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Cytokine expression profiles of bovine lymph nodes: effects of *Mycobacterium bovis* infection and bacille Calmette–Guérin vaccination

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Summary

Cytokine expression in lymph nodes from cattle inoculated intranasally with *Mycobacterium bovis* was compared to that of non-infected animals using real-time polymerase chain reaction. The effect of *M. bovis* infection, 4 months post-challenge, was to suppress the expression of anti-inflammatory cytokines interleukin (IL)-4 and IL-10 as well as the pro-inflammatory cytokines tumour necrosis factor (TNF) and IL-6. Expression of interferon (IFN)- γ and IL-12 was maintained. Animals vaccinated with bacille Calmette–Guérin responded differently to challenge with *M. bovis*. In particular, no decrease in expression of IL-4 or IL-6 was observed following challenge of vaccinated animals and decreased IFN- γ was detected. Also, vaccinated animals had higher levels of IL-4 and IL-10 transcripts compared to unvaccinated animals following challenge. These changes in cytokine expression levels led to a significant shift in the IFN- γ /IL-4 or IFN- γ /IL-10 ratio within the lymph node following challenge. Challenged animals generally showed a strong Th1 bias that was not seen in animals vaccinated prior to challenge. An inverse correlation between the level of pathology and bacterial load within the lymph node and the expression of IL-4, IL-10 and TNF was also observed. These results suggest that in the lymph nodes of cattle with established tuberculosis and a persisting bacterial infection, maintenance of the pro-inflammatory response in combination with a suppressed anti-inflammatory response may control the infection but contribute to host-induced tissue damage. Vaccination, which reduces the bacterial load and consequently the IFN- γ response, may result in less suppression of anti-inflammatory cytokines.

Keywords: BCG, cytokines, interleukins, *Mycobacterium bovis*, vaccines

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Introduction

Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, persists both in countries with active diagnostic and eradication policies and in those that lack these measures. *M. bovis* bacille Calmette–Guérin (BCG), an attenuated form of *M. bovis* utilized as a vaccine against TB in humans, shows variable efficacy (0–80%), due possibly to prior exposure to environmental mycobacteria [1,2]. A similar situation has been described in cattle (reviewed in [3]). Infection with mycobacteria such as *M. bovis* results in the formation of granulomas, comprised of a core of infected and killed macrophages (M ϕ) surrounded and infiltrated by T lymphocytes [4–7], within the lungs and lymph nodes. The granuloma acts to control and constrict the spread of infection, but also results in significant tissue damage [8,9].

Immunity to mycobacteria is dependent mainly on a cell-mediated response involving M ϕ , dendritic cells and an adaptive T cell response. The functions of these cells are modulated by cytokines, and the close proximity of cells within the granuloma is thought to facilitate the immune responses induced [10]. The roles played by cytokines vary widely; some are pro-inflammatory, activating cells of the immune system to kill mycobacteria and inducing a type I immune response, while others, such as interleukin (IL)-4 and IL-10, are anti-inflammatory, down-regulating the pro-inflammatory immune response to control tissue damage [11–13]. Of the pro-inflammatory cytokines, interferon (IFN)- γ is considered to be critical for the control of mycobacterial infection [13,14], although others such as IL-12, IL-6 and tumour necrosis factor (TNF) also play a significant role [15–19].

Several studies have determined cytokine levels at the granulomatous site in humans infected with pathogenic mycobacteria [7,20–25]. These studies include quantitative and semiquantitative measurements of cytokine RNA in tuberculous patients compared to healthy controls [7,20] and immunohistochemical studies of protein levels [24,25]. Results indicated that the expression or presence of IL-12, TNF and IFN- γ was common [7,20–22,25], although one study detected no TNF or IFN- γ protein in samples from tuberculous lesions [24]. IL-10 [20] and transforming growth factor (TGF)- β [25] were also detected in tuberculous lesions, but detection of IL-4, indicative of a Th2 response, was variable [7,20–22,24,25]. By comparing cytokine expression in tuberculous tissue directly to that of controls, a reduction in levels of IL-4 and IL-10 in patients with TB has been demonstrated [7,25]. This suggests that, at least in humans, the immune response to pathogenic mycobacteria in the lung [21–25] and in the lymph node [7,20] is predominantly of the pro-inflammatory Th1 type, promoting killing of infected cells and extracellular bacteria.

Differences in the pathogenicity of *M. tuberculosis*, the causative agent of human TB, and *M. bovis*, manifested in the restricted host-range of *M. tuberculosis*, will be reflected in the host-specific immune response to them. Thus, although many of the principles established for *M. tuberculosis* will hold for *M. bovis*, there must be differences that affect each species' ability to infect their hosts. The cow provides an ideal model for study of *M. bovis* as, unlike the mouse as a model for TB, it is the natural and primary host and develops granulomas that are highly similar in structure to those seen in humans [5,26,27]. Following intranasal exposure of cattle, lymph nodes in the head are known to display lesions characteristic of TB [26,28].

A key objective of this study was to assess quantitatively the expression of key pro- and anti-inflammatory cytokines, known to be involved in immunity to TB, in bovine tissues infected with *M. bovis*. Immune responses were assessed 4 months after challenge, when the acute phase of infection was under control and chronic disease, which occurs in the face of an ongoing immune response, was developing. A second aspect of this study was to determine cytokine expression levels in the tissues of animals vaccinated with BCG prior to challenge. Such animals are known to exhibit a degree of resistance to TB [29–32]. It was proposed that a quantitative examination of the cytokines in the lymph nodes that drain the site of challenge would reveal differences in these groups of animals. This *in vivo* data would complement but may also differ from that obtained from studies involving restimulation of lymphocytes *in vitro*.

