2015

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Veterinary Immunology and Immunopathology 167 (2015) 15–21

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

ZAP-70, CTLA-4 and proximal T cell receptor signaling in cows infected with *Mycobacterium avium* subsp. *paratuberculosis*

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ARTICLE INFO

Article history:
Received 26 March 2015
Received in revised form 18 June 2015
Accepted 29 June 2015

Keywords:
Mycobacterium avium subsp. *paratuberculosis*
T cell function
ZAP-70
CTLA-4

ABSTRACT

Paratuberculosis is a chronic intestinal disease of ruminant animals caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). A hallmark of paratuberculosis is a transition from a cell-mediated Th1 type response to a humoral Th2 response with the progression of disease from a subclinical to clinical state. The objective of this study was to investigate the expression of two crucial molecules in T cell function, ZAP-70 (zeta-chain-associated protein of 70 kDa) and CTLA-4 (cytotoxic T-lymphocyte antigen-4), in cows naturally infected with MAP. Peripheral blood mononuclear cells (PBMCs) isolated from control non-infected cows ($n=5$), and cows in subclinical ($n=6$) and clinical stages of paratuberculosis ($n=6$) were cultured alone (medium only), and with concanavalin A, and a whole cell sonicate of MAP for 24, 72 and 144 h to measure the dynamic changes of ZAP-70 and CTLA-4 expression on CD4, CD8, and gamma delta (γδ) T cells. Flow cytometry was also performed to measure ZAP-70 phosphorylation to examine proximal T cell receptor signaling in animals of different disease status. The surface expression of CTLA-4 was increased in animals in subclinical stage of infection while levels of ZAP-70 were decreased in CD4+ T cells of both subclinical and clinical animals, indicating a change in T cell phenotype with disease state. Interestingly, proximal T cell receptor signaling was not altered in infected animals. This study demonstrated changes in crucial signaling molecules in animals infected with MAP, thereby elucidating T cell alterations associated with disease progression.

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1. Introduction

Paratuberculosis, also called Johne's disease, is a disease of ruminant animals caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Paratuberculosis is a chronic enteric disease with high economic impact in US dairy cattle, where it can cause decreased milk production, lead to early culling of animals, and reduce fertility rates (Lombard, 2011). The disease is primarily subclinical, and clinical signs such as diarrhea and weight loss can take from 2 to 5 years to manifest (Stabel, 1998). The disease has worldwide distribution in most ruminant species, and prevalence of paratuberculosis at the dairy herd level in the US is estimated to be 91.1% (Lombard et al., 2013).

Infection of ruminant animals occurs primarily via exposure to contaminated feces or ingestion of colostrum/milk from infected dams (Stabel, 1998). Upon infection, MAP passes through the M cells and epithelial cells in the small intestine where it is phagocytized by macrophages within the submucosa (Bannantine and Bermudez, 2013). Initially, animals control infection with a Th1 response predominated by the secretion of cytokines such as IFN-γ that activate macrophages to kill the intracellular bacterium (Sweeney, 2011). As disease progresses and clinical manifestations begin to occur, there is a shift from a cell-mediated Th1 to a non-protective Th2 response characterized by antibody titers to MAP. The molecular mechanisms underlying this transition are poorly understood (Stabel, 2006). Some animals that demonstrate clinical signs of disease may have both Th1 and Th2-mediated immune responses, whereas other clinical animals seem to lose Th1-mediated immunity. Previous research has also found that cells isolated from animals with clinical disease neither secrete IFN-γ when stimulated with MAP antigen, nor proliferate or respond to T cell mitogens such as concanavalin A (Weiss et al., 2006; Begg et al., 2011). This suggests that there are other mechanisms

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http://dx.doi.org/10.1016/j.vetimm.2015.06.017
0165-2427/Published by Elsevier B.V.

involved in T cell function during the progression of disease other than simply the shift to a Th2 response, and these may include the induction of tolerance and anergy/hypofunction of T cells.

ZAP-70 (zeta-chain associated protein of 70 kDa) and CTLA-4 (cytotoxic T-lymphocyte antigen-4) are two critical components of the T cell that are involved in modulation of host immune responses (Wang et al., 2010; Walker and Sansom, 2011). Upon engagement of the T cell receptor with an antigen presented by an MHC molecule of an antigen presenting cell, ZAP-70 is directed to ITAMS (immunoreceptor tyrosine-based activation motifs) of the zeta chain of the CD3 receptor, after which it is activated by phosphorylation and invokes downstream signaling molecules of the T cell leading to its full activation (Wang et al., 2010). Depletion studies have demonstrated that in the absence of ZAP-70 a functional immune synapse cannot occur, resulting in retardation of cell activation with subsequent impacts on ability of the cell to secrete cytokines or carry out effector functions (Blanchard et al., 2002). In contrast, CTLA-4 is regarded as a major negative regulator of T cell responses and its coligation with the T cell receptor (TCR) can induce anergy (Schneider et al., 2008). Its inhibitory function occurs by engagement of B7 ligands (CD80/CD86) expressed on antigen presenting cells, resulting in inhibition of cytokine production and T cell proliferation (Rudd et al., 2009). These two molecules work together to either allow T cell activation (ZAP-70) or to abrogate T cell responses (CTLA-4) in the host.

The present study was designed to measure signaling molecules of T cell activation, ZAP-70 and CTLA-4, through flow cytometry in cows in different stages of paratuberculosis. Mechanisms of cellular activation were further explored by quantifying the phosphorylation of ZAP-70 following activation of cells to determine if the signaling cascade was compromised.

