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Article:

Immunohistochemical Expression of MAC387, Arginase 1 and CD163 in Tuberculous Granuloma of Imported Beef Cattle.

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Abstract

Macrophages are the first immune cells to interact with inhaled bacilli, and they have the ability to suppress bacterial growth through phagocytosis. Two distinct phenotypes can be found within granuloma. First, mycobacteria are eliminated more quickly and are killed by proinflammatory (M1). Second, anti-inflammatory macrophages (M2), are intended to preserve tissue integrity and support tissue repair. Therefore, the current study used histopathological examination to classify tuberculous granuloma into four stages (stage I to stage IV) and immunohistochemistry (IHC) to investigate the expression of MAC387, CD163, and Arginase 1 in naturally infected Beef cattle with tuberculosis at different Stages of TB granulomas. Immunohistochemical analysis revealed that the immunolabeling of MAC387$^+$ macrophages significantly reduced as the stage of granuloma increased from stage I to stage IV (P <0.008). However, the immunolabeling of Arg1$^+$ and CD163$^+$ macrophages significantly increased as the stage of granuloma increased from stage I to stage IV (P<0.01, and P<0.001, respectively).

Introduction

The primary cause of bovine tuberculosis (bTB) is *Mycobacterium bovis*, which affects various domestic and wild animal species, humans, and cattle. Animals with TB are significantly less productive, which results in significant economic losses [1, 2]. The most characteristic lesion of bovine tuberculosis is granuloma formation in the tissues and organs, more significantly in the lungs, and lymph nodes [3, 4].

Experimental infections in cattle have made it possible to qualitatively classify granulomas into four stages (I-IV) based on size, cellular composition, and the presence or absence of necrosis, fibrosis, and mineralization in granulomas during the course of bovine tuberculosis infection [5, 6] and more recently in natural infections [7, 8]. In spite of the granuloma's significance as a physical fence in the immune response to M. bovis, little is known about the dynamics of the granuloma-level immunological response [9]. The local cytokine microenvironment in the granuloma can guide macrophage polarization adopting different functional responses according to the stimuli and signals [10, 11]. Thus, two main macrophage subpopulations may be identified, the classically activated (M1) pro-inflammatory macrophages and the alternatively activated (M2) anti-inflammatory macrophages [10]. Mycobacteria replicate in macrophages, which are crucial to the pathophysiology of mycobacterial infections. The quantity and location of macrophages were then observed to vary as the granuloma developed. Macrophages make up a significant portion of the granuloma's cells in stages I and II, however in stages III and IV, they often form a thin rim around the necrotic core and are less prevalent in the outermost layers of the C.T capsule of the granuloma [6].

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Two enzymes associated with key macrophage functions in TB granulomas are inducible nitric oxide synthase (iNos) and arginase-1 (Arg1) which compete for the same substrate, L-arginine. The predominance of either enzyme spatially influences macrophage activation in different granuloma environments [12]. M2 macrophages are characterized by the up-regulation of several surface molecules such as arginase 1 (Arg1), CD163, CD206 [11, 13]. In the current study, immunohistochemistry (IHC) will be used to investigate the expression of MAC387, CD163 and Arginase 1 (Arg1) in spontaneously infected Beef cattle with tuberculosis at various Stages of TB granulomas. The role and location of M2 macrophages will also be discussed.

Materials and Methods

Ethical Considerations

The current study was performed in compliance with the Guidelines of the Egyptian regulations for imported animals. All experimental protocols and procedures were conducted by the ethical committee guidelines of the Faculty of Veterinary Medicine, Sohag University. The ethical approval number is Soh. Un. Vet/00036R

Animals

Imported beef cattle aged from 2 – 3 years from Sudan where they were slaughtered at the Middle East and Wadyna abattoirs, Abu Simbel, Aswan Governorate.

Postmortem examination and Samples collection:

The collection of the samples for this study was carried out during the period from September 2020 to March 2022. Routine postmortem examination of 2150 slaughtered beef cattle were carried out with particular attention to the tuberculous like lesions. Representative samples of the tissues from lymph, liver and lungs of 60 animals grossly showing tuberculous like lesions were collected.

