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Immunohistochemical detection of pro-inflammatory and anti-inflammatory cytokines in granulomas in cattle with natural *Mycobacterium bovis* infection

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ABSTRACT

Cellular immune response was evaluated in lymph nodes and lung with different granulomatous lesions from cattle naturally infected with *Mycobacterium bovis*. For this purpose, we assessed pro-inflammatory and anti-inflammatory cytokines by immunohistochemical assays. Immunoreaction was observed for all the cytokines analyzed. Fourteen animals displayed advanced stage IV granulomas, with intense immunoreactivity to IFN-γ and TGF-β in areas of caseous necrosis, macrophages and lymphocytes. Seven animals showed stage III granuloma, with high immunoreactivity to IFN-γ (average of 44.5% immunoreactive cells) and moderate to TNF-α and to the anti-inflammatory cytokines IL-10 and TGF-β, in relation to the proliferation of fibroblasts in granuloma periphery We found satellite stage I granulomas in 4 bovines and stage II granulomas in 2 bovines, which exhibited low immunostaining response (~13%). Cytokine expression in stage III and IV granulomas was significant, with predominance of immunoreactivity to IFN-γ, thus suggesting a strong, longstanding local immune response mediated by macrophages and epithelioid cells. In addition, these two stages displayed lower reactivity to IL-10; which suggests a deficit of anti-inflammatory cytokines, suppressed immunity and persistence of the infection. High expression of TGF-β could indicate a chronic process with greater tissue damage and fibrosis. Numerous bacilli observed in necrotic areas in stage III and IV granulomas with low expression of IL-1β suggest failure in the immune response with bacterial multiplication. In this study, evidence of in situ presence of cytokines demonstrates these cytokines are involved in the development and evolution of bovine tuberculosis granulomas.

1. Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis, which is an infectious disease that affects particularly cattle, together with several mammals, including humans (Amanfu, 2006). *M. bovis* is transmitted to humans through contaminated milk and dairy products (Kantor et al., 2008) and also through contact with cattle, as may be the case of workers in rural areas and slaughterhouses.

Bovine tuberculosis is present in most countries in the Latin American Region, with variable relevance. In Argentina, on an average of 10 million ovines slaughtered annually, the percentage of bovines with apparent TB lesions at the slaughterhouse inspection decreased from 6.7% to 0.6% between 1969 and 2013 (Pezzone et al., 2011; Torres, 2014). This infection limits livestock production. Argentina produces and exports meat and dairy products and, therefore, bovine tuberculosis is a disease of economic and health concern. The limited understanding of local immune response to *M. bovis* restricts the knowledge of bovine tuberculosis. An effective immune response seems to mainly rely on cell-mediated immune response T-helper type-1 (TH1), and are probably controlled by cytokines released by antigen-specific T cells. Interferon-gamma (IFN-γ) could be a crucial cytokine implicated in this disease (Cooper et al., 1993; Thacker et al., 2007).

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Other pro-inflammatory cytokines, such as Interleukin 12 (IL-12) and Tumor necrosis factor alpha (TNF-α), probably play a role in the TH1 response and granuloma formation; this granuloma is the culmination of the cell-mediated immune response, in which bacilli are walled off, presumably to prevent their spread yet contributing notoriously to tissue damage (Flynn and Chan, 2001; Ulrichs and Kaufmann, 2006). Transforming growth factor-beta 1 (TGF-β1) reduces monocytes ability to limit *M. tuberculosis* in humans. Indeed, TGF-β1 promotes fibrosis and thus increases extracellular matrix deposition (Aung et al., 2000). The balance between controlling bacterial spread and tissue damage may represent the most significant biological challenge to the host immune response.

T-Helper type-2 (TH2) response is characterized by the production of anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 and is believed to inhibit the TH1 response. During the course of infection, cattle show a predominant TH1 response soon after infection that subsequently turns into a TH2-like response; this conversion could be correlated with an increased pathology (Ritacco et al., 1991; Welsh et al., 2005).

Understanding the contribution of TH1 and TH2 responses to protective immunity and pathology of *M. bovis* infection may aid to the development of effective vaccines for cattle (Thacker et al., 2007).

The immune response to *M. bovis* in cattle has been well studied at the systemic level and also by means of the characterization of cell populations in the granulomas (Pollock et al., 1996; Rhodes et al., 2000; Wangoo et al., 2005; Liebana et al., 2007). However, few studies have assessed pro-inflammatory and anti-inflammatory cytokine expression at the tissue level or have investigated the role of these cytokines in granulomatous pathology (Palmer et al., 2007).