2. Methods

2.1. Animals

Holstein dairy cows averaging 6 years of age were used in this study and placed in three groups based upon stage of disease. Treatment groups consisted of noninfected healthy cows ($n = 5$), cows naturally infected with *M. avium* subsp. *paratuberculosis* but asymptomatic (subclinical; $n = 6$), and cows with clinical signs of paratuberculosis (clinical; $n = 6$). Animals were classified as subclinical or clinical based upon results from IFN-γ release assay (Prionics, La Vista, NE) using a whole cell sonicate of MAP (MPS) as the antigen, serum antibody (Herdcheck, IDEXX, Westbrook, ME) and fecal culture. By definition, clinical cows were shedding more than 100 CFU per g of feces and presented with weight loss and intermittent diarrhea. Clinical cows had higher serum antibody titers than subclinically infected cows (Sample/Positive ratio = 2.12 vs 0.11, respectively), but lower IFN-γ responses ($Abs_{450nm} = 0.36$ vs 0.98, respectively). Subclinically infected cows were shedding less than 10 CFU/g of feces and did not present clinical signs of disease. Noninfected control cows were characterized by repeated negative fecal culture performed semi-annually over a 3–5-year period as well as negative results in serum antibody and IFN-γ release assay. All animals were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities and all animal related procedures were approved by the IACUC (National Animal Disease Center, Ames, IA). Cows infected with MAP were housed separately from healthy control cows to prevent cross-contamination between groups.

2.2. Blood collection, culture conditions, and sample collection

Blood was collected from the jugular vein in 2X acid citrate-dextrose solution (1:10) and used for isolation of peripheral blood mononuclear cells (PBMCs) from the buffy coat as previously described (Khalifeh and Stabel, 2013). These cells were resuspended to 2×10^6 cells/ml in complete RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Fungizone® (Gibco). Cell culture was performed in 6-well flat-bottomed plates at 39 °C in 5% CO_2 in a humidified atmosphere. In vitro treatments consisted of no stimulation (NS; medium only), concanavalin A (ConA) (10 μg/ml; Sigma, St. Luis, MO), and a whole-cell sonicate of MAP (MPS; 10 μg/ml) prepared as previously described (Stabel et al., 2011). Plates were incubated for 24, 72 and 144 h and cells were harvested for flow cytometric analyses. During the last 5 h of incubation a cocktail of 10 μg/ml brefeldin A (Sigma), 1 μg/ml ionomycin (Sigma) and 50 ng/ml PMA (Sigma) was added to culture wells to enable intracellular measurement of IFN-γ.

2.3. Flow cytometric measurement of ZAP, pZAP-70, CTLA-4, and IFN-γ

Cells were gently removed from culture plates by pipetting and resuspending cultures in phosphate buffered saline (PBS). Cells were added at a concentration of 1×10^6 cells per well in a 96-well round bottom plate (Corning, Corning, NY), followed by 50 μl of a cocktail of primary antibodies to T cell surface markers CD4, CD8, gamma delta T cell receptor (γδ TCR) (WSU Monoclonal Antibody Center, Pullman, WA) and CTLA-4 (Ancell, Stillwater, MN) (Table 1). After a 15 min incubation at room temperature (RT), plates were centrifuged for 2 min at $400 \times g$, the supernatant was decanted, and 50 μl of a secondary antibody cocktail was added, which included PE-Cy7 anti-mouse IgM (5 μl/ml; Southern Biotech, Birmingham, AL), APC Cy7 anti-mouse $IgG2_b$ (7 μl/ml; Southern Biotech), and Alexa Fluor 350 anti-mouse IgG_1 (25 μl/ml; Life Technologies, Carlsbad, CA). After incubation for 15 min at RT, plates were centrifuged as described above then fixed and permeabilized following manufacturer's instructions with the Cytofix/Cytoperm solution (BD Biosciences San Jose, CA). Cells were washed twice with Perm/Wash buffer (BD Biosciences), followed by intracellular staining for 30 min at 4 °C for the molecules ZAP-70, phosphorylated ZAP-70 (pZAP-70), and IFN-γ. After another two washes with Perm/Wash buffer (BD Biosciences) cells were resuspended in PBS with 0.4% sodium azide for flow cytometric analysis. Samples were evaluated using 10,000 events per sample, using a FACScan flow cytometer (CellQuest software; Becton Dickinson). Analyses were conducted by gating on live cells based upon forward and side scatter characteristics (FlowJo; Tree Star). Due to the low level of live cells in the ConA treatment at 144 h culture this treatment was not considered for further analysis.

Table 1
Primary antibodies used in study.

Target	Ig isotype	Working conc. (μl/ml)	Clone	Fluorochrome used
CD4	IgM	10	CATC83B	PE-Cy7
CD8	IgG_1	10	CACT80C	Alexa 350
CD8	IgG_{2a}	20	MCA837	Alexa 350
γδ TCR	IgG_{2b}	10	GB21A	APC Cy7
CTLA-4[a]	IgG_1	8	ANC152.2/8H5	PerCp
ZAP-70[a]	IgG_1	10	1E7.2	Pacific Blue
IFN-γ[a]	IgG_1	20	MCA1783PE	PE
pZAP-70[a]	Rabbit polyclonal	10	Polyclonal to Y319	Alexa488

[a] Refers to antibodies that were directly conjugated, CTLA-4 to PerCp; ZAP-70 to Pacific Blue, IFN-γ to PE, and pZAP-70 to Alexa Fluor® 488. Surface marker antibodies were diluted in PBS with 0.4% sodium azide whereas intracellular marker antibodies (ZAP-70, pZAP-70, IFN-γ) were diluted in BD Perm Wash buffer.

2.4. Phospho flow cytometric analysis of pZAP-70

To measure ZAP-70 phosphorylation during T cell activation, the technique of phospho flow cytometry was used following a modified protocol (Haas et al., 2008). Isolated PBMCs were plated at 2×10^6/ml in 96-well round bottom plates in RPMI-1640 medium (Life Technologies) and allowed to rest for 1 h at 39 °C in 5% CO_2. No fetal bovine serum was present in the medium in order to reduce baseline ZAP-70 phosphorylation. Antigen-specific T cell activation was performed with a whole-cell sonicate of MAP (MPS; 10 μg/ml). Two positive controls, anti-CD3 (10 μg/ml; MM1A; WSU Monoclonal Antibody Center) and 11 mM hydrogen peroxide (H_20_2) were included to assess assay performance and non-specific responses of cells to stimulation. After addition of different stimulants, cells were incubated for 15 min at 39 °C in 5% CO_2 in a humidified atmosphere, followed by fixation of the cells to halt phosphorylation events with Cytofix/Cytoperm solution (BD Biosciences). After cells were fixed and permeabilized, cell staining followed the protocol of cultured cells for staining of pZAP-70 and ZAP-70 within CD4, CD8 and γδ TCR T cells (Table 1). A substitution of CD8 clone MCA837 (Serotec Raleigh, NC) was made as the clone CACT80C (WSU Monoclonal Antibody Center) does not stain cells that have been previously fixed. A prior time trial determined that maximum ZAP-70 phosphorylation was achieved at 15 min (data not shown), therefore, this was the time selected for cell activation used in the experiments.