Histopathological and Immunohistochemical examinations

The tissues were fixed in 10% neutral buffered formalin for 24 to 72 h, embedded in paraffin, sectioned into 4-m sections, and stained with hematoxylin-eosin (H&E. Granulomas were classified into the different stages (stages I to IV) according to the previously described criteria by [5] and recently by [8]. The Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector Laboratories) was carried out to identify and characterize macrophages (MAC387) and M2 macrophages (Arg1 and CD163). Briefly, 4 μm tissue sections were deparaffinized and rehydrated through graded alcohols, followed by blocking of the endogenous peroxidase activity using 3 % hydrogen peroxide in methanol for 30 minutes (min) in darkness. Table 1 summarizes antigen recovery method, and primary and secondary antibodies details. After antigen retrieval, sections were washed with PBS (pH 7.4) and incubated with blocking solution for 30 min at room temperature in a humidity chamber. Primary antibody was applied either neat or diluted and incubated overnight at 4 °C. Negative controls were included for each antibody; for the negative control, the primary antibody was replaced by the corresponding blocking solution to confirm the lack of non-specific binding. In addition, antibody specificity was verified by substituting the primary antibody by isotype-matched reagents of irrelevant specificity. After washing in PBS, the corresponding biotinylated secondary antibody was applied for 30 min followed by Avidin-Biotin-Peroxidase Complex (Vector Laboratories) for 1 hour at room temperature and darkness. Labeling was visualized by NovaRED™ substrate kit (Vector Laboratories). Finally, slides were counterstained with Harris' hematoxylin.

Image analysis:

Immunolabeled slides were subjected to objective digital image analysis to determine the positive percentage (%) of MAC387$^+$, Arg1$^+$ and CD163$^+$ immunolabeled cells. The whole area of the granuloma was selected as the region of interest (ROI), and the area of immunolabeled cells inside the ROI was determined by (Image J software) after setting thresholds. For stage III and stage IV granulomas, necrotic or mineralized center were not included in the analysis, as described previously [5]. The results were expressed as the percentage of immunolabeled cells within the whole area of the granuloma.

Statistical analysis

The results of the immunohistochemical analyses were expressed as the mean and standard deviation (SD) and the results were compared between the different stages of granuloma. A P value < 0.05 was considered statistically significant. The analyses were conducted using GraphPad Prism 5.0 software (GraphPad Prism software 5.0, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) and Tukey's multiple comparison post hoc test was used to compare expression between different stages of granuloma. In the figures, (*) indicates $P \leq 0.05$, (**) $P \leq 0.01$ and (***) $P \leq 0.001$.

Table 1 Summary of immunohistochemical methodology.

Primary antibody (Clone)	Type	Dilution	Antigen retrieval	Blocking solution	Secondary antibody (dilution)
Myeloid/Histiocyte Antigen (MAC387)	Monoclonal anti-human	1:100	Protease*	BSA 1%	Anti-Mouse (1:200)
Arginase 1	Polyclonal IgG	1:100	Citrate pH 8.5 Mw	BSA 1%	Anti-Goat (1:200)
CD163	Monoclonal anti-human	Neat	Citrate pH 6.0 Ov	NGS 10%	Anti-Mouse (1:200)

*BSA: Bovine serum albumin; NGS: Normal Goat serum; Mw: microwave; Ov: oven; *Protease from Bacillus licheniformis.*