Results indicate that the infected lymph nodes of animals challenged with *M. bovis* show suppression of IL-4, IL-6, IL-10 and TNF expression when compared to healthy animals, but no change was seen in the expression levels of IL-12 or IFN- γ . Vaccination with BCG prior to infection altered the response to challenge; in particular, no decrease in IL-4 or

IL-6 levels were detected but we did observe a decrease in IFN- γ levels.

Materials and methods

Mycobacterial culture, experimental animals and isolation of samples

Procedures for the culture and quantification of bacteria have been described previously [29]. All experiments conformed to local and national guidelines on the use of experimental animals and category III infectious organisms. The animals selected for this study consisted of the following four groups: (1) unvaccinated animals infected with *M. bovis* – four animals challenged intranasally and shown to have lesions at necropsy; (b) unvaccinated unchallenged animals – three animals not exposed to *M. bovis*; (c) vaccinated animals infected with *M. bovis* – five animals vaccinated with BCG and challenged intranasally; and (d) vaccinated unchallenged animals – three animals vaccinated with BCG but not challenged with *M. bovis*. All animals were age-matched and of the same breed. The challenged animals have been reported previously [29]. Briefly, vaccination of challenged animals was carried out at 1 day old with 10^6 BCG Pasteur, and intranasal challenge at 12 weeks with 1.3×10^4 *M. bovis* (AF2122/97) [33]. Control animals were vaccinated with BCG Pasteur at 6 months of age. Pathology and bacteriology relevant to tissues used are summarized in Table 1. At necropsy, 4 months after intranasal exposure, vaccinated animals had significantly lower numbers of bacteria and lesions, where present, were less severe [29].

RNA extraction and synthesis of cDNA

One parotid, one submandibular and both retropharyngeal lymph nodes were removed from all animals post mortem. Tissues were dissected into cubes approximately 0.4 cm^3 for RNA extraction and stored in 4 M guanidine thiocyanate (GTC) for 24 h at 4°C , followed by long-term storage at -20°C . Tissue samples, one per lymph node, were homogenized in buffer RNeasy lysis buffer (RLT; Qiagen, Hilden, Germany) using a Mini-BeadBeater and 1 mm zirconia silica beads (BioSpec Products, Inc., Bartlesville, OK, USA). Samples were homogenized further using Qias shredders (Qiagen). RNA extractions were carried out using the RNeasy mini kit (Qiagen). RNA yields and quality were determined using an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA), and confirmed by agarose gel electrophoresis. RNA was treated with RNase-free DNase I [Ambion (Europe) Ltd, Cambridge, UK] to remove any contaminating genomic DNA prior to reverse transcription into cDNA. Reverse transcription of 200 ng of RNA per sample was performed using 200 units SuperScript II reverse transcriptase, 500 ng oligo dT, 0.5 mM deoxyribonucleoside triphosphate (dNTPs), 10 mM dithiothreitol (DTT), $1\times$ first-strand buffer and 40 units RNasin

Table 1. Lymph node lesion scores and bacterial counts.

Vaccinated + challenged animals	51		53		55		56		60	
	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†
Parotid	0	–	0	–	0	–	0	–	1	–
Submandibular	0	–	0	–	0	–	0	–	2	2.78
Retropharyngeal left	2	3.73	0	–	2	3.67	0	3.44	0	–
Retropharyngeal right	0	–	0	–	2	3.28	0	–	0	–
Unvaccinated + challenged animals	54		57		58		59			
Lymph node	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†	Lesion score*	Log ₁₀ CFU†
Parotid	0	–	3	3.19	1	3.38	4	4.50		
Submandibular	2	3.27	4	3.56	3	3.73	4	4.20		
Retropharyngeal left	4	3.94	4	3.76	4	3.90	4	4.51		
Retropharyngeal right	0	–	4	3.85	4	4.03	4	4.19		

*Score 0–4 indicates increasing severity of lesions. †Log₁₀ colony-forming units (CFU)/g of tissue; – denotes count of < 500 CFU/g tissue.

(Invitrogen Ltd, Carlsbad, CA, USA). cDNA was made up to a total volume of 50 µl by the addition of 30 µl endonuclease-free water prior to use.

Primers and TaqMan® probes

Primers, based on the published sequences of bovine genes, were designed to span introns where possible to ensure discrimination between cDNA and genomic DNA primers and probes for TaqMan® analysis, with the exception of IL-4 and IFN-γ, have been tested previously at the Institute for Animal Health [34,35] and were synthesized by PE oligofactory (Applied Biosystems, CA, USA), Sigma-Genosys Ltd (Haverhill, UK) or Eurogentec Ltd (Romsey, UK). Primer sequences for IL-4 were as follows: forward primer 5'-GCCACACGTGCTTGAACAAA-3', reverse primer 5'-TCTTGCTTGCCAAGCTGTTG-3', probe 5'-TCCTGGGC GGACTTGACAGGAATC-3', and were designed by Dr D. Werling (Royal Veterinary College). The primers and probe used for analysis of IFN-γ were kindly provided by Dr G. Taylor and Dr J. F. Valarcher (Institute for Animal Health) prior to publication. Probes for TaqMan® analysis were labelled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine).

Real-time TaqMan® polymerase chain reaction (PCR) for quantification of cytokine cDNA

Quantitative PCR was carried out using TaqMan® Universal PCR Mastermix (Applied Biosystems), with 100 ng cDNA as a starting template, on an ABI Prism 7700 sequence detection system (Applied Biosystems). The amplification programme consisted of an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was tested in triplicate. Results were quantified by comparison with standard curves produced from known copy numbers of plasmid DNAs containing

sequenced gene fragments for each of the cytokines. The efficiency of RNA extraction and conversion to cDNA was assessed by measuring the expression levels of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each of the samples.