2.5. Tissue immunofluorescent staining for ZAP-70

Immunofluorescence staining was performed on 5 μM frozen ileum sections from non-infected ($n = 2$), subclinical ($n = 5$) and clinical ($n = 5$) animals. Briefly, after fixation in acetone:ethanol (1:1) solution, tissue sections were blocked in 10% normal goat serum (NGS) in PBS solution for 30 min at RT and labeled overnight at 4 °C with anti-ZAP-70 (1:1500; Invitrogen Carlsbad, CA) and anti-CD4 (1:500; VMRD, Inc. Spokane, WA) antibodies. After repeated washing with PBS, the slides were incubated with Alexa Fluor 594 (1:800; Invitrogen) and Alexa Fluor 488 (1:800; Invitrogen) for 1 h at RT. Staining was examined using an E800 Eclipse fluorescence microscope (Nikon, United Kingdom). Images were analyzed (pixel intensity) using SPOT 4.7 Advanced Plus software (SPOT Imaging Solutions, Diagnostic Instruments, Inc.). For overlay, images were adjusted to the same output intensities and merged into a composite image for 2 animals from each infection status.

2.6. Statistical analysis

Statistical analysis was performed using the PROC Mixed function of SAS 9.02 (SAS Institute, Cary, NC). Data obtained from the comparison between treatments, clinical status and day, for each molecule in each cell type were analyzed using a mixed linear model followed by Tukey's range test. Results from 144 h cultures were analyzed in a separate model because of omission of the ConA treatment and higher variance within samples. Two-sided $P < 0.05$ values were considered significant.

3. Results

3.1. T cell expression of ZAP-70

After 24 h culture, ZAP-70 expression on CD4+ T cells was significantly ($P < 0.05$) lower for both subclinical and clinical cows compared to noninfected controls, regardless of in vitro treatment.

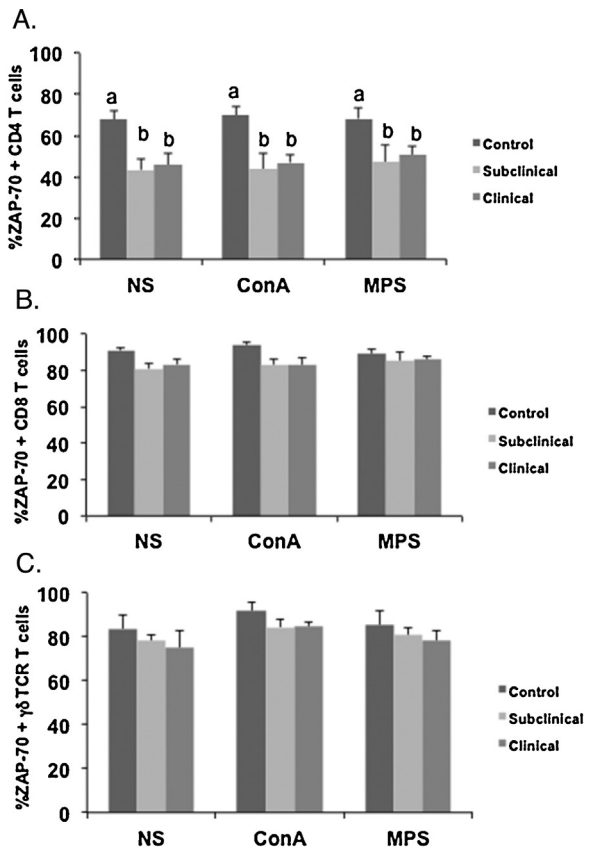

Fig. 1. Expression of ZAP-70 on (A) CD4, (B) CD8, and (C) gd TCR T cells after culture of PBMCs isolated from control cows and cows naturally infected with *Mycobacterium avium* subsp. paratuberculosis. Cells were cultured with medium alone (NS), concanavalin A (ConA), or a whole cell sonicate of MAP (MPS) for 24 h. Data are presented as percentage of ZAP-70+ T cells (mean ± SEM). Significant differences between animal treatment groups are designated by different letters, a, b ($P < 0.05$).

A trend for reduced ZAP-70 expression was also true for CD8 and γδ T cells from infected cattle, although differences were not statistically significant (Fig. 1). Extending PBMC culture times to 72 and 144 h resulted in an increase in the percentage of ZAP-70+ CD4 T cells from 50.8% to 62.7% and 78.8%, respectively for clinically infected cows, reaching levels similar to noninfected animals by 144 h (Fig. 2a). Interestingly, an increase in ZAP-70 expression on CD4 T cells was noted for subclinical cows between 24 and 72 h but no further increases were noted at 144 h, resulting in significantly ($P < 0.05$) lower expression of ZAP-70 for this group. In contrast, pZAP-70 trended toward higher expression on CD4 T cells at all time points for noninfected control cows compared to the infected groups (Fig. 2b). Further, differences in ZAP-70 or pZAP-70 expression were not observed for CD8+ and γδ TCR+ T cells regardless of animal treatment group at extended culture times, although patterns of lower pZAP-70 expression for infected cows paralleled CD4+ T cells (data not shown).