The aim of this study was to evaluate the cellular immune response in different granulomatous lesions of bovines naturally infected with *M. bovis*. For this purpose, we performed immunohistochemistry assays to detect IFN-γ, TNF-α, IL-1β, IL-10 and TGF-β.

2. Materials and methods

2.1. Animal population and samples

A total of 21 naturally infected bovines with positive isolation of *M. bovis* were included in this study. The bovines were Holando-Argentino breed, cows ($n = 16$, >30 months, >350 kg) and steers ($n = 5$, >-18 months, >350 kg).

Samples of tissues with granulomatous lesions were obtained from official slaughterhouses in Santa Fe province. The samples consisted of tissues positive to *M. bovis* by both culture on Stonebrink and Löwenstein-Jensen accordingly to OIE Terrestrial Manual (2009) and to IS6110 PCR as previously described (Zumárraga et al., 2005). The collected tissues were mediastinal lymph nodes (48%), retropharyngeal lymph nodes (33%), tracheobronchial lymph nodes (14%) and lung (5%).

2.2. Histopathology

Tissues with granulomatous macroscopic lesions compatible with bovine tuberculosis from 21 slaughtered bovines were processed according to routine histopathological techniques. The lesions consisted of well encapsulated granulomas of different sizes with caseous necrosis and calcification. After 24 h fixation in 10% neutral buffered formalin, the samples were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin-eosin (H&E) and Ziehl-Neelsen acid-fast staining. Microscopic lesions and presence of acid-fast bacilli were evaluated and the granulomas classified in stages I to IV according to the previously described criteria (Wangoo et al., 2005). All data were registered in individual ad-hoc spreadsheets.

2.3. Immunohistochemical methods

Tissues in paraffin from 21 bovines diagnosed with bovine tuberculosis by histopathological lesions were used to perform the immunohistochemical study as previously described (Liebana et al., 1997). Sections of 4 μm were mounted on ionized slides and were dewaxed, rehydrated and pretreated for antigen retrieval by microwaving in citric acid buffer (10 mM), pH = 6.0 for 3 min at 100 °C. Then, the samples were treated with hydrogen peroxide (1.5 ml) in 97 ml of methanol for 15 min. The immunohistochemical technique performed in this study was streptavidin-biotin-peroxidase (SAB).

The (primary) monoclonal antibodies produced in mouse, anti-IFN-γ, anti-IL-1β, anti-IL-10 and anti-TGF-β, and the polyclonal antibodies produced in rabbit, anti-TNF-α, (Table 1) were applied to the sections overnight at 4 °C. The secondary antibodies used to detect the monoclonal and polyclonal antibodies were equine anti-mouse IgG and a biotinylated goat anti-rabbit IgG, respectively (Table 1). The secondary antibodies were applied for 30 min at room temperature. Streptavidin-HRP conjugate (Ref 434323, Invitrogen, Carlsbad, CA 92008, U.S.A.) was incubated at a 1:400 dilution, for 30 min at room temperature and the development was performed with DAB peroxidase substrate (DAB Substrate Kit, Catalog SK-4100 Vector Laboratories Inc., 30 Ingold Road, Burlingame, CA 94010, U.S.A.). DAB produces a brown color at the site of the reaction. The sections were slightly counterstained with Mayer's hematoxylin. As a negative control, we used a sample from a lymph node of a bovine without gross granulomatous lesions and with negative isolation of *M. bovis*.

Digital image capture was performed using an Olympus® DP 50 digital camera attached to a microscope BX Olympus® 50. For image analysis Image Pro Plus-4® was used counting 10 fields of the granuloma at 40×, and registering the total of cells with positive immunostaining according to Nicol et al. (2008). The percentage of cells with positive reaction was established respect to the total number of cells counted in 10 fields in different sectors of the granuloma.

2.4. Statistical analysis

Data were analyzed by one-way ANOVA with multiple comparisons test and Bonferroni posttest. Any difference with $p < 0.05$ were considered significant. The results shown are the mean of the measurements.

3. Results

3.1. Histopathology

Histopathology with H&E staining revealed different stages of granulomas, mostly of stage IV ($n = 14$) and stage III ($n = 7$), but also satellite granulomas of stage I ($n = 4$) and II ($n = 2$), accordingly to Wangoo et al. (2005) classification.

Stage IV granulomas exhibited mostly necrosis and mineralization. A large fibrous capsule was evident and this capsule shaped an irregular area of large necrosis and mineralization. Peripheral necrotic areas

Table 1
Monoclonal and polyclonal antibodies, manufacturers and dilutions used for immunohistochemistry.

