Cellular and Cytokine Responses in the Granulomas of Asymptomatic Cattle naturally infected with *Mycobacterium bovis* in Ethiopia

Begna Tulu, MSc[1,2]*,

Henny M Martineau, PhD[3],

Aboma Zewude, MSc[4],

Fekadu Desta, MSc[1],

David A Jolliffe, PhD[5]

Markos Abebe, PhD[6],

Taye Tolera Balcha, PhD[6],

Mulugeta Belay, PhD[5],

Adrian R Martineau, PhD[5],

Gobena Ameni, PhD[1,7]

* To whom correspondence should be addressed at Aklilu Lemma Institute of Pathobiology, Sefere Selam Campus, Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia and Department of Medical Laboratory Sciences, Bahir Dar University, PO Box 079, Bahir Dar, Ethiopia. Tel:+251 911 263 679, Email: tulubegna@gmail.com

1. Aklilu Lemma Institute of Pathobiology, Sefere Selam Campus, Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia

2. Department of Medical Laboratory Sciences, Bahir Dar University, PO Box 079, Bahir Dar, Ethiopia

3. Department of Pathology, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield AL9 7TA, UK

4. Ethiopian Public Health Institute, PO Box. 1242/5456, Addis Ababa, Ethiopia

5. Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London E1 2AB, UK8.

6. Armauer Hansen Research Institute, Jimma Road, Addis Ababa, Ethiopia

7. Department of Veterinary Medicine, College of Food and Agriculture, United Arab Emirates University, PO Box 1551, Al Ain, UAE

ABSTRACT

Cells (CD3+ T cell and CD68+ macrophages), cytokines (IFN-γ+ and TNF-α+) and effector molecule (iNOS+) responses were evaluated in the lymph nodes and tissue of cattle naturally infected with *Mycobacterium bovis*. Detailed post mortem and immunohistochemical examinations of lesions were performed on 16 cows positive for single intradermal cervical comparative tuberculin (SICCT) test which were identified from dairy farms located around the Addis Ababa City. The severity of the gross lesion was significantly higher ($p=0.003$) in *M. bovis* culture positive (n=12) cows than in culture negative (n=4). Immunohistochemical techniques showed that in culture positive cows, the mean immunolabeling fraction of CD3+ T cells decreased as the stage of granuloma increased from stage I to stage IV ($p<0.001$). In contrast, the immunolabelling fraction of CD68+ macrophages, IFN-γ+, TNF-α+ and iNOS+ increased from stage I to stage IV ($p< 0.001$). In culture negative cows, early stages showed a significantly higher fraction of CD68+ macrophages ($p=0.03$) and iNOS+ ($p=0.007$) when compared to culture positive cows. Similarly, at advanced granuloma stages, culture negative cows demonstrated significantly higher mean proportions of CD3+ T cells ($p< 0.001$) compared to culture positive cows. Thus, this study demonstrates that following natural infection of cows with *M. bovis*, as the stage of granuloma increases from stage I to stage IV, the immunolabelling fraction of CD3+ cells decreases while the immunolabeling fraction of CD68+ macrophages, IFN-γ+, TNF-α+ and iNOS+ increases.

Key words: Immune response, Granuloma, *Mycobacterium bovis*, Immunohistochemistry, Asymptomatic cows, Natural infection

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle mainly caused by *M. bovis*, a member of the *Mycobacterium tuberculosis* complex (MTBc). *M. bovis* has a wide host range that includes domestic animals, wildlife and humans (1, 2). With over 50 million infected cattle worldwide, bTB causes significant economic loss to the agricultural industry, costing US$3 billion annually (3). Effects on human morbidity and mortality are also considerable. In 2019 alone, it was reported that *M. bovis* was responsible for 143, 000 new human TB cases and 12, 300 deaths. Over 91.0% of the deaths were from African and Asian countries (4).

In some developed countries, the introduction of test and slaughter of bTB infected cattle together with continuous surveillance systems and movement restrictions, has achieved dramatic results in lowering the prevalence and even eradicating the disease (5, 6). However, these control programs are costly, and in countries like Ethiopia where bTB is an endemic disease and the agricultural economy relies on traditional farming practices (7, 8), new tools like effective vaccination and immunodiagnostic are urgently needed (2, 9, 10).

The single intradermal cervical comparative tuberculin (SICCT) test is the most widely used test for the diagnosis of bTB in live cattle (11). SICCT test measures the delayed hypersensitivity reaction to the tuberculin antigen-purified protein derivative (PPD) of *Mycobacterium bovis* (PPDb) and *Mycobacterium avian* (PPDa). In infected animals, there is swelling and indurations at both injection sites 72 hours later (11, 12). However, SICCT test has lower sensitivity when there is co-infection with certain parasites like *Fasciola hepatica and Strongylus sp* (13, 14) which are widely distributed in Ethiopia (15, 16) .

The second feasible bTB control option for developing countries like Ethiopia is through the vaccination program. However, presently, there are no effective vaccines that exist for the control of bTB in cattle. Bacillus Calmette Guerin (BCG) which is used in humans has certain limitations in cattle, including interference with the SICCT test.