3.2. Analysis of CTLA-4 and IFN-γ expression in T cells of 24, 72 and 144 h cultures

Surface expression of CTLA-4 was measured in CD4, CD8, and γδ T cell subsets at 24, 72 and 144 h time points. At 24 h, cows in the subclinical group had a higher ($P < 0.05$) percentage of CD4+ T cells expressing CTLA-4 for all in vitro treatments when compared to control and clinical cows (Fig. 3A). This effect was also observed in CD8+ T cells but was not statistically significant (Fig. 3B). In contrast, surface expression of CTLA-4 was negligible on γδT cells

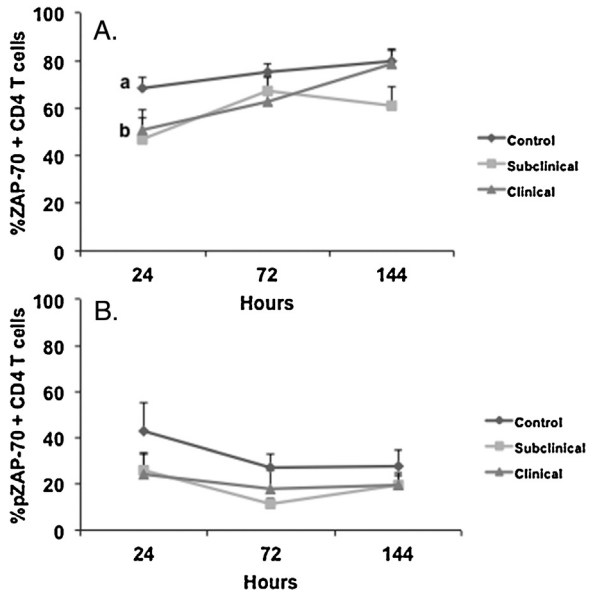

Fig. 2. Expression of ZAP-70 (A) and pZAP-70 (B) on CD4+ T cells after culture of PBMCs isolated from control cows and cows naturally infected with *Mycobacterium avium* subsp. paratuberculosis. Cells were cultured with a whole cell sonicate of MAP (MPS) for 24, 72, or 144 h. Data are presented as percentage of ZAP-70+ or pZAP-70+ T cells (mean ± SEM). Significant differences between infection group are designated by different letters, a, b ($P < 0.05$).

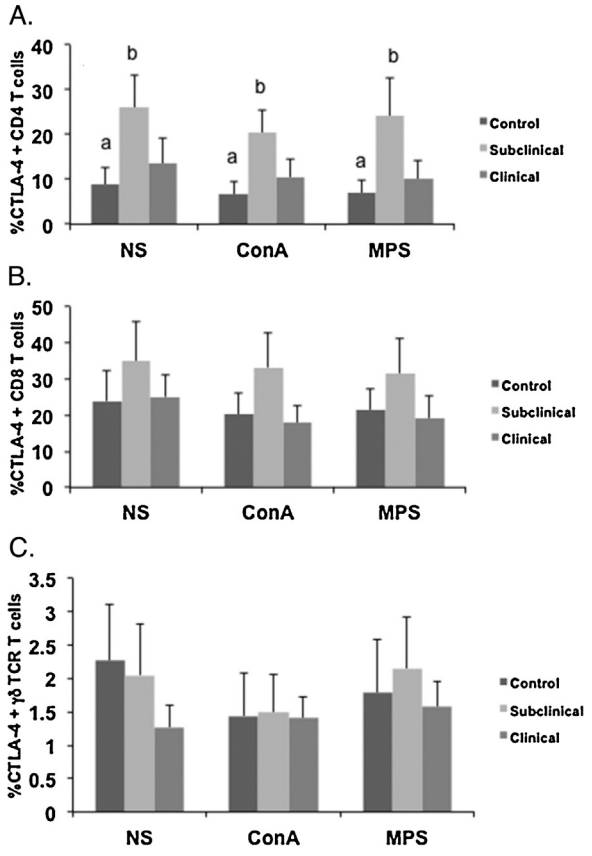

Fig. 3. Expression of CTLA-4 on (A) CD4, (B) CD8, and (C) γδ TCR T cells after culture of PBMCs isolated from control cows and cows naturally infected with *Mycobacterium avium* subsp. paratuberculosis (MAP). Cells were cultured with medium alone (NS), concanavalin A (ConA), or a whole cell sonicate of MAP (MPS) for 24 h. Data are presented as percentage of ZAP-70+ T cells (mean ± SEM). Significant differences between animal treatment groups are designated by different letters, a, b ($P < 0.05$).

after 24 h of culture, regardless of in vitro treatment (Fig. 3C). Differences noted in CTLA-4 expression on CD4+ T cells between infection groups was also demonstrated in a representative flow cytometry density plot, averaging 3.9, 22.4, and 12.8% for control, subclinical and clinical cows, respectively (Fig. 4). Upon extending culture times to 72 and 144 h, marginal differences were observed between treatment groups with the number of CTLA-4+ CD4 T cells decreasing from 24.1 to 12.5% between 24 and 72 h of culture for subclinical cows (Fig. 5A). Upon stimulation of PBMC with MPS, levels of intracellular IFN-γ did tend to be higher for infected cows compared to controls over time (Fig. 5B), however, the high degree of variability precluded any significance. Measurement of intracellular IFN-γ in ConA-stimulated PBMC demonstrated an opposite trend with higher overall expression in control cows compared to infected cows (data not shown).

3.3. Phospho flow cytometry and measurement of ZAP-70 phosphorylation

To address proximal T cell receptor signaling and T cell activation, phospho flow cytometry was performed to measure phosphorylation of ZAP-70 following TCR (T cell receptor) engagement. Hydrogen peroxide and anti-CD3 antibody were included