Antibodies	Code and manufacturer[a]	Dilution
Primary antibodies		
Mouse anti-bovine IFN-γ	MCA 1964 – Serotec, Oxford, UK	1:25
Mouse anti-sheep IL-1β	MCA 1658 – Serotec, Oxford, UK	1:25
Mouse anti-bovine IL-10	MCA 2110 – Serotec, Oxford, UK	1:25
Mouse anti-human TGF-β	MAB 1032 – Chemicon, CA, USA	1:700
Rabbit anti-bovine TNF-α	AMP 852 – Serotec, Oxford, UK	1:300
Secondary antibodies		
Biotinylated equine anti-mouse IgG	Code BA 2000 - Vector Laboratories Inc., CA, U.S.A.	1:400
Biotinylated goat anti-rabbit IgG	Code BA 1000 - Vector Laboratories Inc., CA, U.S.A.	1:400

[a] Serotec, Oxford, UK; Chemicon, CA, USA; Vector Laboratories Inc., 30 Ingold Road, Burlingame, CA 94010, U.S.A.

consisted of macrophages, epithelioid cells, lymphocytes and Langhan's giant cells (Fig. 1, 4a–b). Stage III granulomas exhibited epithelioid and Langhan's giant cells in the peripheral areas of the central caseous necrosis, with central calcification. Near the fibrous capsule, the inflammatory cell population consisted of macrophages, lymphocytes and scattered neutrophils (Fig. 1, 3a–b). Stage II granulomas exhibited limited necrosis with neutrophils, lymphocytes, macrophages and scant Langhan's giant cells. These granulomas appeared as satellites of stage IV lesions in two animals. In addition, few fibroblasts delimited these granulomas (Fig. 1, 2a–b). Finally, stage I granulomas displayed irregular unencapsulated cluster of epithelioid macrophages, dispersed lymphocytes and small number of Langhan's cells. In four animals, these granulomas were satellites of stage III and IV granulomas (Fig. 1, 1a–b).

Ziehl-Neelsen staining revealed acid-fast bacilli (data not shown), with 5 and >5 bacilli (≥5) in 13 bovines and only few (0–1) acid-fast bacilli in 7 animals with stage IV granulomas, 1 animal with stage III granulomas and in six bovines with satellites stage I and II granulomas (Table 2).

3.2. Immunohistochemistry analysis of the four different stage of granuloma in bovine tuberculosis

We observed immunostaining positive reaction to the different cytokines in all sections of tissues with granulomatous lesions of 21 slaughtered bovines analyzed (Tables 2, 3 and Figs. 1 and 2). Table 3 also shows significant ($p < 0.05$) differences found in comparisons of cytokine immunostaining across stages and of all cytokines per stage.

Granulomas classified as stage IV ($n = 14$) typically showed strongest reactivity to IFN-γ than to IL-10 ($p > 0.05$). This reactivity was evident in necrotic and peripheral zones with strong reactivity in lymphocytes, macrophages and epithelioid cells, and lower intensity in Langhan's giant cells (Fig. 1, 4c–d). Furthermore, bovines with generalized tuberculosis showed a high grade of immunostaining for IFN-γ (cases # 8 and # 10), except for bovine # 19. This bovine showed stronger staining for IL-10 than for IFN-γ and in this case all Langhan's giant cells were negative to IFN-γ immunohistochemistry assay. Five bovines exhibited >50% of immunostained positive TNF-α cells (macrophages,