Statistical analysis

All analysis was carried out using Microsoft® Excel 2002 (Microsoft Co., Redmond, WA, USA), Minitab™ version 13.32 (Minitab Inc., State college, PA, USA) and R (<http://www.r-project.org>). Error bars indicate ± standard deviation (s.d.). For each cytokine, differences between the four animal groups were assessed using a two-way factorial analysis of variance (ANOVA) using a model that included the interaction term (vaccinated:challenged). This interaction term determines whether prior vaccination has an effect on the host response to infection and vice versa. The effects of each factor were tested independently using type III sums of squares. Where the interaction term was not significant, the significance of each factor was taken from the ANOVA. Where the interaction term was significant, within-group two-sample *t*-tests were used to assess the significance of each factor. Mann-Whitney tests were used for the comparison of IFN-γ/IL-4 or IFN-γ/IL-10 ratios. Correlations between cytokine expression levels were assessed by Pearson's correlation coefficient. Correlations between cytokine expression levels and lesion scores or bacterial counts were assessed using Spearman's rank correlation coefficient.

Results

Suppression of cytokine expression in cattle infected with *M. bovis* compared to uninfected animals

Preliminary screening carried out by standard PCR indicated that differences may exist between the cytokine profiles in the lymph nodes of the four groups of animals (data

not shown). Real-time PCR was used to assess quantitative expression of IL-4, IL-6, IL-10, IL-12 (p40 subunit), TNF and IFN- γ transcripts. For all cytokines, transcripts were detected in the majority of lymph node samples examined (Fig. 1), indicating that the tissues sampled for analysis were transcriptionally active and not subject to levels of necrosis inhibitory to gene expression. Differences between groups were analysed to determine whether infection, vaccination or an interaction between infection and vaccination had a significant effect on the cytokine expression level (Table 2). Where the interaction effect was significant, differences between groups were analysed further by within-group two-sample *t*-tests. In the unvaccinated group, challenge with *M. bovis* had a significant effect on the cytokine expression levels within the lymph node (Fig. 1, Table 2). We detected a significant decrease in expression levels of IL-4 ($P < 0.05$), IL-6 ($P < 0.001$), IL-10 ($P < 0.001$) and TNF ($P < 0.001$) in challenged animals compared to unchallenged animals. No significant effect of challenge was seen for IL-12 p40 or IFN- γ expression levels in unvaccinated animals.

Vaccinated animals display an altered response to challenge compared to unvaccinated animals

We also studied the response to challenge in animals that were vaccinated with BCG prior to infection with *M. bovis*. As indicated by the interaction effects seen (Table 2), vaccination had a significant effect on the response to challenge. Unlike the unvaccinated animals, vaccinated animals did not show a decrease in IL-4 or IL-6 levels following challenge, and showed a decrease in IFN- γ levels ($P < 0.05$). A comparison of vaccinated challenged and unvaccinated challenged animals indicates significantly higher levels of IL-4 in vaccinated animals.

Similar responses to challenge in vaccinated and unvaccinated animals were seen for the expression of IL-12 p40 (no change following challenge), TNF and IL-10 (decrease in expression following challenge). However, significant differences were detected between the expression levels of IL-10 in vaccinated challenged animals compared to unvaccinated challenged animals. Thus, although significant decreases were seen in IL-10 following challenge in both groups