Results

1.Histopathological examination

According to the histopathological examination of tissue samples from lymph nodes which were used for immunohistochemistry study, the granuloma was classified into 4 stages (I, II, III and IV) **(Fig. 1 A, B, C** and **D).** Stage I: Irregular unencapsulated clusters of epithelioid macrophages, with interspersed lymphocytes and few admixed neutrophils. Langhan's multinucleated giant cells may be present, but necrosis is not present. Stage II: Granulomas composed primarily of epithelioid macrophages and enclosed partly or completely by a thin capsule. Infiltration of lymphocytes, neutrophils and often Langhan's multinucleated giant cells with minimal necrotic areas are sometimes present, generally composed of necrotic inflammatory cells. Stage III: The granuloma is fully encapsulated, with central necrotic areas, which are caseous and minimally mineralized. Epithelioid macrophages admixed with Langhan's multinucleated giant cells surrounded the necrotic areas. A peripheral zone of macrophages mixed with clusters of lymphocytes and scattered neutrophils extended to the fibrous capsule. Stage IV: Thickly encapsulated, large, irregular, multicentric granulomas with prominent caseous necrosis and extensive islands of mineralization comprising the greatest area of the lesion. Epithelioid macrophages and multinucleated giant cells surrounded the necrosis, with particularly dense clusters of lymphocytes near the peripheral fibrotic capsule.

2.Immunohistochemical examination:

2.1 MAC387+ macrophages:

MAC387 was used as a pan or general marker for macrophages and multinucleated giant cells, MAC387-positive macrophages were very abundant and increase the area of immunolabeling in stage I and II granulomas while stage III and IV showed fewer positive cells (**Fig. 2**). cells were diffusely distributed within the granulomas of early stages **(Fig. 3, A and B)** while they were present in the outer layers in more advanced stage

in the development of the granulomas **(Fig. 3, C and D).**

2.2 Arginase1+ macrophages:

Anti-Arginase1 antibody was used to identify epithelioid, macrophages and multinucleated giant cells (MNGCs). Arginase1 was considered as M2 marker. The Arginase1 immunolabeling fraction increased from stages I to IV **(Fig. 4)**. The majority of Arginase1-expressing cells were macrophages, including epithelioid cells and multinucleated giant cells. The positive reaction was lower in stage I/II granulomas, occasionally forming clusters of positive cells **(Fig. 5, A and B).** Stage III/IV granulomas showed positive macrophages with strong immunolabeling in the outer internal layers of the granulomas and surrounding the necrotic foci with mineralization **(Fig. 5, C and D)**.
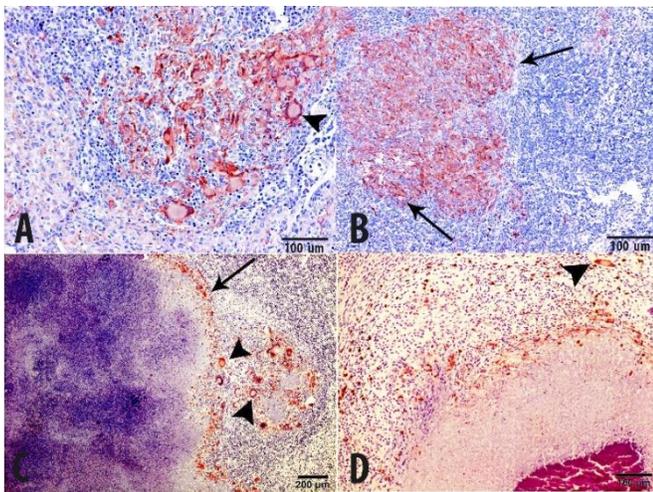
2.3 CD163+ Macrophages