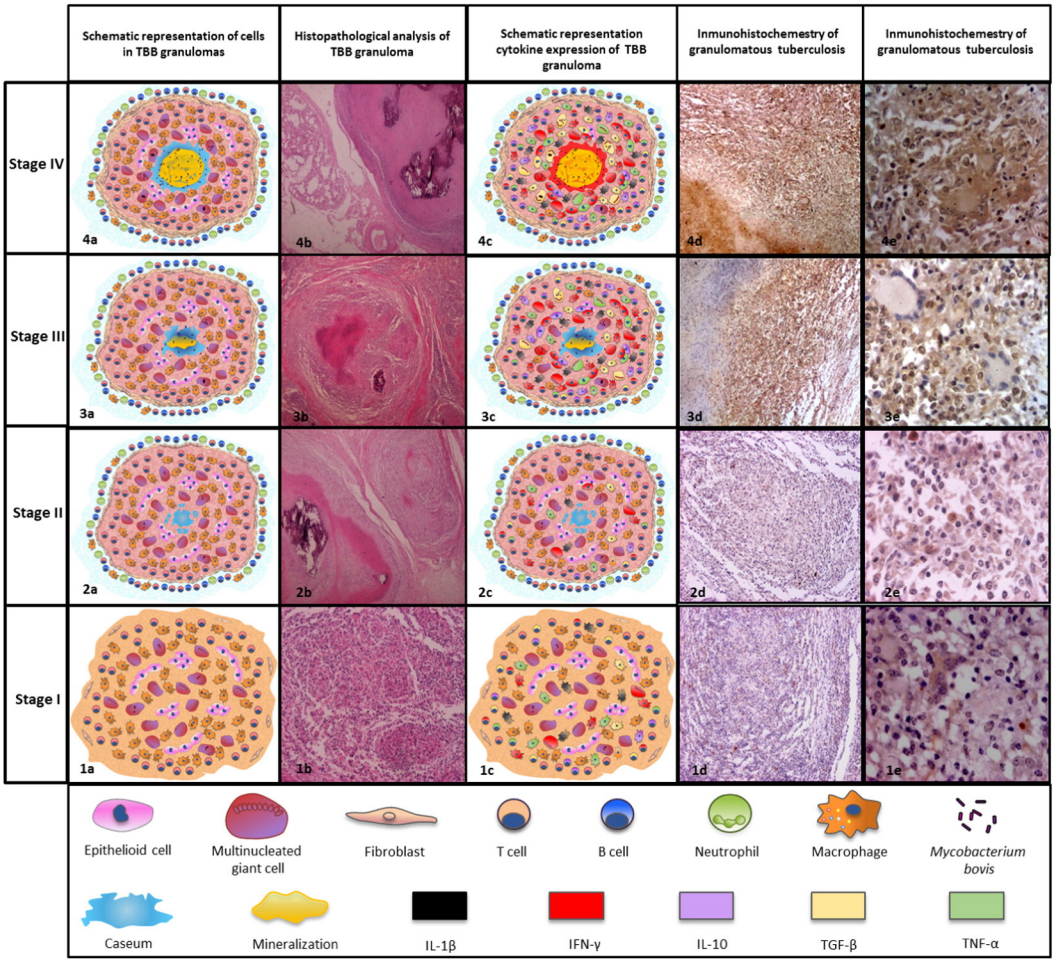

Fig. 1. 4a: Schematic representation of different cells in stage IV granuloma. 4b: Section of a lung with stage IV granuloma. Bovine 17. H&E. ×20. 4c: Schematic representation of cytokine expression in stage IV granuloma. 4d: Demonstration by IHC of IFN-γ in stage IV granuloma. Immunostaining in central necrosis and peripheral cells (Langhan's giant cells, epithelioid cells, macrophages and lymphocytes) (31,15% of cell with immunostaining). Case # 17. Lung. ×100. 4e: Demonstration by IHC of TGF-β in stage IV granuloma. Many macrophages and epithelioid cells show a strong positive cytoplasmic immunostaining (66,38%). # No. 17. Lung. ×400. 3a: Schematic representation of different cells in stage III granuloma. 3b: Section of a tracheobronchial lymph node with stage III granuloma. Bovine 3. H&E. ×20. 3c: Schematic representation of cytokine expression in stage III granuloma. 3d: Demonstration by IHC of IL-10 in stage III granuloma in lymphocytes and negative in central necrosis. Case no. 3. Tracheobronchial lymph node. ×100. 3e: Demonstration by IHC of IL-10 in stage III granuloma. Langhan's giant cells are negative, while many lymphocytes, macrophages and epithelioid cells are positive to IL-10 (31,24%). Case # 3. Tracheobronchial lymph node. ×400. 2a: Schematic representation of different cells in stage II granuloma. 2b: Section of a lung with satellite stage II granuloma. Bovine 17. H&E. ×20. 2c: Schematic representation of cytokine expression in stage II granuloma. 2d: Demonstration by IHC of IFN-γ in scattered cells of satellite granuloma stage II. Case no. 17. Lung. ×40. 2e: Demonstration by IHC of IFN-γ in scattered macrophages and lymphocytes (7,54% of cell with immunostaining) of satellite granuloma stage II. Case # 17. Lung. ×400. 1a: Schematic representation of different cells in stage I granuloma. 1b: Section of a tracheobronchial lymph node with satellite stage I granuloma, with irregular unencapsulated cluster of epithelioid macrophages, dispersed lymphocytes and scattered Langhan's cells. Bovine 15. H&E. ×100. 1c: Schematic representation of cytokine expression in stage I granuloma. 1d: Demonstration by IHC of TNF-α in scattered cells of satellite granuloma stage I. Case no. 15. Tracheobronchial lymph node. ×100. 1e: Demonstration by IHC of TNF-α on epithelioid and macrophages (4,3% of cell with immunostaining) of satellite granuloma stage I. Case # 15. Tracheobronchial lymph node. ×400.