Hence, understanding the local immunological responses is of paramount importance in the effort to develop new vaccines and diagnostic tools (2, 9). During mycobacteria infection, granuloma formation is the main mechanism of host immune response to contain the spread of bacterial dissemination, but this can result in significant tissue damage (17, 18). Immunity against mycobacteria is primarily a cell mediated immune (CMI) response, which involves recruitment of macrophages, dendritic cells, and helper T cell type-1 (TH1) modulated by cytokines (17, 19, 20). Cytokines like interferon gamma (IFN- γ) (20), interleukin-12 (IL-12) (21), IL-6, and tumor necrosis factor (TNF) play a significant role in activating immunological cells to kill mycobacteria and inducing TH1 responses (22). In addition, the production of molecules like nitric oxide (NO) by macrophages or phagocytic cells during mycobacterial infection play a crucial role in the intracellular killing of mycobacteria as it is cytotoxic at high concentrations. NO release is enhanced by inflammatory stimuli via the up regulation of inducible forms of NOS (iNOS or NOS2) with in inflammatory macrophages (23, 24). Conversely, cytokines such as IL-4 (25) and IL-10 (26), known as the anti-inflammatory cytokines, are responsible for down-regulating the role of pro-inflammatory immune responses to control the tissue damage (17).

Existing studies on the immune response of cattle against *M. bovis,* largely focus on the experimental infections generated through the respiratory route (10, 17, 27-29).

Through characterization of gross and microscopic lesion development, these studies have shown host immune response related factors to influence bTB disease outcome (19, 30). Susceptibility to *M. bovis* infection has also been shown to be influenced by host genetic makeup and age related factors (31, 32).

However, there are few studies on the fundamental aspects of host immune response in a natural infection setup (33, 34). Menin *et al*., (2013) describe that during natural infection with bTB, the lesion severity, measured using a pathology severity score (33), correlates positively with viable bacterial loads. Similarly, neutrophil numbers in the granuloma are associated with increased *M. bovis* proliferation (33). Another study shows that as the stage of granuloma increases, macrophages and epithelioid cells mediate an increase in expression of cytokines (35). Still, little is known about the local immune response of CD3+ T cells, CD68+ macrophages, IFN-γ, TNF-α and iNOS in cattle naturally infected with *M. bovis*. Thus, the objective of this study was to evaluate the responses of selected immune cells (CD3+ T cells and CD68+ macrophages), pro-inflammatory cytokines (IFN-γ, TNF-α) and the effector molecule (iNOS) across stages of granuloma development in cattle with natural *M. bovis* infection.

RESULTS

Animal signalment, body condition and *M. bovis* culture status

Samples were taken from 16 cows with positive SICCT tests (≥ 4 mm cut off). All cows were female, and ranged in age from 2.5 to 9 years, with a mean of 5.8 years. Seven (44.0%) were in poor body condition, 6 (37.5%) were medium and 3 (18.7%) in good body condition. Twelve (75.0%) of the cows were positive for *M. bovis* culture and 4 (25.0%) were negative (Table S1).

Gross pathology

All 16 cows had gross lesion suggestive bTB, characterized by caseous necrosis. Lymph node lesions were detected in 99/176 (56.3%) samples from the head and neck region, thorax and abdomen. More specifically lesions were found in the 16/16 (100.0%) caudal mediastinal lymph nodes, 15/16 (94.5%) bronchial lymph nodes, 13/16 (81.3%) cranial mediastinal lymph nodes, 11/16 (68.7%) hepatic lymph nodes, 6/16 (37.5%) mesenteric lymph nodes and 5/16 (31.3%) tracheal lymph nodes. Lung lesions were found in 6/16 (37.5%) cows, and 33/96 (34.4%) lung samples.

The total gross pathology score was significantly greater ($p=0.004$) in M. bovis culture positive than in culture negative animals (Fig. 1C). Within culture positive cows the lymph node gross pathology score was significantly higher in the thoracic lymph nodes ($p < 0.05$) as compared to head and abdominal lymph nodes (Fig. 1A).

Histopathology

A total of 37 tissues were examined from both culture positive and culture negative animals. Representative microscopic findings are shown below (Fig. 2). Culture positive animals had more granulomas in stages I to IV when compared to culture negative animals. The four culture negative cows had granulomas in their cranial and caudal mediastinal lymph nodes only. The majority of samples examined microscopically in this study were from caudal and cranial mediastinal lymph nodes (Table S2).

Acid fast bacillus staining

A modified Zeihl Nelseen histochemical stain was used to detect the presence of intralesional acid-fast bacilli (AFB). There was no correlation between the stage of the granuloma and the AFB positivity (Fig. S1).

Immunohistochemistry

Immunohistochemistry was used to detect CD3+ T cells , CD68+ macrophages , interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS). Antigen expression was compared between culture positive and culture negative animals and different stages of granuloma. The positive labeling was expressed as a fraction of the total area examined. All positive and negative controls stained appropriately.

Macrophages (CD68+)

Anti- CD68+ antibody was used to identify epithelioid macrophages and multinucleated giant cells (MNGCs). In both culture positive and negative animals, the CD68+ immunolabeling fraction within the granulomas increased from stage I to IV (Fig. 3). In culture positive animals, a one-way ANOVA analysis showed this change to be statistically significant ($p < 0.001$), which was also the case when different granuloma stages were compared; stage I vs. stage III ($p = 0.006$), stage I vs. stage IV ($p = 0.001$), stage II vs. IV ($p < 0.001$) and stage III vs. IV ($p = 0.009$). When the immunolabeling fraction of CD68+ cells compared between culture positive and negative cows, in early granuloma stage (I) culture negative cows showed a higher ($p = 0.037$).

T cells (CD3+)

In culture positive animals, the CD3+immunolabeling fraction decreased from stages I to IV ($p < 0.001$) (Fig. 4). In culture negative animals, the same fraction increased from stages I to IV, but this was not statistically significant ($p > 0.05$). However, when culture negative and culture positive cows with advanced stage granulomas (III and IV) were compared to early stage (I and II), the CD3+ immunolabelling fraction was higher in the early stage ($p<0.001$).