Anti-CD163 antibody was used to identify epithelioid, macrophages and multinucleated giant cells (MNGCs). CD163 was considered as M2 marker, immunohistochemical staining for CD163 showed the location of macrophages within all stages of the granuloma but the expression was different in distribution of immunolabeled cells and percentage of positive area in each stage of granuloma. Generally, immunohistochemical staining for CD163 showed a higher mean percentage staining in the late stages of granulomas compared to the early stages of granuloma, being statistically significant in stages I and IV. The positive area of CD163+ cells increased as granuloma development occurred **(Fig. 6)**. In early stages of granuloma (Stage I and stage II), the positive cells were few and scattered through the granuloma **(Fig. 7, A and B)** compared to the late stages (stage III and Stage IV), the positive cells were numerous surrounding the necrotic area and near to the fibrous C.T capsule **(Fig. 7, C and D)**.
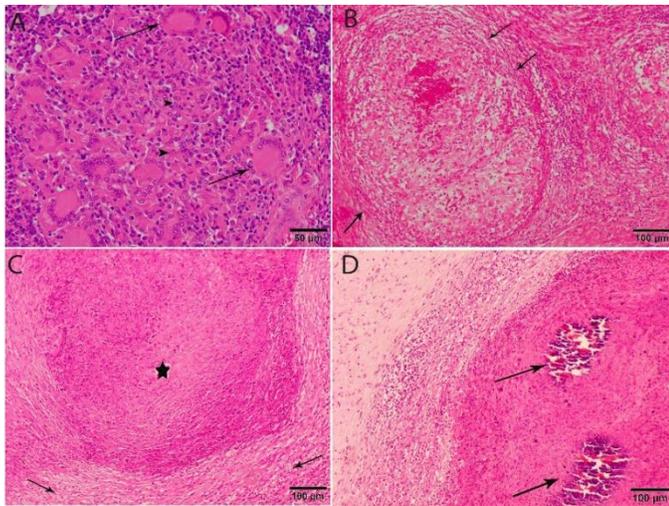
Fig.1: Histological sections showing the four stages of granulomas from naturally infected beef cattle with bovine tuberculosis. A) Stage I granuloma, epithelioid cell, and multinucleated giant cells (arrows) surrounded by lymphocytes. (Hx & E. Bar = 50
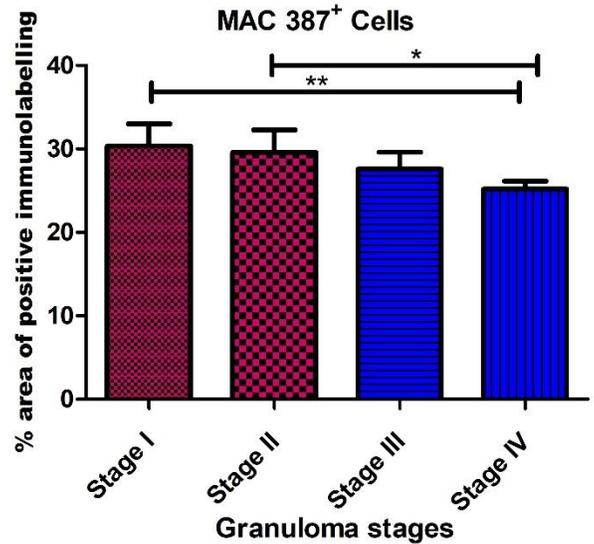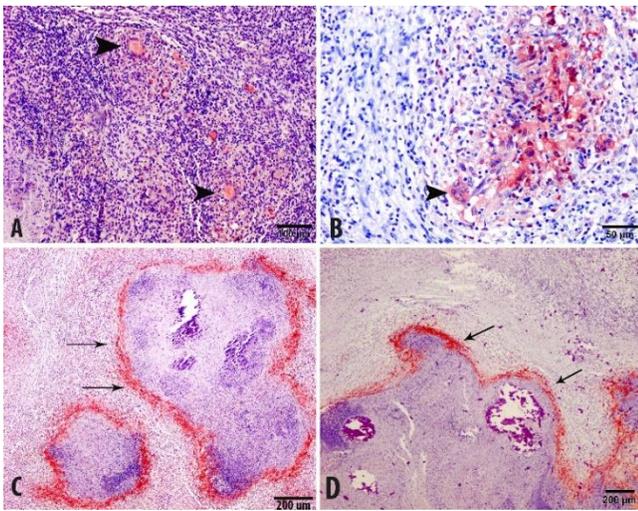

Fig. 2: The MAC387 + immunolabeling fraction was higher in the early-stage granulomas compared with late stages, with significant difference between stage I and stage IV) (P <0.008).