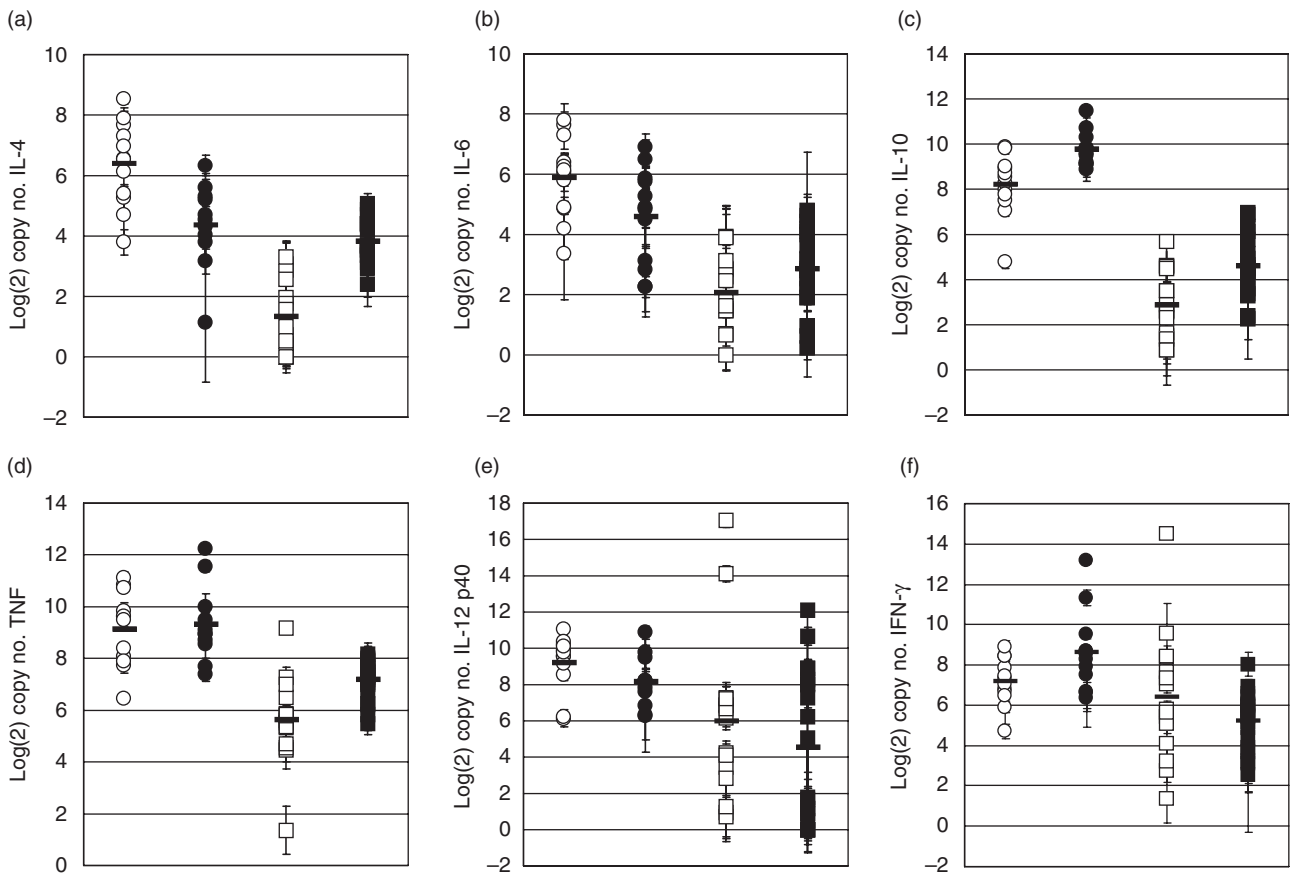


Fig. 1. Real-time polymerase chain reaction (PCR) analysis of cytokine production by bovine lymph nodes. Interleukin (IL)-4 (a), IL-6 (b), IL-10 (c), tumour necrosis factor (TNF) (d), IL-12 p40 (e) and interferon (IFN)- γ (f) log(2) copy number per 100 ng lymph node cDNA starting template. \circ , Unvaccinated, unchallenged animals; \bullet , vaccinated, unchallenged animals; \square , unvaccinated, challenged animals; \blacksquare , vaccinated, challenged animals. Results are averaged triplicate values for each lymph node sample. Error bars indicate standard deviation (s.d.) of each sample.

Table 2. Effect of infection and vaccination on cytokine expression levels.

Cytokine	Infection effect	Vaccination effect	Interaction effect
IL-4	< 0.001	0.484	< 0.001
IL-6	< 0.001	0.633	0.048
IL-10	< 0.001	0.004	0.917
TNF	< 0.001	0.105	0.193
IL-12 p40	0.059	0.308	0.847
IFN- γ	0.007	0.942	0.040

Figures indicate *P*-values for the effect of infection, vaccination or an interaction between the two factors on the cytokine expression level per animal. Bold type indicates significance ($P < 0.05$). IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.

($P < 0.001$), there were higher levels of the cytokine in the vaccinated animals compared to unvaccinated animals ($P = 0.004$).

Correlation between cytokine gene expression levels in bovine lymph nodes

Results shown in Fig. 1 suggest a similar pattern of gene expression in bovine lymph nodes following infection with *M. bovis* for several of the cytokines studied. Correlation analysis (Table 3) indicates a significant positive correlation between the expression levels of IL-4, IL-6, IL-10 and TNF ($P < 0.05$). Thus, for these cytokines, infected lymph nodes with a high expression of one of these cytokines also showed a high level of expression of the other three cytokines. A significant positive correlation was also detected between the expression levels of TNF and IFN- γ ($P < 0.05$).

The balance of Th1 and Th2 cytokines has a significant impact on the immune response elicited against mycobacterial infection. It was therefore important to determine the ratio of the Th1 cytokine IFN- γ , compared to the anti-inflammatory cytokines IL-4 and IL-10. As shown in Fig. 2, the IFN- γ /IL-10 ratio increased significantly in both vaccinated ($P < 0.01$) and unvaccinated animals ($P < 0.001$) following challenge. However, although a significant increase in ratio of IFN- γ /IL-4 was observed following challenge in

unvaccinated animals ($P < 0.01$), a significant decrease in IFN- γ /IL-4 ratio was observed following challenge in vaccinated animals ($P < 0.001$).

The extent of pathology following infection correlates with cytokine expression levels within the lymph node

Correlations can be detected between the cytokine expression levels and the lesion score of the lymph nodes from infected animals (Table 4). Assessment by Spearman's rank correlation coefficient showed that there was a significant negative correlation between the expression levels of IL-4, IL-10 and TNF and the lesion score for the lymph nodes from infected animals. No significant correlation between expression level and lesion score was found for IL-6, the p40 subunit of IL-12 or IFN- γ (Table 4). Similar results can be seen for the relationship between cytokine expression levels and the number of mycobacteria isolated from the lymph node (Table 4). As was found for the lesion score data, there was a significant negative correlation between bacterial count and the transcript levels of IL-4, IL-10, and TNF. As for the lesion scores, no significant correlation was found between the levels of IL-6, IFN- γ or IL-12 p40 expression and the bacterial count.

Table 3. Correlation of cytokine expression levels in bovine lymph nodes.

	IL-4	IL-6	IL-10	TNF	IL-12 p40
IL-6	0.409 0.020				
IL-10	0.557 0.001	0.432 0.014			
TNF	0.741 < 0.001	0.466 0.007	0.597 < 0.001		
IL-12 p40	-0.163 0.371	-0.198 0.278	-0.053 0.774	0.078 0.673	
IFN- γ	-0.182 0.319	-0.053 0.772	-0.009 0.962	0.366 0.040	0.258 0.154

Top value: Pearson correlation score; bottom value: *P*-value. Bold type indicates significance ($P < 0.05$). IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.