Cytokines IFN-γ+ and TNF-α+

For both culture positive and negative cows, the IFN-γ+ immunolabeling fraction increased from stages I to IV ($p < 0.001$) (Fig. 5). For the TNF-α+ immunolabeling fraction, in culture positive cows, there was a statistically significant increase from stage I to IV ($p < 0.001$) (Fig. 6). In culture negative cows, the immunolabeling fraction increased from stage I to IV granulomas, with differences between stage I and II reaching statistical significance ($p = 0.034$).

Inducible nitric oxide synthase (iNOS+)

For culture positive cows only, the iNOS immunolabeling fraction increased from stage I to IV ($p=0.0001$) (Fig. 7).

DISCUSSION

This study used gross pathology, histological scoring and immunohistochemical techniques, to further understand the role of the immune response in cattle naturally infected with *M. bovis*. Initial gross and microscopic examination of lymph nodes and lungs, found the most numerous and severe lesions within thoracic lymph nodes. Immunohistochemical techniques were used to demonstrate that as the stage of

granuloma increased from I to IV, the immunolabeling fraction of CD3+ cells decreased, while the immunolabeling fraction of CD68+ macrophages, IFN-γ+, TNF-α and iNOS+ increased. Some of these changes were also shown to vary between *M. bovis* culture status, with the granulomas of culture negative animals showing a higher expression of CD68+, CD3+ (stage III and IV), IFN-γ+ and iNOS+ (stage I) when compared to culture positive animals.

Gross and microscopic examination demonstrated that characteristic TB lesions were most frequently identified in the caudal mediastinal, bronchial and cranial mediastinal lymph nodes of the thorax, and that the severity of these lesions was greater when compared to other lymph nodes. This result supports the respiratory tract as the most common route of infection (31), which is similar to the findings in a study on naturally infected *M. bovis* cattle from a comparable geographical area (36). Ameni found that when these cattle were exposed to an intensive husbandry system, they demonstrated a higher frequency and severity of bTB-lesions in the respiratory tract, but cattle kept on pasture showed a higher severity of bTB lesions in their abdominal lymph nodes (33, 36). In this study, the culture positive group showed a greater involvement of head and abdominal lymph nodes than the culture negative group, supporting the potential role of oral and other infection routes for this cohort.

Using immunohistochemical techniques, it was observed that in culture positive animals, the immunolabeling fraction of CD68+ macrophages increased with the granuloma stage from I to IV ($p<0.001$). An increase was also shown in culture negative animals, but this was not statistically significant. Similar findings have been shown in experimental infections, where CD68+ cell numbers increase as the level of granuloma increases (27). In culture positive animals, the presence of increased

MNGCs in advanced granulomas could be an indication of the active multiplication of the *M. bovis* bacteria, when the immune response is not able to contain the microorganism (37). Conversely, in culture negative animals higher CD68+ immunolabeling fractions were found at the early granuloma stages when compared to culture positive animals. This could be associated with the role of MNGCs in the early immune response, geared towards protection and elimination of the bacteria.

In contrast to CD68+ macrophages, in culture positive cows the immunolabeling fraction of CD3+ cells decreased from granuloma stage I to stage IV, but showed no decrease in culture negative animals. This finding is similar to an experimental study designed to evaluate the role of CD3+ cells response in BCG vaccinated and non-vaccinated groups during *M. bovis* infection (28), and supports the role of an adaptive immune response mediated by T cells in containment of *M. bovis* infection. Most importantly, the cell-mediated immune response effected by CD4+ T cells by producing Th1 cytokines, such as IFN-γ, and the cytolytic activity of CD8+ cells toward infected macrophages is crucial (38).

In culture positive animals the immunolabeling fraction of IFN-γ+, TNF-α+ and iNOS+ shows the same trend as CD68+ macrophages, increasing with the granuloma stage from I to IV. Evidence from natural *M. bovis* infection from other species has shown that the presence of CD68 macrophages and CD3 T cells in and surrounding granuloma correlates with the high level expression of pro-inflammatory cytokines like IFN-γ and TNF-α and iNOS effector molecules (34). These pro-inflammatory cytokines are important in promoting the formation and function of the granuloma. Previous studies (27, 28, 35) observed a significant increase in the level of pro-inflammatory cytokine, mainly IFN-γ+, as the stage of granuloma advances. Nitric

oxide (NO) production by macrophages during mycobacterial infection has also been shown to play a crucial role in the intracellular killing of mycobacteria, as it is cytotoxic at high concentrations (23). This observed increase in pro-inflammatory cytokines (IFN-γ and TNF-α) and effector molecules (iNOS) seems likely to have contributed to the regulation of the bovine immune response during *M. bovis* infection (35).

Evidence from this study provides basic information on the host immune response during natural infection with *M. bovis* which could be used for future studies in the investigation of biomarkers necessary for diagnostics and vaccines in the fight against bTB. Limitations that could affect generalization of these findings to other countries include the effects of regional influence on farming practices and cattle genetics, and the small number of culture negative animals for comparison with results from culture positive animals.

CONCLUSION

This study highlighted the role of macrophages, T cells and chemical mediators like IFN-γ, TNF-α and iNOS during naturally infected asymptomatic cows with *M. bovis* from intensive dairy farms in central Ethiopia. For *M. bovis* culture positive animals, the activity of CD68 macrophages, IFN-γ, TNF-α and iNOS were more intense as the level of granuloma increases while CD3+ T cells population decreases as the stage of granuloma increases. Thus, the activity of CD68+, IFN-γ+, TNF-α+ and iNOS+ could play a protective role in the immune defense against *M. bovis* during naturally infected asymptomatic cows.

