterns of lesions of bovine tuberculosis in wild red deer and wild boar. *Vet. Rec.* 163, 43–47. <https://doi.org/10.1136/vr.163.2.43>.