Fig. 2. Th1/Th2 bias in bovine lymph nodes following challenge with *Mycobacterium bovis*.

Ratio of interferon (IFN)- γ /interleukin (IL)-4 (a) and IFN- γ /IL-10 (b) copy number per 100 ng lymph node cDNA starting template. \circ , Unvaccinated, unchallenged animals; \square , unvaccinated, challenged animals; \bullet , vaccinated, unchallenged animals; \blacksquare , vaccinated, challenged animals. Results are averaged triplicate values for each lymph node sample. * $P < 0.01$, ** $P < 0.001$ by Mann-Whitney tests.

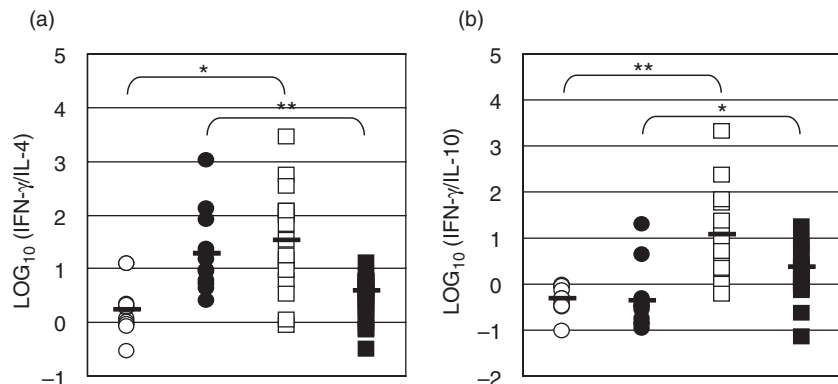


Table 4. Correlation of cytokine expression levels and pathology in infected animals.

Cytokine	Lesion score		Bacterial count	
	Spearman's rank correlation coefficient	<i>P</i> -value	Spearman's rank correlation coefficient	<i>P</i> -value
IL-4	-0.715	< 0.001	-0.715	< 0.001
IL-6	-0.261	0.130	-0.261	0.130
IL-10	-0.400	0.018	-0.400	0.018
TNF	-0.571	< 0.001	-0.571	< 0.001
IL-12 p40	0.139	0.423	0.139	0.423
IFN- γ	0.18	0.299	0.18	0.299

Bold type indicates significance ($P < 0.05$). IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.

Discussion

In this study we analysed the cytokine gene expression profiles of lymph nodes draining the area of infection in cattle challenged intranasally with *M. bovis* and compared them to levels in uninfected cattle. In addition, the effects of vaccination with *M. bovis* BCG on cytokine expression in both healthy and challenged animals were examined. The model allowed the analysis of events in local tissues at a time when a chronic infection was becoming established, after the acute early stage of infection when bacterial replication is extensive and after the induction of an immune response, evident from about 2–3 weeks post-infection [36]. There are few quantitative data describing cytokine gene expression from models of TB examined at this disease point. In human studies it is impossible to establish the length of time for which disease has been established. Studies have been carried out in mice, but here granuloma formation does not involve caseation and necrosis [27]. The model used here provided data from a natural host and pathogen that is relevant to understanding the development of chronic and persistent granulomas.

Using quantitative PCR, we detected a significant decrease in the expression levels of IL-4, IL-6, IL-10 and TNF in challenged animals compared to unchallenged animals, indicating suppression of these elements of the immune response as a result of the infection. A reduction or complete absence of cytokines in tissue infected with mycobacteria has been observed previously both in cattle, for example during *M. avium* ssp. *paratuberculosis* infection [37], and in other species [24,25]. One study of tissue biopsies from the lungs of TB patients detected no TNF, IFN- γ or IL-4 in the samples [24], while another showed a marked reduction in IL-10 and IL-4 expression [25]. These and other studies indicate, therefore, that detection of cytokine expression in healthy tissue is not unusual [25,37,38].

The number of transcripts encoding the pro-inflammatory cytokines IFN- γ and IL-12 were not reduced in the lymph nodes of challenged animals. This may relate to the time-point at which samples were taken. Seventy days after BCG infection of mice, the initial increases seen in IFN- γ and IL-12 mRNA were returning to baseline levels [39]. A second

study of IFN- γ levels in the peripheral blood of cattle infected with *M. bovis* showed that at 12 weeks post-infection, while IFN- γ protein levels were still elevated, mRNA levels were similar to those seen prior to infection [40]. IFN- γ and IL-12 are crucial for immunity to mycobacteria as they activate M ϕ to produce other cytokines and release nitric oxide to kill intracellular mycobacteria [10,41,42] and promote phagosomal acidification, counteracting intracellular persistence [43,44]. The maintenance of this response is therefore of obvious benefit to the host, and results in a strong Th1 bias in infected lymph nodes, as was observed. Whether down-regulation of IL-4 and IL-10 expression by the host supports the maintenance of IL-12 and IFN- γ synthesis, or if the active maintenance of IL-12 and IFN- γ responses by the host results in the suppression of IL-4 and IL-10 expression cannot be determined. The suppressed levels of IL-4, IL-6, IL-10 and TNF in infected animals in comparison to maintenance of IL-12 and IFN- γ indicates a specificity in the suppression, not a generalized consequence of infection and necrosis due to the developing chronic immune response.