Table 2
Percentage of immunostaining reaction to different cytokines in bovine tissues with positive isolation of *Mycobacterium bovis*.

Case no.	Generalized lesions[a]	Tissue sample	Pro-inflammatory cytokines (%)[b]			Anti-inflammatory cytokines (%)[b]		Micro stage[c]	No. bacilli[d]	Animal
			IFN-γ	TNF-α	IL-1β	IL-10	TGF-β			
2	Yes	Retropharyngeal LN	32.0	9.1	8.8	33.1	32.3	IV	0–1	Cow
4	Yes	Mediastinal LN	45.5	12.5	20.6	42.5	32.0	IV	0–1	Steer
6	Yes	Mediastinal LN	47.5	27.5	19.1	27.0	11.8	IV	0–1	Cow
8	Yes	Retropharyngeal LN	52.2	29.4	18.7	13.3	54.0	IV	>5	Cow
9	Yes	Mediastinal LN	46.3	26.7	4.7	7.1	29.8	IV	1–5	Cow
10	Yes	Retropharyngeal LN	48.7	8.3	9.4	31.3	31.8	IV	>5	Cow
11	No	Retropharyngeal LN	46.3	52.0	22.2	10.2	41.2	IV	0–1	Cow
12	No	Mediastinal LN	57.2	56.1	15.6	25.8	24.8	IV	>5	Cow
13	No	Mediastinal LN	23.5	55.4	7.6	28.5	28.2	IV	0–1	Steer
16	Yes	Retropharyngeal LN	32.4	40.5	18.9	29.4	16.3	IV	0–1	Cow
17	No	Lung	31.2	57.3	12.6	43.0	66.4	IV	1–5	Cow
19	Yes	Retropharyngeal LN	13.0	7.7	7.7	30.8	15.5	IV	>5	Cow
20	No	Mediastinal LN	59.8	15.0	22.0	45.0	8.1	IV	1–5	Cow
21	Yes	Tracheobronchial LN	18.8	53.9	30.9	13.8	17.4	IV	0–1	Cow
1	No	Retropharyngeal LN	27.7	11.1	11.9	29.0	3.1	III	1–5	Cow
3	No	Tracheobronchial LN	13.2	14.0	5.2	31.2	2.8	III	0–1	Steer
5	No	Mediastinal LN	35.9	8.8	5.5	20.1	12.5	III	1–5	Steer
7	No	Mediastinal LN	72.9	3.8	14.9	10.5	15.6	III	1–5	Cow
14	No	Mediastinal LN	64.5	62.2	11.6	23.8	16.2	III	1–5	Cow
15	Yes	Tracheobronchial LN	49.4	9.2	19.3	24.3	45.1	III	>5	Cow
18	No	Mediastinal LN	48.1	17.3	1.7	27.2	24.6	III	>5	Cow
17[e]	No	Lung	7.54	5.1	2.70	1.6	12.72	II[f]	0–1	Cow
6[e]	Yes	Mediastinal LN	10.0	2.0	4.6	4.0	3.0	II[f]	0–1	Cow
5[e]	No	Mediastinal LN	12.2	13	4.45	10.5	8	I[f]	0–1	Steer
18[e]	No	Mediastinal LN	9.78	7.36	5.1	12.1	11.0	I[f]	0–1	Cow
15[e]	Yes	Tracheobronchial LN	7.1	4.3	7.75	5.7	8.5	I[f]	0–1	Cow
16[e]	Yes	Retropharyngeal LN	11.40	5.56	8.25	15.4	12	I[f]	0–1	Cow

[a]Presence of macroscopic lesions in 21 animals detected at slaughterhouse.
[b]Percentage of cells with positive staining at immunohistochemical study with different cytokines.
[c–d]Classification according to criteria described by Wangoo et al., 2005.
[e]No. case with satellites granuloma.
[f]Microscopic stage of satellite granuloma.

epithelioid and Langhan's giant cells). Staining for IL-1β revealed scattered positive cells (an average of 15,6%); three animals with <10% positive cells had numerous bacilli positive to Ziehl-Neelsen staining (cases #. 9, 10 and 19). All the animals exhibited evident macroscopic lesions with fibrotic capsule yet only 7 bovines showed 30% or higher positive immunostaining cells for TGF-β and 4 of them presented generalized disease (cases #. 2, 4, 8 and 10). Some Langhan's giant cells strongly expressed TGF-β (Fig. 1, 4c–e). Positive immunostaining for TGF-β was evident in necrotic areas of granulomas (cases # 2, 8, 11 and 17).

