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Research paper

Time dependent expression of cytokines in *Mycobacterium bovis* infected cattle lymph nodes

J. Witchell[a,1], S.V.P.K. Maddipatla[a], A. Wangoo[b,2], M. Vordermeier[c], M. Goyal[a,*]

[a] School of Life Sciences, University of Hertfordshire, College Lane, Hatfield, Herts AL10 9AB, UK
[b] Department of Pathology, Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey KT15 3NB, UK
[c] TB Group, Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey KT15 3NB, UK

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ABSTRACT

Advancements in the current diagnostic and vaccination protocols employed against bovine tuberculosis rely heavily upon a sound knowledge of the bovine immunological response. Central to this is the importance of timing in the cellular immune profile and how this dynamic process evolves post-*Mycobacterium bovis* challenge. In the present study, we quantitatively analysed mRNA expression of interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukins (IL) 4 and 10 within select thoracic lymph nodes of cattle infected with *M. bovis* for 5, 12 and 19 weeks as compared to non-infected bovine tissues. The *M. bovis* infected lymph nodes displayed significantly higher expression levels of IFN-γ and TNF-α as compared to the non-infected lymph node tissues. This, in conjunction with undetectable levels of IL4, suggests a pro-inflammatory cytokine response. However a significant increase was also detected in IL10 mRNA which is consistent with a described aspect of T_H1 type T cells in *Leishmania* infection, a 'self-limiting' process in which cells produced both IFN-γ and IL10 with the aim of controlling the heightened immunopathological responses. This was further reflected when comparing the cytokine profiles of the individual lymph node types, as those displaying a higher IFN-γ/IL10 ratio also had a greater level of gross pathology. This data highlights the important role of IL10 in the bovine response to *M. bovis* infection and supports its involvement as an immunological marker of disease progression.

1. Introduction

Bovine tuberculosis (bTB) poses a significant economic and veterinary threat, with cases within the UK increasing substantially over the past 20 years (Reynolds, 2006; Jalava et al., 2007). Enhanced knowledge on the progression of the bovine immune response following *Mycobacterium bovis* infection would greatly aid the development of future diagnostic and vaccination procedures. Without reliable correlates of a protective response, it is substantially more difficult to assess the effectiveness of potential vaccination candidates. In addition, the current diagnostic tests for bTB rely heavily on the formation of a cell mediated/IFN-γ response (Vordermeier et al., 2001; Cockle et al., 2006; Sopp et al., 2008) and therefore an understanding on the dynamics of this response post-challenge may have consid-

Abbreviations: bp, base pairs; CP value, crossing point value; FFPE, formalin-fixed, paraffin embedded; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon gamma; IL, interleukin; MHC, major histocompatability complex; mRNA, messenger ribonucleic acid; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RNA, ribonucleic acid; TNF-α, tumour necrosis alpha.

* Corresponding author. Tel.: +44 1707 284624; fax: +44 1707 285046.
E-mail address: m.goyal@herts.ac.uk (M. Goyal).
[1] Present address: The Wellcome Trust, 215 Euston Road, London NW1 2BE, UK.
[2] Present address: Veterinary Medicines Directorate, Addlestone, Surrey KT15 3LS, UK.

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Table 1
Primer and probe sequences for quantitative dual labelled probe PCR (sequences designed at the Veterinary Laboratory Agency, VLA).

PCR product	Forward primer 5′–3′	Reverse primer 5′–3′	Fluorescent probe (5′ FAM and 3′ BHQ-1 labelled)
TNF-α	CGGTGGTGGGACTCGTATG	GCTGGTTGTCTTCCAGCTTCA	CAATGCCCTCGTGGCCAACGG
IFN-γ	CAGAAAGCGGAAGAGAAGTCAGA	CAGGCAGGAGGACCATTACG	TCTCTTTCGAGGCCGGAGAGCATCA
IL10	GGTGATGCCACAGGCTGAG	AGCTTCTCCCCCAGTGAGTTC	CACGGGCCTGACATCAAGGAGCA
IL4	GCCACACGTGCTTGAACAAA	TCTTGCTTGCCAAGCTGTTG	TCCTGGGCGGACTTGACAGG

Oligonucelotide sequences were commercially manufactured by Biomers.net (Germany).

erable importance on the timing of diagnostic testing. The majority of previously performed time dependent immune studies have been based on whole blood cultures due to the ease of collecting samples (Rhodes et al., 2000; Thacker et al., 2007; Joardar et al., 2002; Welsh et al., 2005). However, as bovine tuberculosis infection in cattle is centred primarily in the lungs and associated lymph nodes, the representation of whole blood serum immunological assays for the actual infection has been a strong topic of debate (Badell et al., 2009). In the present study, we quantitatively measured the level of IFN-γ, TNF-α, IL10 and IL4 mRNA expression within select bovine thoracic lymph nodes, at three time points post-experimental *M. bovis* challenge (5, 12 and 19 weeks) and compared to non-infected cattle lymph node samples. The key focus of this study was to provide a unique view of the time dependent change in immune cytokine profile at the actual site of infection.