Fig. 3: MAC387+ staining in (A): stage I and (B): stage II granulomas in the lymph node of beef cattle naturally infected with Mycobacterium. Heavy positive staining can be observed within the cytoplasm of macrophages (arrows) and multi-nucleated giant.
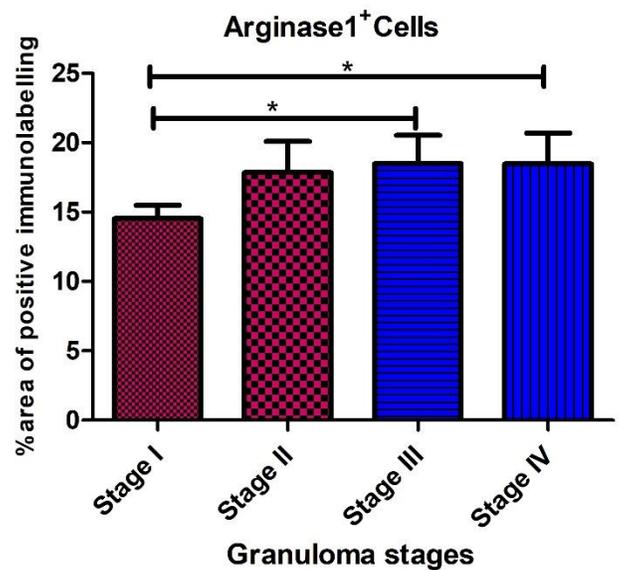

Fig. 4: The Arginase 1 + immunolabeling fraction was higher in the late-stage granulomas compared with early stages, with a significant difference between stage I and stage IV. (P <0.01).

Fig. 5: Arginase 1+ immunolabeling in stage I and II granulomas (A&B) showing: positive expression observed in a few scattered multinucleated giant and macrophages cells (arrowheads). (IHC, Bar = 100 μm). (C): Arginase 1+ macrophages immunolabeling form
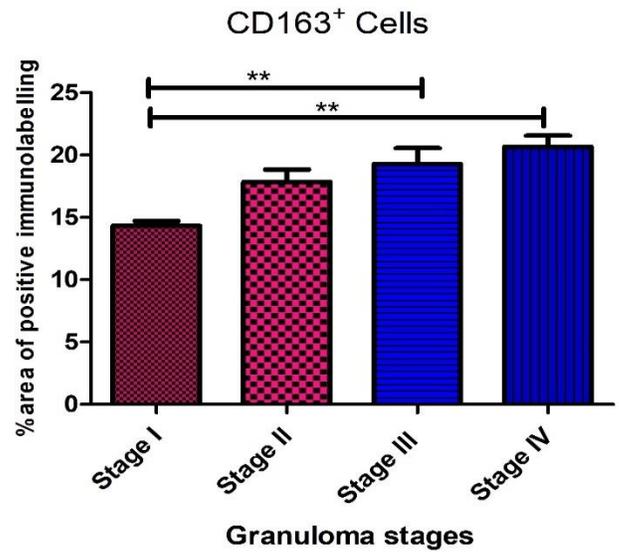
Fig. 6: The CD163 + immunolabeling fraction was higher in the late-stages granulomas compared with early stages, with significant difference between stage I and stage IV. (P <0.001).
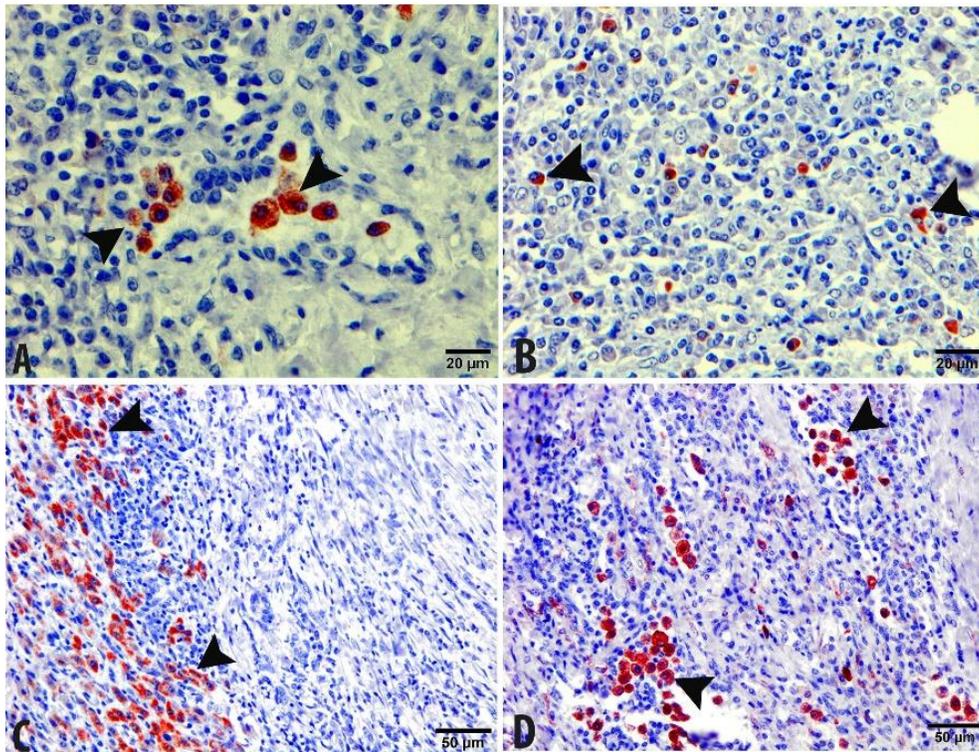
Fig. 7: CD163+ immunolabeling in stage I and II granulomas (A&B) showing: cytoplasmic and cell surface strong positive expression observed in a few scattered macrophages cells (arrowheads). (IHC, Bar = 20 μm). (C): CD163+ macrophages immunolabeling for