MATERIAL AND METHODS

267 **Study setting and ethical statement**

268 The study was conducted on semi-urban intensive dairy farms situated in central
269 Ethiopia, Oromia Special Zone surrounding Addis Ababa City, the capital of Ethiopia.
270 The study obtained ethical approved from the Armauer Hansen Research Institute
271 (AHRI) Ethics Review Committee (Ref P018/17), from the Ethiopian National
272 Research Ethics Review Committee (Ref 310/253/2017), the Queen Mary University
273 of London Research Ethics Committee, London UK (Ref 16/YH/0410); and by the
274 Aklilu Lemma Institute of Pathobiology, Addis Ababa University (Ref
275 ALIPB/IRB/011/2017/18). Written informed consent was obtained from all the owners
276 of the farms.

277 **Animals**

278 A total of 16 single intradermal cervical comparative tuberculin test (SICCT) test
279 positive cows suspected to be naturally infected with *M. bovis* were obtained from 16
280 different farms. Sex and body condition score (BCS) were recorded. A method
281 developed by Nicholson and Butterworth (39) was used to determine the BCS. Poor
282 BCS was considered with extremely lean cattle with projecting dorsal spines pointed
283 to the touch and individual noticeable transverse processes. Medium BCS was
284 considered with cattle with usually visible ribs having little fat cover and barely visible
285 dorsal spines. Good BCS was considered with Fat cover is easily observed in critical
286 areas and the transverse processes were not visible or felt.

287 ***SICCT test***
288 Briefly, SCCIT test was performed as follows. Two sites on the right side of the mid-
289 neck, 12 cm apart, were shaved, and the skin thicknesses were measured with
290 calipers. One site was injected with an aliquot of 0.1ml containing 2,500 IU/ml bovine

PPD (PPDb) (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom). Similarly, 0.1ml of 2,500 IU/ml avian PPD (PPDa) (Veterinary Laboratories Agency, Addlestone, Surry, United Kingdom) was injected into the second site. After 72 h, the skin thicknesses at the injection sites were measured (11). Then the difference between the swellings of PPDa and PPDb were calculated and the positive result was determined at cut off $\geq$ 2 mm.

Culture

Isolation of mycobacteria was performed according to World Organization for Animal Health protocols (40). Briefly, tissue specimens for culture were collected into sterile universal bottles in 5ml of 0.9 % saline solution, and then transported to Aklilu Lemma Institute of Pathobiology (ALIPB) TB laboratory. The tissues were sectioned, homogenized and the sediment was neutralized by 1% (0.1N) HCl using phenol red as an indicator. Thereafter, 0.1ml of suspension from each sample was spread onto a slant of two Löwenstein Jensen (41) medium tubes one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37^{o}C for at least eight weeks and with weekly observation of the growth of colonies. In order to report culture negative, the tissues were repeatedly cultured three times.

Postmortem examination

The cows were humanely slaughtered by personnel of the local abattoirs in the study area. The post-mortem examination was performed by an experienced meat inspector. From all the 16 animals, a total of a total of 176 lymph nodes and 96 lung tissues were examined by slicing the tissue into 0.5-1cm sections, and assigning a pathology severity score, as developed by Vordermeier *et al*., 2002 (30) shown in Table S2. Both lymph node and lung pathology score were added to determine the

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total pathology score per animal. In order to maintain the scoring consistency, all scoring was performed by a single person.

Histopathology

A total of 37 tissue samples (27 culture positive and 10 culture negative) with high gross pathology scores were selected from lymph nodes and lung tissues. Lesions were carefully selected to include the encapsulated granulomas of different sizes with caseous necrosis and mineralisation.

The tissues were fixed in 10% neutral buffered formalin for 24-72 hours, embedded in paraffin, sectioned in 4µm sections and stained with hematoxylin-eosin (H&E) and Ziehl Neelsen acid fast stain. Granulomas were classified into different stage I to IV according to the previously described criteria (Table S3) (27). The granulomas were scored experienced Veterinary Pathologist before the result of *M. bovis* culture was known. Acid fast bacilli (AFB) were recorded as being present or not.

Immunohistochemistry

For the immunohistochemistry experiment, 4 µm formalin fixed tissue samples were stained with avidin-biotin-complex (ABC Vector Elite; Vector Laboratories) method. Tissue sections were first either deparaffinized or dewaxed and rehydrated. Antigen retrieval was induced by heat (Microwave) or enzymes (trypsin /chymotrypsin) (Sigma, Poole, UK) (Table 1) and adjusted to pH 9 or 6 using 0.1N sodium hydroxide. Tissue sections were washed in running tap water, and then incubated with a blocking buffer (normal goat/horse serum in 10 ml PBS) for 30 minutes. Slides were incubated with primary antibody overnight at room temperature and with the secondary antibody for 20 minutes. The labelling was amplified using avidin-biotin-peroxidase conjugate (ABC elite; Vector Laboratories) and visualized using 3, 30-

diaminobenzidine tetrahydrochloride. The unbound conjugates were removed prior to DAB application with two buffer washes. Finally, the slides were washed in tap water and stained by Mayer's Haematoxylin counterstain, and mounted for analysis. For negative control tissue we used a bovine lymph node with no gross lesion and no isolation of *M. bovis* with culture. For each experiment we included a slide with secondary antibody but no primary antibody.