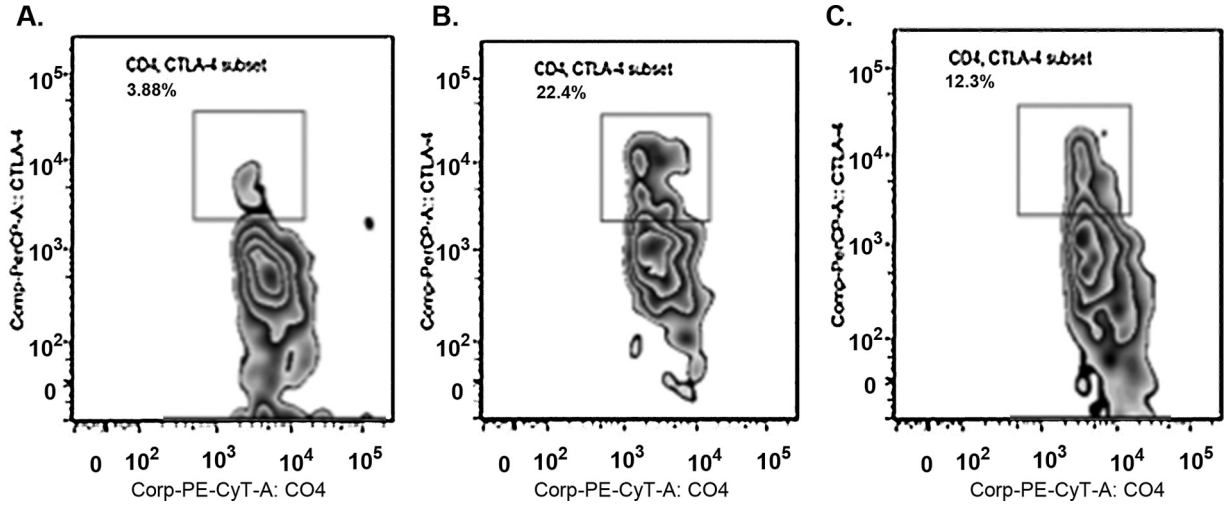

Fig. 4. Histogram demonstrating representative flow cytometric staining for surface expression of CTLA-4 on CD4+ T cells from a control cow (A), subclinical cow (B), and a clinical cow (C) after culture of PBMCs for 24 h with a whole cell sonicate of MAP (MPS).

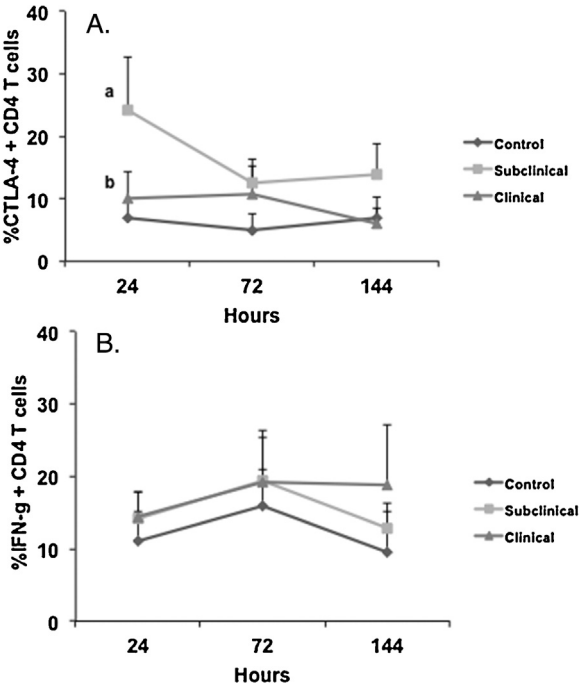

Fig. 5. Expression of CTLA-4 (A) and IFN-γ (B) on CD4+ T cells after culture of PBMCs isolated from control cows and cows naturally infected with *Mycobacterium avium* subsp. paratuberculosis. Cells were cultured with a whole cell sonicate of MAP (MPS) for 24, 72, and 144 h. Data are presented as percentage of CTLA-4+ or IFN-γ+ T cells (mean ± SEM). Significant differences between animal treatment groups are designated by letters, a, b ($P < 0.05$).

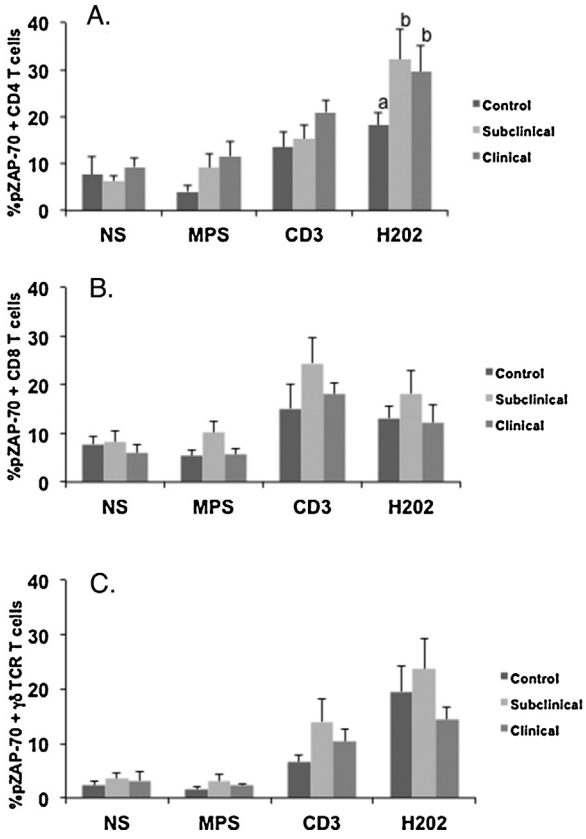

Fig. 6. Phopho flow analysis of pZAP-70 on (A) CD4, (B) CD8, and (C) γδ TCR T cells after stimulation of PBMCs isolated from control cows and cows naturally stimulated with medium alone (NS), a whole cell sonicate of MAP (MPS), anti-CD3 antibody, and hydrogen peroxide (H_2O_2). Data are presented as percentage of pZAP-70+ T cells (mean ± SEM). Significant differences between animal treatment groups are designated by designated by different letters, a, b ($P < 0.05$).

as positive controls to measure the activation capacity of the cell in an antigen-independent manner, via non-receptor- and receptor-mediated events. Within the CD4+, CD8+, and γδ TCR+ T cell subsets, treatment with H_2O_2 or anti-CD3 yielded significant ($P < 0.05$) increases in pZAP-70 expression for all cows in relation to the NS treatment (Fig. 6). In contrast, pZAP-70 expression in T cells was not different between the MPS and NS treatments, regardless of animal infection status. However, a pattern of higher ($P < 0.05$) pZAP-70 expression in CD4+ T cells from infected cows was observed after in vitro treatment of cells with H_20_2 (Fig. 6A). This pattern of expression across treatment groups was repeated after treatment of cells with either MPS or anti-CD3, albeit at a lower level. Additionally, a trend toward higher expression of pZAP-70 in CD8+ T cells was observed for subclinical cows, regardless of in vitro treatment (Fig. 6B), and in γδ TCR+ T cells to a lesser extent (Fig. 6C).