Discussion

Bovine TB is a chronic, progressive, infectious, and contagious disease caused by *M. bovis* [14]. bTB is characterized by gross and microscopic lesions (tubercles), however, the antemortem diagnosis of this disease is difficult due to the chronicity of the infection and the modulation of the immune response. The complex interaction between mycobacteria and the host immune system makes it difficult to understand the pathogenesis of animal tuberculosis, affecting its diagnosis and control. Therefore, deepening in the mechanisms involved in the onset and progress of the TB granuloma along the infection may increase valuable information to advance the understanding of this disease. Based on the histopathological assessment of tissue sections in this study, the granulomas were classified into four stages (Stage I to Stage IV). Different developmental stages of granuloma formation found within the same organ, during histological assessment of tissue sections in this study. Presence of different microenvironments within the same tissue has been previously suggested in studies with cattle experimentally infected with *M. bovis* [15, 16] or with cattle naturally infected with mycobacterium tuberculosis complex [7, 8]. Stage I, distinguished by irregular, unencapsulated clusters of primarily epithelioid macrophages, lymphocytes, and multinucleated giant cells that lack necrosis. Stage II, formed by primarily epithelioid macrophages, Langhans giant cells, and lymphocytes around limited areas of necrosis, was partially or thinly encapsulated. Encapsulated granulomas with central caseous necrosis were present at stage (III). Stage IV consisted of enormous, thickly encapsulated, multicentric granulomas with significant caseous necrosis and highly mineralized areas surrounded by epithelioid cells, giant cells, and lymphocytes. Also, these descriptions in our result agreed with those reported by many authors such as [7, 8]. The expression of different markers involved in myeloid cell differentiation and macrophage polarization were evaluated in granulomas located in lymph nodes from cattle naturally infected with tuberculosis. Firstly, specific marker for myeloid cells (MAC387) were used to characterize the macrophage population along the different granuloma stages, and secondly, a specific marker for M2 macrophages (Arginase1, CD163) was evaluated. Results show that MAC387 is expressed in cattle granulomas, decreasing slightly its expression in advanced stage granulomas (III and IV), similar to that reported by [17, 18] in fallow deer and wild boar, in which the percentage of MAC387^{+} cells decreased towards the more advanced stages.