2. Materials and methods

Experimental infection of large animals such as cattle involves extensive ethical and financial consideration. Archival tissue therefore provides a unique source on which further experimental procedures can be performed many years after the initial studies and without having to infect more animals. The *M. bovis* infected tissues used were formalin-fixed, paraffin embedded and sourced from three historical studies in which the parameters met the aims of this study.

2.1. Experimental infection

Non-vaccinated Friesian Holstein heifers and bullocks of approximately 6 months of age and with no history of tuberculosis infection were experimentally infected intratracheally with *M. bovis* (reference strain AF2122/97, $0.8–1.0 \times 10^4$ colony forming units) as described by Dean et al. (2005). The animals were euthanized by intravenous injection of sodium pentobarbitonic at either 5 (three cattle), 12 (three cattle) or 19 (four cattle) weeks post-infection. Tissue sections of the cranial mediastinal, caudal mediastinal, left bronchial and cranial tracheobronchial lymph nodes were fixed in neutral-buffered formalin (10% formaldehyde) for 7 days as detailed previously by Witchell et al. (2008). Gross pathology was represented by the percentage area coverage of granulomatous lesions within each individual *M. bovis* infected lymph node tissue sample, evaluated using 4 μm sections of the formalin-fixed, paraffin embedded tissue blocks stained with haematoxylin and eosin. This was estimated by counting the number of fields of view that contained lesions (100× magnification) and calculating the corresponding percentage area coverage in relation to the entire lymph node section. Similar scoring techniques have also been used previously by Widdison et al. (2006, 2009) and Vordermeier et al. (2002). Bacterial culture performed on each lymph node sample of the infected animals displayed positive results for *M. bovis*. Tissue samples of the left bronchial, caudal mediastinal and cranial mediastinal lymph nodes taken from five non-infected Friesian Holstein heifers and bullocks were used as a control group.

2.2. Total RNA extraction

The published protocol for total RNA extraction from *M. bovis* infected, formalin fixed paraffin embedded lymph node tissue using the Optimum™ FFPE kit (Ambion Ltd, UK) has been previously described (Witchell et al., 2008). Briefly, samples were deparaffinised using xylene at room temperature and dissolved in Proteinase K (60 U/μl) solution at 37 °C for 6 h. RNA extraction buffer was added to the supernatant, passed through a micro filter cartridge by centrifugation and transferred to a micro elution tube into which 2×10 μl volumes of pre-heated (70 °C) RNA elution solution were added. This was then left at room temperature for 1 min before centrifuging ($16{,}500 \times g$) for 1 min to elute the RNA. The concentration and purity of the total RNA samples were analysed using a Biophotometer (Eppendorf, Germany) and run on tris-acetate EDTA (TAE) agarose gel electrophoresis. The total RNA samples were finally treated with DNase I to remove contaminating DNA, according to the manufacturer's instructions (Ambion Ltd, UK).

2.3. cDNA synthesis and real-time polymerase chain reaction

The protocol was carried out as per the manufacturers' guidelines for the ImProm RT kit (Promega, UK). Briefly, 1 μg of total RNA for each sample was added to 0.5 μg oligo$(dT)_{15}$, 1× ImProm-IT reaction buffer, 4 mM Magnesium chloride, 0.5 mM deoxyribonucleoside triphosphate (dNTPs) and 1× ImProm-RT mix. The reaction mixture was incubated for 5 min at 25 °C followed by 60 min at 42 °C to synthesize the cDNA.

Quantitative PCR was performed using a dual labelled probe and primer method (sequences displayed in Table 1) and the Quantitect™ Probe PCR (Qiagen, UK) kit. For each sample, 1500 ng of cDNA was added to a 1× solution of QuantiTect RT-PCR mastermix, 0.4 μm forward primer, 0.4 μm reverse primer, 0.2 μm probe and RNase-free water to a final volume of 25 μl. The quantitative PCR reaction