TNF is known to act in conjunction with IFN- γ to induce the release of reactive oxygen and nitrogen species in infected M ϕ [16,17]. However, although TNF has many beneficial effects and is thought to limit inflammation early on in disease [16,45], it can become toxic at high concentrations during disease progression, through an increase in necrosis and tissue damage, particularly in sites with a Th0 or Th2 profile characterized by the production of IL-4 [46,47]. The observed down-regulation of TNF following challenge may thus be a response initiated by the host to attempt to control the level of tissue destruction occurring, although it could also benefit mycobacterial persistence as a result of decreased production of reactive oxygen and nitrogen intermediates. Down-regulation of the anti-inflammatory IL-4 and IL-10 response in infected animal tissues is likely to relate to the role of these two cytokines in the regulation of granuloma size and necrosis, as well as preventing tissue damage through the regulation of type I cytokine production [48–54]. IL-4 can be transcribed as a splice variant, IL-4 δ 2, which acts as an antagonist of IL-4 activity in mice and humans [55,56]; however, its role in cattle immunity is unknown and was not included as part of this study.

The observed negative correlation between the Th2 cytokines IL-4 and IL-10 and the pathology score is unsurprising given their role in limiting tissue destruction, thus smaller, less necrotic lesions show the highest levels of these anti-inflammatory cytokines. The inverse correlation between bacterial load and Th2 cytokine expression may relate to their role in suppressing pro-inflammatory cytokine expression [11–13]. These cytokines may be down-regulated actively in lesions with a high bacterial load to allow maintenance of the pro-inflammatory response and killing of mycobacteria. We also detected an inverse correlation between TNF expression and pathology score. This is more surprising as TNF, like other pro-inflammatory cytokines, has a role in mycobacterial killing [16,45]. However, an inverse correlation between increased pathology and TNF production has been described previously in a mouse model of tuberculosis [38]. This is thought to relate to an increase in the levels of TGF- β , which acts to decrease TNF production [38,57].

A number of studies have related the extent of lesions at necropsy to the amount of IFN- γ detected by enzyme-linked immunosorbent assay in restimulated blood cells from cattle infected in the respiratory tract with *M. bovis* [29,40,58–60]. Studies have shown that IFN- γ levels in restimulation assays of blood cells correlate with that in infected lymph nodes [29,58–60]. However, no positive or negative correlation has been shown for IL-4 or IL-10 protein levels in the periphery and at the site of the disease; in fact, IL-10 appears to correlate broadly with IFN- γ in supernatants from restimulated lymphocytes [61], and IL-4 is not always detected [29,58,62]. Discrepancies in immune responses to mycobacteria at the site of disease and in the periphery have been reported elsewhere [13,63].

Vaccination of cattle prior to infection with pathogenic mycobacteria lowered significantly the extent of granuloma formation and the number of bacteria isolated from the node [29]. We hypothesized that the differences seen in pathology would be reflected in the cytokine expression profiles of vaccinated animals and found that vaccinated animals did not show the decrease in IL-4 and IL-6 levels observed in unvaccinated animals following challenge. Instead, the levels of these two cytokines were maintained. Following challenge, IL-4 and IL-10 expression levels were also higher in the vaccinated animals compared to unvaccinated animals. In addition, contrary to what was found in unvaccinated animals following challenge, vaccinated animals showed decreased levels of IFN- γ expression in response to infection. These changes in expression of pro- and anti-inflammatory cytokines in vaccinated animals are reflected in the Th1/Th2 bias of the immune response, as evidenced in the IFN- γ /IL-4 ratio. Unlike the unvaccinated animals, which developed a strong Th1 bias in response to *M. bovis* infection, the vaccinated animals had lower levels of IFN- γ expression in comparison to IL-4. The down-regulation of the pro-inflammatory response and up-regulation of

the anti-inflammatory response correlates with the reduced bacterial load and restricted granuloma formation observed in vaccinated animals. Thus a likely scenario is that the decreased bacterial load results in a less potent IFN- γ response and consequently less suppression of IL-4 synthesis occurs.

In this study we measured cytokine expression levels only at 16 weeks post-infection. The time of sampling can have a significant impact on the levels of cytokines present, as the cytokine response to mycobacterial infection, at least in other species, is dynamic. During the early Th1 response to mycobacterial infection in mice IFN- γ is the predominant cytokine, then the response shifts to a combined IFN- γ and IL-4 mediated response, and subsequently an IL-4 dominant Th2 response as infection progresses [14,64]. Patterns of cytokine expression have also been described in cattle [40,62,65] and an antibody response is evident in the later stages of chronic disease that may be related to a Th0/Th2 shift in the immune response [36,40]. Results reported here support this, as at 4 months post-infection unvaccinated cattle show a strong Th1 bias, whereas vaccinated cattle exhibit a more Th0-type profile.

We propose that in unvaccinated cattle challenged with *M. bovis* the cytokine response in the lymph nodes is predominantly pro-inflammatory, as indicated by the decreased expression levels of the anti-inflammatory cytokines IL-4 and IL-10 compared to the maintenance of IFN- γ and IL-12. In the case of IL-4 and IL-10, the levels of suppression can be correlated directly with the level of pathology and bacterial burden of the lymph node, indicating a direct response to infection. The pro-inflammatory response, while necessary for infection control, is deleterious to the host as increased tissue-damage occurs. In contrast, animals vaccinated with BCG prior to *M. bovis* challenge show reduced pathology in the lymph nodes, relating to a decreased bacterial burden and a consequent Th0-type immune response.

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References

- 1 Fine PE. Variation in protection by BCG. implications of and for heterologous immunity. *Lancet* 1995; **346**:1339–45.
- 2 Fine PEM, Carneiro IAM, Milstein JB, Clements CJ. Issues relating to the use of BCG in immunization programmes. WHO/V&B/9923. Geneva: World Health Organization, 1999.
- 3 Buddle BM, Pollock JM, Skinner MA, Wedlock DN. Development of vaccines to control bovine tuberculosis in cattle and relationship to vaccine development for other intracellular pathogens. *Int J Parasitol* 2003; **33**:555–66.