3.4. Immunofluorescence analysis of ZAP-70 in ileum CD4+ T cells

To evaluate the presence of ZAP-70 at the site of infection in a known target tissue, immunofluorescence was performed on sections of ileum obtained from control, subclinical and clinical cows at necropsy. Upon staining for ZAP-70, differences in staining intensity were observed between the control, subclinically and clinically infected cows, averaging 38.9 ± 6.8, 15.2 ± 3.4, and 21 ± 3 pixel intensity, respectively. Interestingly, mean staining intensity for CD4+ T cells in the ileum was also lower for infected cows compared to control cows (22.3 ± 3.3 vs 53.8 ± 0.9). In tissue from control cows, CD4+ T cells were found to be co-localized with ZAP-70 throughout the entire section of ileum (Fig. 7). In contrast, ileal tissue from clinical cows showed a marked reduction in the number of CD4+ T cells expressing ZAP-70 (Fig. 7).

4. Discussion

Studies on animals with paratuberculosis have provided evidence suggesting that anergy or T cell hypofunction occurs in the advanced stages of disease. Lymphocytes isolated from the ileum of infected cows did not proliferate after incubation with MAP antigen, demonstrating a hyporesponsive state of these cells correlating with anergy (Weiss et al., 2006). Similarly, investigation of the cellular immune response to MAP in naturally and experimentally infected sheep yielded attenuated responses in animals in more advanced stages of disease (Begg et al., 2011). These observations are part of an immune shift that occurs in paratuberculosis that is marked by a loss of cell-mediated immunity corresponding with lower IFN-γ and increased IL-10 levels and a subsequent rise in humoral immunity (Stabel, 2006). In paratuberculosis there are a limited number of studies that have addressed potential alterations within T cells and the events leading to clinical disease. Yet this information is critical to providing a better understanding of the disease as well as the possibility of more targeted approaches for vaccine and therapeutic development.

In the present study, both flow cytometric and immunofluorescent staining demonstrated that infected cows had fewer ZAP-70+ T cells, particularly within the CD4+ T cell subset. Interestingly, regardless of treatment, clinical and subclinical animals had fewer ZAP-70 positive cells at 24 h. This may indicate a broad change in these cells which cannot be reversed by strong T cell stimulation, as fewer positive cells were also observed after treatment with the T cell mitogen concanavalin A. It has been shown that patients with advanced forms of leprosy have significantly less

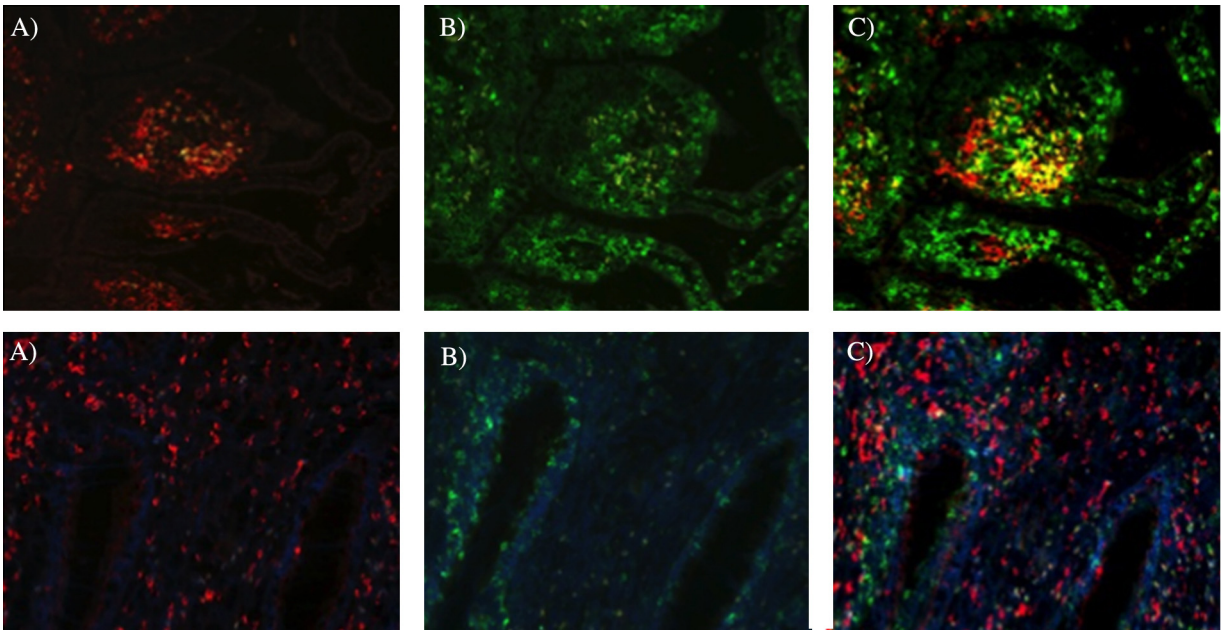

Fig. 7. Immunofluorescent staining of ileal sections from a control cow (top panel) and a cow clinically infected with *Mycobacterium avium* subsp. paratuberculosis (bottom panel) for CD4+ T cells (A), ZAP-70+ cells (B), and merged images of CD4 and ZAP-70 stains (C). Dual positive cells are indicated by yellow color in merged images.

pZAP-70 and ZAP-70 present in their T cells (Kumar et al., 2011). Similar to paratuberculosis, patients in the early stages of infection (tuberculoid leprosy) possess a cell-mediated immunity (CMI) that is considered to be effective in controlling disease. This later transitions to more severe lepromatous leprosy phase in which clinical signs of disease become evident and T cell hypofunction has been observed (Walker and Lockwood, 2006). Considering the importance of ZAP-70 in T cell signaling, it would be expected that the T cells identified as being ZAP-70 negative have a decreased potential for activation and effector function, potentially being rendered anergic.