Granulomas classified as stage III ($n = 7$) showed stronger reactivity to IFN-γ than to other cytokines ($p < 005$) and three bovines presented over 50% of IFN-γ reactive cells (Fig. 1, 3c–d). No positive TNF-α immunostaining was observed in necrotic areas of granulomas, with the exception of case # 14. This bovine showed <25% of positive cells to TNF-α and this reactivity was evident in macrophages and scattered Langhan's giant cells. Positive reactivity to IL-10 was evident in lymphocytes, macrophages and epithelioid cells, but absent in necrotic areas and most Langhan's giant cells (Fig. 1, 3c–e). Indeed, <30% of Langhan's giant cells displayed a positive reaction to IL-10 in 4 bovines (cases # 1, 5, 7 and 14). Bovines with stage III granulomas (except case # 15)

Table 3
Median of percentage of immunostaining of different cytokines in bovine tissues with tuberculosis microscopic granulomatous lesions stage I to IV. Data are presented as median ± SD. Multiple comparisons are shown under the table when significant, except when only non-significant data were found.

	Stage I	Stage II	Stage III	Stage IV
IFN-γ	10.59 ± 2.2	8.77 ± 1.74	48.1 ± 20.7	45.9 ± 14.5
TNF-α	6.46 ± 3.8	3.55 ± 2.19	11.1 ± 19.9	28.45 ± 19.8
IL-1β	6.42 ± 1.9	3.65 ± 1.34	11.6 ± 6.2	17.15 ± 7.4
IL-10	11.3 ± 4.0	2.8 ± 1.70	24.3 ± 6.9	28.95 ± 12.2
TGF-β	9.75 ± 1.9	7.86 ± 6.87	15.6 ± 14.5	29.00 ± 16.2

Differences along cytokines in a given stage:
Stage 1 nonsignificant
Stage 2 nonsignificant
Stage 3: IFN-γ significantly higher than TNF-α, IL-1β, TGF-β
Stage 4: IFN-γ and s TNF-α significantly higher than IL-1β.
Differences along stages in a given cytokine:
IFN-γ: stage III and IV significantly higher than stage I and II
TNF-α: stage IV significantly higher than stage I
IL-1β, IL-10: stage IV significantly higher than stage I
TGF-β: nonsignificant differences.

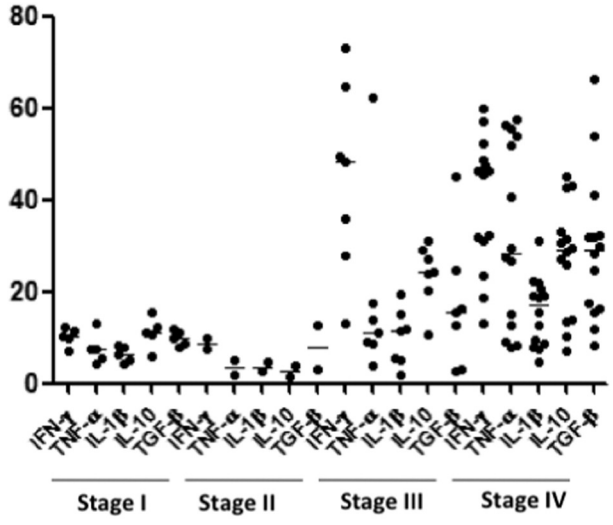

Fig. 2. Dot column representation of the percentage of immunostaining of different cytokines in individual granulomatous lesions in stages I to IV.

showed a low immunostaining reaction to TGF-β (<25%). Similar to stage IV, numerous bacilli identified with Ziehl-Neelsen staining were evident in a bovine with poor reactivity to IL-1β (case # 18), whereas a low number of bacilli (1–5) was present in <20% IL-1β positive cells in several other bovines ($n = 5$).

Granulomas classified as stage II ($n = 2$) were observed as satellites of granulomas of stage IV in 2 bovines, and showed poor immunostaining, with 2.0 to 12.7% immunoreactive cells (Table 2 and Fig. 1, 2c–d). Pro-inflammatory cytokines (IFN-γ and IL-1β) were evident in lymphocytes, macrophages and few Langhan's cells with higher percentage of positive cells to these cytokines than to the anti-inflammatory IL-10 (Fig. 1, 2c–e). TGF-β immunostaining showed minimal expression in the early stage of granuloma formation. Bacilli were scarce (0–1) and located inside macrophages and giant cells.