- 4 Gonzalez-Juarrero M, Turner OC, Turner J, Marietta P, Brooks JV, Orme IM. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *Mycobacterium tuberculosis*. *Infect Immun* 2001; **69**:1722–8.
- 5 Neill SD, Pollock JM, Bryson DB, Hanna J. Pathogenesis of *Mycobacterium bovis* infection in cattle. *Vet Microbiol* 1994; **40**:41–52.
- 6 Neill SD, Bryson DG, Pollock JM. Pathogenesis of tuberculosis in cattle. *Tuberculosis (Edinb)* 2001; **81**:79–86.
- 7 Bergeron A, Bonay M, Kambouchner M *et al.* Cytokine patterns in tuberculous and sarcoid granulomas: correlations with histopathologic features of the granulomatous response. *J Immunol* 1997; **159**:3034–43.
- 8 Fayyazi A, Eichmeyer B, Soruri A *et al.* Apoptosis of macrophages and T cells in tuberculosis associated caseous necrosis. *J Pathol* 2000; **191**:417–25.
- 9 Orme IM. The immunopathogenesis of tuberculosis: a new working hypothesis. *Trends Microbiol* 1998; **6**:94–7.
- 10 Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* 2001; **19**:93–129.
- 11 Wallis RS, Ellner JJ. Cytokines and tuberculosis. *J Leukoc Biol* 1994; **55**:676–81.
- 12 Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; **383**:787–93.
- 13 Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL. Cytokine production at the site of disease in human tuberculosis. *Infect Immun* 1993; **61**:3482–9.
- 14 Orme IM, Roberts AD, Griffin JP, Abrams JS. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. *J Immunol* 1993; **151**:518–25.
- 15 Saunders BM, Frank AA, Orme IM, Cooper AM. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to *Mycobacterium tuberculosis* infection. *Infect Immun* 2000; **68**:3322–6.
- 16 Mohan VP, Scanga CA, Yu K *et al.* Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. *Infect Immun* 2001; **69**:1847–55.
- 17 Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol* 1988; **141**:2407–12.
- 18 Wang J, Wakeham J, Harkness R, Xing Z. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest* 1999; **103**:1023–9.
- 19 Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. *Infect Immun* 1997; **65**:5149–56.
- 20 Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominent Th2 cytokine response in human tuberculosis. *Infect Immun* 1996; **64**:1351–6.
- 21 Fenhalls G, Wong A, Bezuidenhout J, van Helden P, Bardin P, Lukey PT. *In situ* production of gamma interferon, interleukin-4, and tumor necrosis factor alpha mRNA in human lung tuberculous granulomas. *Infect Immun* 2000; **68**:2827–36.
- 22 Fenhalls G, Stevens L, Bezuidenhout J *et al.* Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas. *Immunology* 2002; **105**:325–35.
- 23 Kaplan G, Post FA, Moreira AL *et al.* *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun* 2003; **71**:7099–108.
- 24 Aung H, Toossi Z, McKenna SM *et al.* Expression of transforming growth factor-beta but not tumor necrosis factor-alpha, interferon-gamma, and interleukin-4 in granulomatous lung lesions in tuberculosis. *Tuber Lung Dis* 2000; **80**:61–7.
- 25 Bai X, Wilson SE, Chmura K, Feldman NE, Chan ED. Morphometric analysis of Th (1) and Th (2) cytokine expression in human pulmonary tuberculosis. *Tuberculosis (Edinb)* 2004; **84**:375–85.
- 26 Cassidy JP, Bryson DG, Pollock JM, Evans RT, Forster F, Neill SD. Early lesion formation in cattle experimentally infected with *Mycobacterium bovis*. *J Comp Pathol* 1998; **119**:27–44.
- 27 Ehlers S. Immunity to tuberculosis: a delicate balance between protection and pathology. *FEMS Immunol Med Microbiol* 1999; **23**:149–58.
- 28 Stamp JT. Bovine pulmonary tuberculosis. *J Comp Pathol* 1948; **58**:9–23.
- 29 Hope JC, Thom ML, Villarreal-Ramos B, Vordermeier HM, Hewinson RG, Howard CJ. Vaccination of neonatal calves with *Mycobacterium bovis* BCG induces protection against intranasal challenge with virulent *M. bovis*. *Clin Exp Immunol* 2005; **139**:48–56.
- 30 Buddle BM, Keen D, Thomson A *et al.* Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae*. *Res Vet Sci* 1995; **59**:10–6.
- 31 Buddle BM, de Lisle GW, Pfeffer A, Aldwell FE. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG. *Vaccine* 1995; **13**:1123–30.
- 32 Buddle BM, Wards BJ, Aldwell FE, Collins DM, de Lisle GW. Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* 2002; **20**:1126–33.
- 33 Garnier T, Eiglmeier K, Camus JC *et al.* The complete genome sequence of *Mycobacterium bovis*. *Proc Natl Acad Sci USA* 2003; **100**:7877–82.
- 34 Collins RA, Howard CJ, Duggan SE, Werling D. Bovine interleukin-12 and modulation of IFN-gamma production. *Vet Immunol Immunopathol* 1999; **68**:193–207.
- 35 Werling D, Collins RA, Taylor G, Howard CJ. Cytokine responses of bovine dendritic cells and T cells following exposure to live or inactivated bovine respiratory syncytial virus. *J Leukoc Biol* 2002; **72**:297–304.
- 36 Hanna J, Neill SD, O'Brien JJ. Use of PPD and phosphatide antigens in an ELISA to detect the serological response in experimental bovine tuberculosis. *Res Vet Sci* 1989; **47**:43–7.
- 37 Tanaka S, Sato M, Onitsuka T, Kamata H, Yokomizo Y. Inflammatory cytokine gene expression in different types of granulomatous lesions during asymptomatic stages of bovine paratuberculosis. *Vet Pathol* 2005; **42**:579–88.
- 38 Hernandez-Pando R, Orozco H, Arriaga K, Sampieri A, Larriva-Sahd J, Madrid-Marina V. Analysis of the local kinetics and localization of interleukin-1 alpha, tumour necrosis factor-alpha and transforming growth factor-beta, during the course of experimental pulmonary tuberculosis. *Immunology* 1997; **90**:607–17.
- 39 Fulton SA, Martin TD, Redline RW, Henry Boom W. Pulmonary immune responses during primary *Mycobacterium bovis* – Calmette-Guérin bacillus infection in C57Bl/6 mice. *Am J Respir Cell Mol Biol* 2000; **22**:333–43.