Image analysis

For each granuloma, a total of 10 fields from different areas of the granuloma, avoiding necrotic and mineralized areas, were analyzed using a Fiji-ImageJ software (https://imagej.net/Fiji/Downloads). All images were examined at X400 magnification, and captured with an Olympus®DP74 digital camera attached to a microscope BX Olympus®63. Briefly, after image was imported to Fijii-Image J software actual color was deconvulated into three different colors (green, gray and blue) using H DAB vector. The second color (gray) used for further processing and converted into black and white contrast using "Make Binary" tool, color threshold was adjusted at default (0 scale for min and 255 for max). Next the mean (including minimum and maximum) value of area of fraction was taken and percent area was determined (42). For each antibody, the total area of positive labeling was given as a percentage of the total area examined in 10 fields.

Statistical Analysis

The results of the histopathological and the immunohistochemical analysis were expressed in mean and standard deviation, and the results were compared between the stages of granuloma and between culture results. A nonparametric statistical analysis employing Mann Whitney test was used to compare the means and $p<0.05$

was considered statistically significant. The analyses were conducted using GraphPad Prism 8.0 (San Diego, CA, USA).

CONTRIBUTORS

BT and GA conceived the study. BT, AZ, FD, HMM, ARM and GA contributed to study design and development of laboratory assays. BT, AZ, FD, HMM, MB, MA, TTB, DAJ, ARM and GA contributed to implementation of the study and contributed to the data acquisition. BT did statistical analyses, wrote the first draft of the manuscript and had final responsibility for the decision to submit for publication. All authors reviewed the final draft and agree with its content and conclusions.

DECLARATION OF INTERESTS

All authors have no competing interests to declare. The views expressed are those of the authors and not necessarily those of the United Kingdom Medical Research Council or the United Kingdom Department of Health.

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Fig. 1: Gross pathology severity score of lymph nodes and lung tissues of cattle positive for *M. bovis* culture (n=12) compared to negative for *M. bovis* culture (n=4). A) Gross pathology severity score of the *M. bovis* culture positive animals. B) Gross

pathology severity score of the *M. bovis* culture negative animals. C) Gross pathology severity score vs. culture result of both culture positive and culture negative animals. P values from Mann Whitney test. Proportions of animals positive for TB like lesion are also displayed. CP: culture positive, CN: culture negative, LN: Lymph nodes, LRM: left retropharyngeal medial, RPH: retropharyngeal, MCR: medial cranial, MCD: medial caudal, BRC: bronchial, TB: tracheobronchial, and MS: Mesenteric, HEP: hepatic, and LUN: lung.

Fig. 2: The four stages of granulomas in lymph nodes from naturally infected asymptomatic cows with *M. bovis*. A) Stage I (Initial). Clustered epithelioid macrophages are typical of this stage. HE 10*10. B) Stage II (Solid). Increased number of epitheliod macrophages including Langharn's giant cells (arrow). Encapsulation is complete and central caseous necrosis is lacking. HE 10*10. C) Stage III (Minimal necrosis) thinly encapsulated with epitheliod macrophages and caseous necrosis. HE 10*10. D) Stage IV (Necrosis and mineralization). Large, irregular, encapsulated granuloma, often with multiple centers of caseuous necrosis and mineralization. HE 10*10.

Fig. 3: Macrophages (CD68+). A) Mean percentage of area of positive immunolabeling within granulomas of stage I to IV for CD68+ within the lymph nodes and lung tissue. The mean percentage of immunolabeling fraction of culture positive animals significantly increase as the stage of granuloma increases from stage I to stage IV ($p<0.05$). Similarly for culture negative animals, as the stage of granuloma increases from stage I to stage IV an increased immunolabeling fraction was observed although it was not statistically significant ($p>0.05$). *Culture negative animals showed significantly higher immunolabeling fraction at stage I of the granuloma ($p=0.037$). The results are expressed as means and SD. Fiji-ImageJ

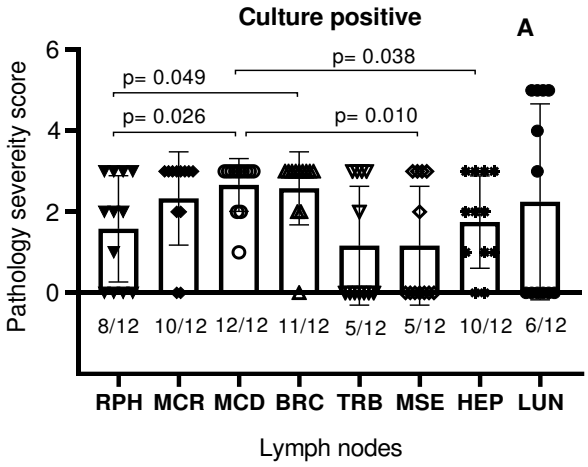
553 software was used to measure the % area of positive labeling. P values from Mann
554 Whitney test. Immunlabeling of CD68+ macrophages of the lymph nodes of *M. bovis*
555 culture positive (B, C) and (D, E) culture negative animals. Higher percentages of
556 CD68+ macrophages can be seen in stage IV granulomas (C, E) compared to stage
557 I (B, D).

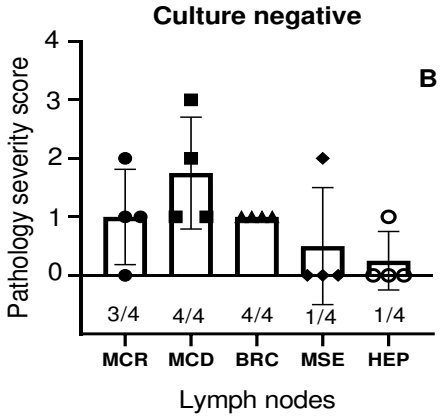
558 Fig. 4: T cells (CD3+). Mean percentage area of positive immunolabeling within
559 granulomas of stage I to IV for CD3+ T cells within the lymph nodes and lung tissue.
560 For culture positive animals, the mean percentage of CD3+ immunolabeling fraction
561 decreases as the stage of granuloma increases from stage I to stage IV ($p<0.05$). On
562 the other hand, for culture negative animals the immunolabeling fraction stayed the
563 same as the stage of granuloma increases. *At stage IV, culture negative animals
564 showed an increased CD3+ immunolabeling fraction as compared to culture positive
565 animals ($p<0.05$). The results are expressed as mean and SD. Fiji-ImageJ software
566 was used to measure the % area of positive labeling. P values from Mann Whitney
567 test.