M2 polarization was explored by the expression of Arg 1 and CD163. Interestingly, the Arginase1 immunolabeling fraction increased from stage I to IV. The majority of Arginase1- expressing cells were macrophages, including epithelioid cells and multinucleated giant cells. The positive reaction was lower in stage I/II granulomas, occasionally forming clusters of positive cells. Stage III/IV granulomas showed positive macrophages with strong immunolabeling in the outer internal layers of the granulomas and surrounding the necrotic foci with mineralization. There are scarce number of studies analyzing this marker in TB in livestock, but similar results have been previously observed in mice [19], macaques [12]and human lungs [20]. Mycobacteria escapes the host immune response by manipulating multiple host cell signaling pathways. For example, *mycobacteria* are able to survive and multiply within phagosomes, reducing its exposure to toxic antibacterial agents produced by the host. One of the most important host antimycobacterial mechanisms is the production of nitric oxide (NO), which is toxic to various intracellular pathogens, including Mtb. In mouse models of Mtb infection, it has been shown that the ability to escape NO toxicity is essential for bacterial survival [21]. In activated macrophages, NO is a product of L-arginine conversion of L-citrulline by inducible NO synthase (iNOS). Besides iNOS, L-arginine is also a substrate for arginase 1 (Arg1) enzyme, which converts L-arginine into urea and L-ornithine, the precursor of polyamines. Mtb may increase the expression of Arg1, leading to competition with iNOS for its substrate (L-arginine) and drastic reduction in NO production [19]. Our results agree with similar suppressive roles of Arg1-expressing macrophages in other chronic infectious diseases granuloma [22, 23]. Moreover, Arg1-expressing cells accumulate in and around *S. mansoni* granulomas [22] and in skin lesions in case of cutaneous leishmaniasis [24] ; these lesions often are necrotic and hypoxic [25] similar to tuberculous granuloma [26]. Therefore, increased Arg1 expressing macrophages in late granuloma stages lead to the failure of healing and persistence of lesions, which is a consequence of impaired T-cell responses resulting from local depletion of L-arginine by arginase1 [20, 26, 27]. These observations indicate that the control of T-cell responses by Arg1 is a common mechanism of immunosuppression in chronic tuberculous infections. In the present study, CD163 was considered as M2 marker, immunohistochemical staining for CD163 showed a higher mean percentage staining in the late stages of granulomas compared to the early stages of granuloma, being statistically significant in stages I and IV.

CD163 has been classically categorized as M2, anti-inflammatory, marker [28, 29] but unfortunately poorly studied during TB in domestic animals. Regarding this, Mattila et al., (2013) observed that granulomas with a necrotic core have a high percentage of CD163^{+} cells close to the periphery of the granuloma in humans and macaques,

as well as in the surrounding fibrous capsule, similarly to our findings in advanced stages of granulomas in cattle. This suggests that in cattle there is also a tendency towards the M2 profile, particularly at end stage granulomas. In a previous study, a higher histopathology score was observed for macrophages expressing CD163 in diffuse multibacillary lesions (late-stage disease) [30]. The results of these previous studies are consistent with increased expression of CD163 observed in late granuloma stages in the current study. A recent report showed that TB-modulated immune environments are sufficient to polarize macrophages toward the M2-like phenotype. These polarized macrophages, characterized as CD16$^+$ CD163$^+$, showed pathogen permissively by secreting anti-inflammatory cytokines, more susceptibility to intracellular bacterial growth, and inhibition of T-cell proliferation, resulting in allowing persistence of infection and deterioration of host defenses [31, 32]. In this line, although a pro-inflammatory status might be beneficial to control the development of the granuloma, once established, the anti-inflammatory response could act to control tissue damage generated by the granuloma itself.

Conclusion

This study decorated the role and expression of MAC387$^+$, CD163$^+$ and Arginase1$^+$ macrophages in different stages of the granuloma. Increase expression of CD163$^+$, and Arginase1$^+$ macrophages in late stages of granuloma (III, IV) compared with early stages (I, II). Therefore, we can conclude that Anti-inflammatory macrophages (M2) are more polarized in late stages of granuloma (III, IV) resulting in allowing persistence of infection and deterioration of host defenses.

Authors' contribution

The work was equally distributed between authors. All authors have read and approved the final version of the manuscript.

Conflict of interest

There is no conflict of interest.

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