- 40 Welsh MD, Cunningham RT, Corbett DM *et al.* Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology* 2005; **114**:101–11.
- 41 Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* 1992; **175**:1111–22.
- 42 Flesch I, Kaufmann SH. Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. *J Immunol* 1987; **138**:4408–13.
- 43 Via LE, Fratti RA, McFalone M, Pagan-Ramos E, Deretic D, Deretic V. Effects of cytokines on mycobacterial phagosome maturation. *J Cell Sci* 1998; **111**:897–905.
- 44 Hostetter JM, Steadham EM, Haynes JS, Bailey TB, Cheville NF. Cytokine effects on maturation of the phagosomes containing *Mycobacteria avium* subspecies paratuberculosis in J774 cells. *FEMS Immunol Med Microbiol* 2002; **34**:127–34.
- 45 Zganiacz A, Santosuosso M, Wang J *et al.* TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. *J Clin Invest* 2004; **113**:401–13.
- 46 Moreira AL, Tsenova L, Aman MH *et al.* Mycobacterial antigens exacerbate disease manifestations in *Mycobacterium tuberculosis*-infected mice. *Infect Immun* 2002; **70**:2100–7.
- 47 Hernandez-Pando R, Rook GA. The role of TNF-alpha in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. *Immunology* 1994; **82**:591–5.
- 48 Sugawara I, Yamada H, Mizuno S, Iwakura Y. IL-4 is required for defense against mycobacterial infection. *Microbiol Immunol* 2000; **44**:971–9.
- 49 Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. *J Exp Med* 1991; **174**:1549–55.
- 50 Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991; **147**:3815–22.
- 51 Fiorentino DF, Zlotnik A, Vieira P *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; **146**:3444–51.
- 52 Mendez-Samperio P, Hernandez-Garay M, Nunez Vazquez A. Inhibition of *Mycobacterium bovis* BCG-induced tumor necrosis factor alpha secretion in human cells by transforming growth factor beta. *Clin Diagn Lab Immunol* 1998; **5**:588–91.
- 53 Oswald IP, Wynn TA, Sher A, James SL. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. *Proc Natl Acad Sci USA* 1992; **89**:8676–80.
- 54 Jacobs M, Fick L, Allie N, Brown N, Ryffel B. Enhanced immune response in *Mycobacterium bovis* bacille Calmette Guérin (BCG)-infected IL-10-deficient mice. *Clin Chem Lab Med* 2002; **40**:893–902.
- 55 Arinobu Y, Atamas SP, Otsuka T *et al.* Antagonistic effects of an alternative splice variant of human IL-4, IL-4delta2, on IL-4 activities in human monocytes and B cells. *Cell Immunol* 1999; **191**:161–7.
- 56 Atamas SP, Choi J, Yurovsky VV, White B. An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL-4-stimulated T cell proliferation. *J Immunol* 1996; **156**:435–41.
- 57 Hirsch CS, Yoneda T, Averill L, Ellner JJ, Toossi Z. Enhancement of intracellular growth of *Mycobacterium tuberculosis* in human monocytes by transforming growth factor-beta 1. *J Infect Dis* 1994; **170**:1229–37.
- 58 Rhodes SG, Buddle BM, Hewinson RG, Vordermeier HM. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. *Immunology* 2000; **99**:195–202.
- 59 Vordermeier HM, Chambers MA, Cockle PJ, Whelan AO, Simmons J, Hewinson RG. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infect Immun* 2002; **70**:3026–32.
- 60 Villarreal-Ramos B, McAulay M, Chance V, Martin M, Morgan J, Howard CJ. Investigation of the role of CD8⁺ T cells in bovine tuberculosis *in vivo*. *Infect Immun* 2003; **71**:4297–303.
- 61 Hope JC, Thom ML, McCormick PA, Howard CJ. Interaction of antigen presenting cells with mycobacteria. *Vet Immunol Immunopathol* 2004; **100**:187–95.
- 62 Rhodes SG, Palmer N, Graham SP, Bianco AE, Hewinson RG, Vordermeier HM. Distinct response kinetics of gamma interferon and interleukin-4 in bovine tuberculosis. *Infect Immun* 2000; **68**:5393–400.
- 63 Coussens PM, Verman N, Coussens MA, Elftman MD, McNulty AM. Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*: evidence for an inherent pro-inflammatory gene expression pattern. *Infect Immun* 2004; **71**:409–22.
- 64 Jiao X, Lo-Man R, Winter N, Deriaud E, Gicquel B, Leclerc C. The shift of Th1 to Th2 immunodominance associated with the chronicity of *Mycobacterium bovis* bacille Calmette-Guérin infection does not affect the memory response. *J Immunol* 2003; **170**:1392–8.
- 65 Joardar SN, Ram GC, Goswami T. Dynamic changes in cellular immune responses in experimental bovine tuberculosis. *Med Sci Monit* 2002; **8**:BR471–80.