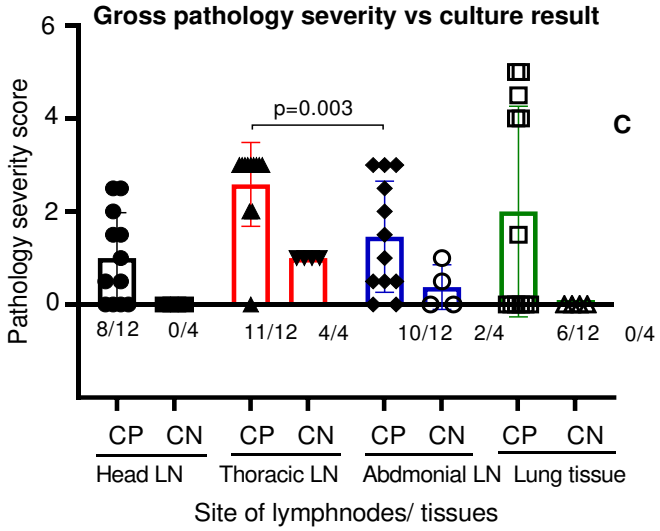
568 Fig. 5: Interferon gamma (IFN-γ+). A) Mean percentage area of positive
569 immunolabeling within granulomas of stage I to IV for IFN-γ+ within the lymph nodes
570 and lung tissue. Both culture positive and culture negative animals showed a
571 statistically significant increase in the mean percentage of immunolabeling fraction
572 ($p<0.05$). The results are expressed as means and SD. Fiji-ImageJ software was
573 used to measure the % area of positive labeling. P values from Mann Whitney test.
574 Immunolabeling of IFN-γ+ cells of the lymph nodes of *M. bovis* culture positive (B, C)
575 and (D, E) culture negative animals. Higher percentages of IFN-γ+ cells can be seen
576 in stage IV granulomas (C, D) compared to stage I (B, E).

577 Fig. 6: Tumor necrosis factor- alpha (TNF-α+). The mean percentage area of positive
578 immunolabeling for TNF-α+ within the lymph nodes and lung tissue of both culture
579 positive and negative animals showed an increase from stage I to IV granuloma
580 ($p<0.05$). The results are expressed as means and SD. Fiji-ImageJ software was
581 used to measure the % area of positive labeling. P values from Mann Whitney test.

582 Fig. 7: Inducible nitric oxide synthase (iNOS+). The mean percentage area of
583 positive immunolabeling for iNOs+ within the lymph nodes and lung tissue for culture
584 positive animals showed significant increase as the stage of granuloma increase
585 from stage I to IV ($p<0.05$). For culture negative animals the iNOS+ immunolabeling
586 fraction did not show any variation as the granuloma increases from stage I to IV.
587 The results are expressed as means and SD. Fiji-ImageJ software was used to
588 measure the % area of positive labeling. P values from Mann Whitney test.







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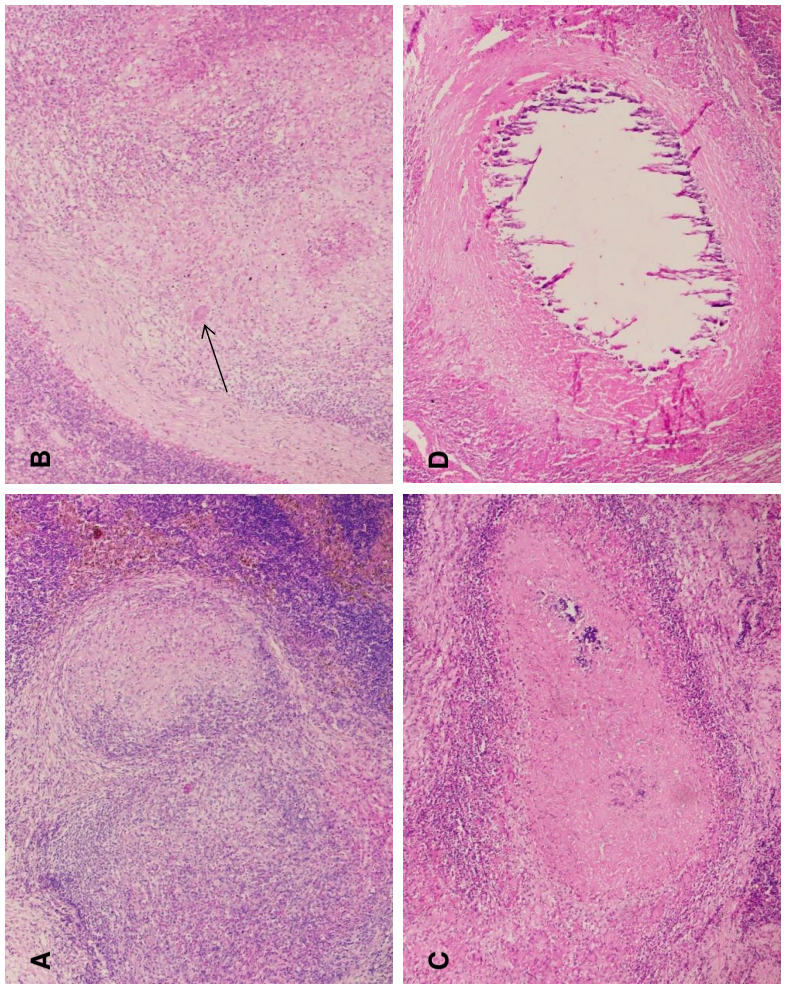