Granulomas classified as stage I ($n = 4$) were observed as satellites of granulomas of stage III and IV in four bovines, and showed poor immunostaining, with 4,3 to 15,4% immunoreactive cells (Table 2) in epithelioid, macrophages and giant cells, in contrast to the immunoreaction observed in stage III and IV granulomas (Fig. 1, 1c–d–e). Bacilli were scarce (0–1) within macrophages and giant cells.

4. Discussion

For this study, granulomatous bovine tuberculosis lesions were classified microscopically into 4 stages, following the criteria stated by Rhoades et al. (1997) and Wangoo et al. (2005). This method is based on lesion expansion and cellular composition of granulomas. We sampled macroscopically visible granulomatous lesions, which correspond to histopathological stage III ($n = 7$) and IV ($n = 14$).

Cassidy et al. (1998) observed similar lesions after 35 days of *M. bovis* inoculation in calves. In their study, these lesions with large necrotic centers and mineralization were evident mostly in both diaphragmatic lung lobes and lymph nodes. These researchers find no differences between macroscopic lesions in experimentally and naturally infected bovines; cattle displayed evident granulomatous lesions predominantly in retropharyngeal and mediastinal lymph nodes and lungs. Microscopic lesions reported in that study are similar to the lesions described in our study, with aggregates of neutrophils surrounding necrotic areas and in satellite lesions. This finding suggests a possible role of neutrophils in the expansion of the disease. Palmer et al. (2007) observed stage I and II granulomas as satellites that were placed in adjacent areas of stage III and IV granulomas at 60 days after experimental inoculation of *M. bovis* in calves, with reduction after 270 days. Liebana et al. (2007) also observed satellite stage I and II granulomas in various lymph nodes of cattle inoculated with high doses of *M. bovis* and euthanized between 5 and 12 weeks after infection. Coincidentally with the results in our study, Menin et al. (2013) revealed high frequency of chronic lesions (stage III and IV) in different tissues in naturally infected animals. This could be due to an increased resistance to *M. bovis* infection (Thoen et al., 2006). Furthermore, most of the lesions found are in advanced/chronic stage of development. The thickly encapsulation lesion might suggest decrease or lower dissemination and an active anti *M. bovis* immune response during natural infection. We observed correlation between six animals with satellite granuloma and lower TGF-β immunolabeling. Importantly, it should be noted that the stage I and II granulomas measured here were only associated with more advanced lesions and that cell composition and cytokine patterns may be different to that of stage I representing the initiation of the infection.

We observed a high number of bacilli with Ziehl-Neelsen staining in stage III and IV granulomas. These bacilli were scattered in necrotic area and located inside the cells. These findings suggest that bacteria replicate rather than remaining in a persistent state (Ahmad et al., 2006; Alvarez et al., 2009).

Using immunohistochemistry, we found high expression of IFN-γ, TNF-α, and IL-10 mainly in stage III and IV granulomas and of TGF-β only in stage IV, with significant differences with I and II stage granulomas and the negative control (Table 3, Fig. 2). Wangoo et al. (2005) evaluated cytokines and cells by immunohistochemistry in granulomas of experimentally inoculated bovines. They observed that macrophages and Langhan's giant cells expressed TGF-β mainly in advanced stages of granulomas, coincident to our findings. Indeed, we also found strong TGF-β staining in advanced stages of granulomas: stage IV granulomas and in one stage III granuloma. They attributed the high number of positive immunostaining cells to the large fibrosis detected in stage IV granulomas. Nevertheless, beside its participation in the fibrosis process, TGF-β could have an active role in bovine tuberculosis pathogenesis. In fact, TGF-β suppresses T-Cell response and reduces bactericide function of macrophages by decreasing the production of IFN-γ, IL-12, TNF-α, IL-6, IL-1, oxygen reactive and nitric oxide intermediates (Barnes and Wizel, 2000).

Wangoo et al. (2005) also investigated CD79+, TCD3+, γδ and CD68+ in bovine tuberculosis granulomas, and observed that γδ cells, which release cytokines IFN-γ, TNF-α and IL-2, were scarce in stage I granulomas but numerous in stage III and IV lesions. This is similar to our findings. Indeed, we detected many immunoreactive cells to IFN-γ and TNF-α in stage IV granulomas, but not in stage I and II (Table 3).

We found positive immunostaining to IFN-γ in necrotic areas of stage III and IV granulomas and to TGF-β in some stage IV granulomas. The result observed for TGF-β is coincident with what was reported by Mustafa et al. (2006), who described positive immunostaining to TGF-β in *M. tuberculosis* granulomas in humans. This could be attributed to the damage suffered by macrophages and Langhan's giant cells, with loss of cell membranes and release of cytokines in necrotic area, as reported by Wangoo et al. (2005).