There was an increase in the relative number of CTLA-4 + CD4 T cells in animals with subclinical paratuberculosis and differences in expression between subclinical and clinical animals as well as control non-infected animals were more prominent within the CD4+ population. Several studies on the expression of CTLA-4 in ruminants have focused on γδ T cells with inconsistent observations (Hanrahan et al., 1997; Blumerman et al., 2007; Fikri et al., 2000). However, these studies did not measure CTLA-4 on the surface of cells as performed in this study, rather mRNA levels were measured; measurement of CTLA-4 on the cellular surface is important since it is tightly regulated due to its impacts on T cell function (Valk et al., 2008).

A decrease in CTLA-4 expression over time has previously been observed in the different T cell subsets in mice and humans, demonstrating that upon T cell activation the maximal surface expression of CTLA-4 occurs following 48–72 h, followed by reduced expression with longer incubation periods (Walunas et al., 1994; Wang et al., 2001). In this study, maximal expression was found at 24 h, possibly due to a difference in host species and/or stimulation conditions.

Perhaps it is not a coincidence that the CD4+ T cells were the subset with the highest CTLA-4 expression. This is the same population that would be expected to play a bigger role in directing a Th1 type response through macrophage activation. MAP may induce a higher expression of CTLA-4 in T cells of infected animals as an evasion strategy from the immune system to promote chronic infection. Interestingly, patients with leprosy generated similar patterns of CTLA-4 expression whereby patients with tuberculoid leprosy expressed significantly more CTLA-4 compared to lepromatous patients (Palermo et al., 2012). As suggested in that study and in corroboration with data from the present study, CTLA-4 expression may be involved in the induction of T cell hypofunction during early and mid-stages of infection, whereas it is likely other molecules could be involved in the maintenance of an anergic T cell state in the later stages of chronic infection. Programmed death-1, a cell surface molecule that regulates adaptive immune response and is related to T cell exhaustion, may be one candidate, as it induces a state that is progressive, with dysfunction worsening overtime (Wherry, 2011).

Also pertinent to paratuberculosis infection are other roles that CTLA-4 has been demonstrated to have in Th1 cells. These include T cell differentiation and migration to sites of inflammation and antigenic challenge (Oosterwegel et al., 1999; Knieke et al., 2009). This may provide an explanation of how animals in the subclinical stage can control infection if T cells expressing high levels of CTLA-4 are efficiently migrating to target sites of infection. In addition, cells that express CTLA-4 have been shown to have extrinsic functions causing downregulation in other cells. This includes the induction of indoleamine 2,3-dioxygenase (IDO) activity in APCs, which leads to tryptophan catabolism that can inhibit other T cells from proliferating (Walker and Sansom, 2011). This may also be an immune alteration caused by the increased CTLA-4 + T cells in animals infected with MAP as increased IDO activity has been described in animals with paratuberculosis (Plain et al., 2011).

The lack of changes in proximal T cell receptor signaling as measured by TCR activation and ZAP-70 phosphorylation between infected and control animals in this study is not necessarily surprising. Phospho flow cytometry was used to compare T cell receptor signaling among CD4+ T cells, and found that T cell activation by anti-CD3 antibody induced a higher magnitude of ZAP-70 phosphorylation in Tregs compared to non-Treg CD4+ T cells (Hanschen et al., 2012). Also, it was found that antigen-specific TCR stimulation did not significantly activate these signaling pathways, similar to what is reported here. An elevated number of Treg cells in PBMCs of animals infected with MAP have been reported and may explain the lack of alteration in proximal T cell signaling in this study, with the clinical group yielding the highest phosphorylated ZAP-70 response in CD4+ T cells following anti-CD3 activation (Coussens et al., 2012). A subject for future investigation is if MAP

has the ability to directly inhibit ZAP-70 phosphorylation as has been described in studies with *M. tuberculosis*. It has been shown that pre-incubation of T cells with *M. tuberculosis* mannosylated lipoarabinomannan (Man-LAM) inhibits ZAP-70 phosphorylation, a mechanism likely due to insertion of Man-LAM into the membrane of target T cells (Mahon et al., 2012).

5. Conclusion

This study investigated crucial molecules involved in T cell function in the context of paratuberculosis. Very few studies have addressed T cell function in paratuberculosis, especially T cell signaling events. Surprisingly, findings from this study correlate very well with findings from studies with *M. leprae*, suggesting the etiological agents of both diseases share similar mechanisms of immune evasion to cause chronic infection. This study for the first time discovered alterations in two crucial signaling molecules of the T cell in animals infected with MAP, shedding light into immune alterations that occur and possible triggers that lead to clinical paratuberculosis.

Acknowledgements

The authors wish to acknowledge the excellent technical assistance of Ami Frank and Margaret Walker, as well as the animal caretakers involved in this study.

References

Bannantine, J.P., Bermudez, L.E., 2013. No holes barred: invasion of the intestinal mucosa by *Mycobacterium avium* subsp. *paratuberculosis*. Infect. Immun. 81, 3960–3965.

Begg, D.J., de Silva, K., Carter, N., Plain, K.M., Purdie, A., Whittington, R.J., 2011. Does a Th1 over Th2 dominancy really exist in the early stages of *Mycobacterium avium* subspecies *paratuberculosis* infections? Immunobiology 216, 840–846.

Blanchard, N., Di Bartolo, V., Hivroz, C., 2002. In the immune synapse, ZAP-70 controls T cell polarization and recruitment of signaling proteins but not formation of the synaptic pattern. Immunity 17, 389–399.

Blumerman, S.L., Herzig, C.T., Wang, F., Coussens, P.M., Baldwin, C.L., 2007. Comparison of gene expression by co-cultured WC1+ gammadelta and CD4+ alphabeta T cells exhibiting a recall response to bacterial antigen. Mol. Immunol. 44, 2023–2035.

Coussens, P.M., Sipkovsky, S., Murphy, B., Roussey, J., Colvin, C.J., 2012. Regulatory T cells in cattle and their potential role in bovine paratuberculosis. Comp. Immunol. Microbiol. Infect. Dis. 35, 233–239.