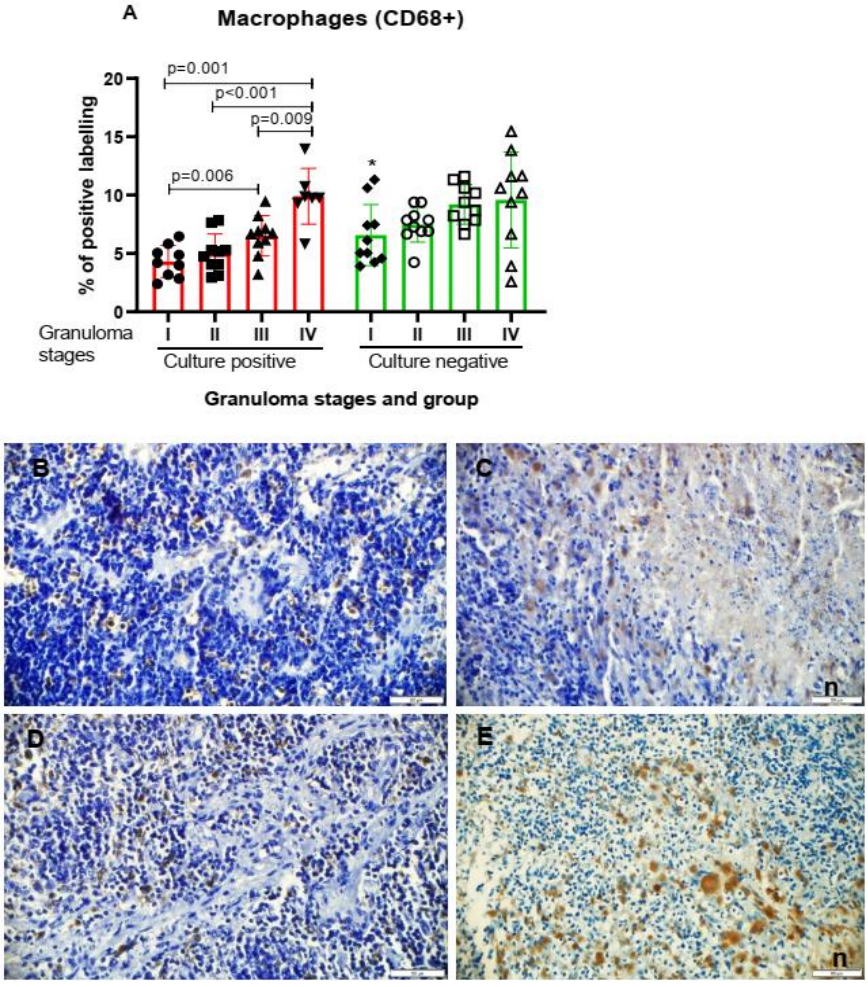

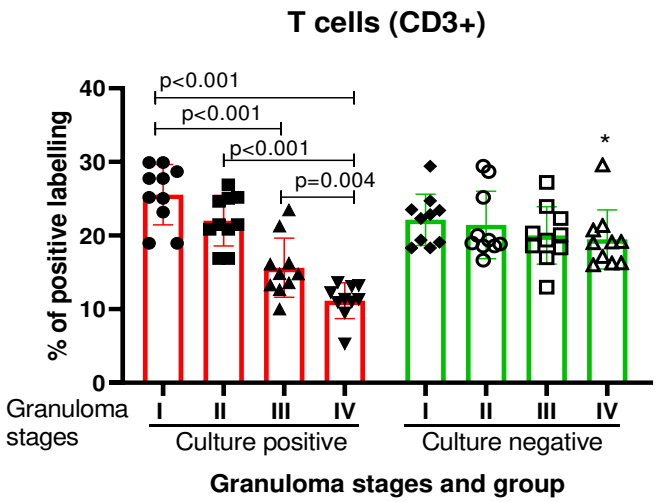

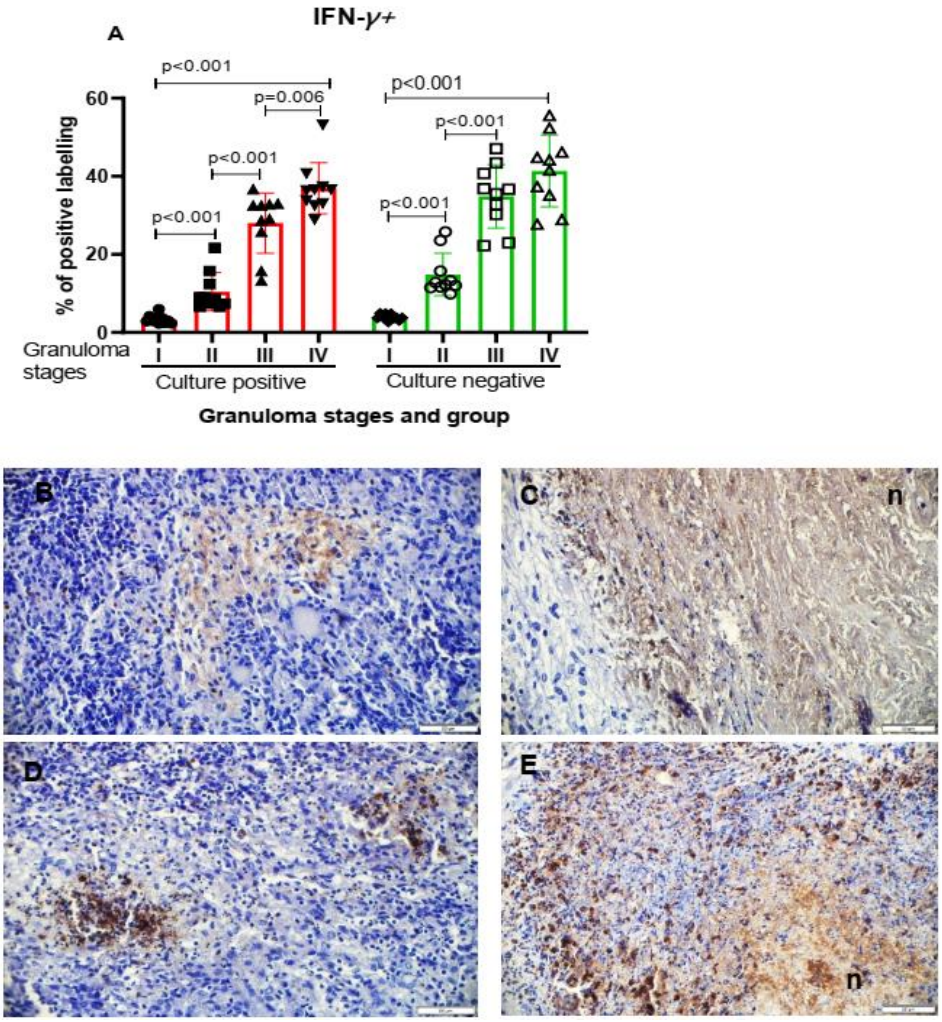

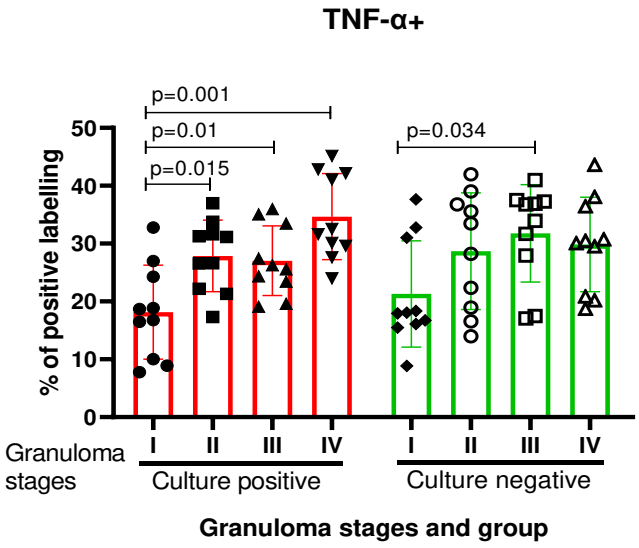

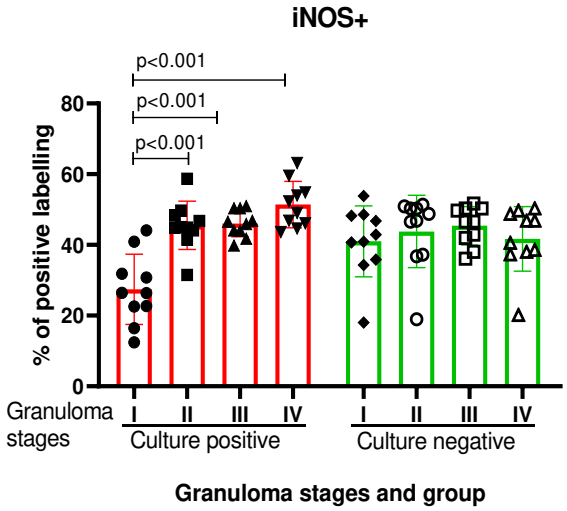

Table 1: Antibodies used for immunohistochemistry

Primary antibody	Antibody type	Supplier	Dilution	Antigen retrieval method	Secondary antibody	Buffer
CD68	Mouse versus human CD68(monoclonal)	Dako, M0718	1:50	Trypsin/chymotrypsin	Goat versus mouse (1/200)	TBS
CD3	Rabbit versus human CD3(polyclonal)	Dako, A0452 (Ely, UK)	1:400	Trypsin/chymotrypsin*	Goat versus rabbit (1/1000)	TBS
IFN-γ	Mouse versus bovine IFN-γ(monoclonal)	Serotec CC330	1:200	Microwave, 396 min at 100°C, in citric acid buffer, pH6	Goat versus mouse (1/200)	TBS
TNF-α	Mouse versus bovine TNF- α	Serotec MCA (Ab/15-3)	1:100	Trypsin/chymotrypsin	Goat versus mouse (1/200)	TBS
iNOS	Rabbit versus mouse iNOS(polyclonal)	Millepore 06-573 (Billerica, MA, USA)	1:400	Microwave, high-pH buffer, 295 min at 100°C	Goat versus rabbit (1/1000)	TBS

*Trypsin/chymotrypsin was prepared by measuring 0.5g of trypsin and 0.5g of chymotrypsin and 1g of CaCl2 were dissolved in 1L of distilled water and the resulting solution titrated to pH 7.8 using 0.1M sodium hydroxide solution.