Liebana et al. (2007) analyzed the presence of CD4+, CD8+ and CD25+ cells by immunohistochemistry in bovine tuberculosis granulomas (stage I, II, III and IV), and found immunoreactivity in all of the stages. In stage I and II, they observed CD4+ scattered cells in all areas of the granuloma, thus indicating a relation between granuloma maturation with its ability to release IL-2 and IFN-γ. However, they failed in demonstrating the presence of the cytokines. In our study, we observed poor immunoreactive cells in stage I and II granulomas. Nevertheless, pro-inflammatory cytokine immunostaining was stronger than in the control cuts (Table 3), in accordance with Liebana et al.'s (2007) findings. This result suggests recruitment, retention and proliferation of cells, which leads to enlarged granulomas. However, the scarce number of stage I and II granulomas make it difficult to draw firm conclusions.

IL-1β is released in response to TNF-α (Sasindran and Torrelles, 2011). In our study, all animals with stage III granulomas showed <15% reactive IL-1β cells (except case # 14, which showed high levels of TNF-α immunostaining). This finding is consistent with a low TNF-α immunostaining. Lack of IL-1β could indicate failure of immune response in the development of mature granuloma and, as a consequence, intensive bacterial multiplication.

Mustafa et al. (2006) studied human tuberculosis lymphadenitis, but failed to demonstrate significant differences in the expression of TNF-α and IL-10. They found positive immunostaining for these cytokines and their reactivity was higher than for TGF-β and IFN-γ. Coincidentally, we detected similar results in 5 animals (cases #. 2, 8, 13, 17 and 19). In Mustafa's study, unlike epithelioid cells, more Langhan's giant cells expressed IL-10 than TNF. In contrast, we observed the same result only in 3 animals (cases #. 17, 10 and 4). In general, only few Langhan's giant cells were positive to cytokine immunostaining, most of them exhibiting an intracytoplasmic weak reaction. These authors found strongest expression of TGF-β instead of IL-10 and TNF-α in necrotic centers, whereas IFN-γ was not detected in these regions. In contrast, we observed higher expression of IFN-γ and TGF-β in lymphocytes, macrophages and in necrotic areas.

Similar to Palmer et al. (2015), we observed significantly higher expression of TNF-α, TGF-β, IFN-γ in lesions than in lungs without lesions. This finding suggests a potential role for these cytokines and chemokines in the regulation of bovine immune response to *M. bovis* and granuloma evolution. Also Shu et al. (2014) observed that mRNA

expression of IFN-γ, IL-17A, IRF5(1) and arginase 1 (Arg1) was significantly up-regulated in the lung compared to that in pulmonary lymph nodes. They also observed that mRNA expression of IFN-γ, IL-12p40, TNF-α and iNOs in pulmonary lymph nodes was significantly higher than that of non-lesioned prescapular lymph nodes. These results suggested that a stronger proinflammatory immune response in the lesioned lung may be consequence of enhanced expression of IRF5 thus promoting IFN-γ and IL-17 production.

In our study, we observed a lack of reactivity to IL-10 in advanced granulomas. This finding is similar to that of Thacker et al. (2007). These researchers observed a progressive decrease of IL-10 expression in bovines with severe pathology by *M. bovis*. Moreover, coincidentally with these authors, in our study infected and uninfected (control) bovines expressed similar amounts of IL-10.

Altogether, cytokine expression in stage III and IV granulomas was significant (Fig. 2), predominantly IFN-γ expression. These findings are consistent with the results of other reports, thus suggesting that IFN-γ expression correlates with increased pathology (Hope et al., 2005; Palmer et al., 2007; Thacker et al., 2007; Villarreal-Ramos et al., 2003; Vordermeier et al., 2002; Palmer et al., 2015).

These data could demonstrate a strong longstanding immune response, mediated by lymphocytes, macrophages and epithelioid cells, and a lower IL-10 response; which suggest a shortfall of anti-inflammatory cytokines in the environment. Pro-inflammatory cytokines play a critical role in the initiation of infection. The described lesions indicate a failure in the initial immune response, which was unable to limit the spread of infection in these animals; in fact, 10 of these animals displayed generalized lesions and 6 of them had satellite granulomas. However, pro-inflammatory cytokine activity could be associated with a strong immune response in advanced granulomas releasing growth factors such as TGF-β, which promotes collagen accumulation.

In this study, evidence of in situ presence of cytokines demonstrates their role in the development and evolution of bovine tuberculosis granulomas. It is crucial to identify the released cytokines or their absence in bovine tuberculosis granulomas, for they can be related with the progress of the disease. These aspects should be taken into consideration to develop control and eradication plans and to implement different types of vaccines.

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