Fikri, Y., Nyabenda, J., Denis, M., Pastoret, P.P., 2000. Purification and characterization of bovine WC1+ gammadelta T lymphocytes from peripheral blood. Vet. Res. 31, 229–239.

Haas, A., Weckbecker, G., Welzenbach, K., 2008. Intracellular Phospho-Flow cytometry reveals novel insights into TCR proximal signaling events. A comparison with Western blot. Cytometry A 73, 799–807.

Hanrahan, C.F., Kimpton, W.G., Howard, C.J., Parsons, K.R., Brandon, M.R., Andrews, A.E., Nash, A.D., 1997. Cellular requirements for the activation and proliferation of ruminant gammadelta T cells. J. Immunol. 159, 4287–4294.

Hanschen, M., Tajima, G., O'Leary, F., Hoang, K., Ikeda, K., Lederer, J.A., 2012. Phospho-flow cytometry based analysis of differences in T cell receptor signaling between regulatory T cells and CD4+ T cells. J. Immunol. Methods 376, 1–12.

Khalifeh, M.S., Stabel, J.R., 2013. Clinical disease upregulates expression of CD40 and CD40 ligand on peripheral blood mononuclear cells from cattle naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Clin. Vaccine Immunol. 20, 1274–1282.

Knieke, K., Hoff, H., Maszyna, F., Kolar, P., Schrage, A., Hamann, A., Debes, G.F., Brunner-Weinzierl, M.C., 2009. CD152 (CTLA-4) determines CD4 T cell migration *in vitro* and *in vivo*. PLoS ONE 4 (5), e5702, http://dx.doi.org/10.1371/journal.pone.0005702

Kumar, S., Naqvi, R.A., Khanna, N., Rao, D.N., 2011. Disruption of HLA-DR raft, deregulations of Lck-ZAP-70-Cbl-b cross-talk and miR181a towards T cell hyporesponsiveness in leprosy. Mol. Immunol. 48, 1178–1190.

Lombard, J.E., 2011. Epidemiology and economics of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 27, 525–535.

Lombard, J.E., Gardner, I.A., Jafarzadeh, S.R., Fossler, C.P., Harris, B., Capsel, R.T., Wagner, B.A., Johnson, W.O., 2013. Herd-level prevalence of *Mycobacterium avium* subsp. paratuberculosis infection in United States dairy herds in 2007. Prev. Vet. Med. 108, 234–238.

Mahon, R.N., Sande, O.J., Rojas, R.E., Levine, A.D., Harding, C.V., Boom, W.H., 2012. *Mycobacterium tuberculosis* ManLAM inhibits T-cell-receptor signaling by interference with ZAP-70, Lck and LAT phosphorylation. Cell Immunol. 275, 98–105.

Oosterwegel, M.A., Mandelbrot, D.A., Boyd, S.D., Lorsbach, R.B., Jarrett, D.Y., Abbas, A.K., Sharpe, A.H., 1999. The role of CTLA-4 in regulating Th2 differentiation. J. Immunol. 163, 2634–2639.

Palermo, M., de, L., Trindade, M.Â., Duarte, A.J., Cacere, C.R., Benard, G., 2012. Differential expression of the costimulatory molecules CD86, CD28, CD152 and PD-1 correlates with the host-parasite outcome in leprosy. Mem. Inst. Oswaldo Cruz 107, 167–173.

Plain, K.M., de Silva, K., Earl, J., Begg, D.J., Purdie, A.C., Whittington, R.J., 2011. Indoleamine 2,4-dioxygenase, tryptophan catabolism, and *Mycobacterium avium* subsp. *paratuberculosis*: a model for chronic mycobacterial infections. Infect. Immun. 79, 3821–3832.

Rudd, C.E., Taylor, A., Schneider, H., 2009. CD28 and CTLA-4 coreceptor expression and signal transduction. Immunol. Rev. 229, 12–26.

Schneider, H., Valk, E., Leung, R., Rudd, C.E., 2008. CTLA-4 activation of phosphatidylinositol 3-kinase (PI 3-K) and protein kinase B (PKB/AKT) sustains T-cell anergy without cell death. PLoS ONE 3 (12), e3842, http://dx.doi.org/10.1371/journal.pone.0003842

Stabel, J.R., 1998. Johne's disease: a hidden threat. J. Dairy Sci. 81, 283–288.

Stabel, J.R., 2006. Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. Anim. Health Res. Rev. 7, 61–70.

Stabel, J.R., Waters, W.R., Bannantine, J.P., Lyashchenko, K., 2011. Mediation of host immune responses after immunization of neonatal calves with a heat-killed *Mycobacterium avium* subsp. *paratuberculosis* vaccine. Clin. Vaccine Immunol. 18, 2079–2089.

Sweeney, R.W., 2011. Pathogenesis of Paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 27, 537–546.

Valk, E., Rudd, C.E., Schneider, H., 2008. CTLA-4 trafficking and surface expression. Trends Immunol. 29, 272–279.

Walker, L.S., Lockwood, D.N., 2006. The clinical and immunological features of leprosy. Br. Med. Bull. 77–78, 103–121.

Walker, L.S., Sansom, D.M., 2011. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. Nat. Rev. Immunol. 25, 852–863.

Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., Bluestone, J.A., 1994. CTLA-4 can function as a negative regulator of T cell activation. Immunity 1, 405–413.

Wang, H., Kadlecek, T.A., Au-Yeung, B.B., Goodfellow, H.E., Hsu, L.Y., Freedman, T.S., Weiss, A., 2010. ZAP-70: an essential kinase in T-cell signaling. Cold Spring Harb. Perspect. Biol. 2, a002279, http://dx.doi.org/10.1101/cshperspect.a002279

Wang, X.B., Zheng, C.Y., Giscombe, R., Lefvert, A.K., 2001. Regulation of surface and intracellular expression of CTLA-4 on human peripheral T cells. Scand. J. Immunol. 54, 453–458.

Weiss, D.J., Evanson, O.A., Souza, C.D., 2006. Mucosal immune response in cattle with subclinical Johne's disease. Vet. Pathol. 43, 127–135.

Wherry, E.J., 2011. T cell exhaustion. Nat. Immunol. 12, 492–499.