

Characterisation of
***Mycobacterium avium* subspecies *paratuberculosis* reactive**
T cells in goats

By

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Master Thesis

60 study points

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Main field of study in Immunology

June 2012

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Acknowledgment

This work was performed at the Norwegian Veterinary Institute, Section of Immunology, in collaboration with the University of Oslo in the period from January 2011 to June 2012.

My heartfelt gratitude and thanks must be firstly offered to God for his merciful support and guidance to me to complete this study.

I wish to express my sincere thanks and deepest appreciation to my supervisor Dr Ingrid Olsen and Co-supervisor Stig Tollefsen Ph.D. researcher, for their kind supervision advice and help during the practical work and in the process of writing this thesis.

Also my thanks are due to my internal supervisor, Professor Inger Sandlie, for valuable feedback during the final steps of the writing process.

Special thanks goes to Siri Kulberg, Ph.D., researcher at the Norwegian Veterinary Institute, Section of Immunology, for her help to construct the primers and probes for RT-PCR, and her help for analysing my samples and learning me all there is to know about flow cytometry.

I most grateful to head and all members of Section of Immunology at the Norwegian Veterinary Institute for their help and for providing adequate facilities to complete my study.

I would like to thanks Tone Johansen, Ph.D. researcher in Norwegian Veterinary Institute, Section for Bacteriology, for her help to providing MAP culture and PCR results, also thanks to Kari Lybeck Ph.D. student for in Norwegian Veterinary Institute, Immunology Department, for her help for setting up intracellular cytokine staining flow cytometry.

I am grateful to my husband Adel Al-Jumaily for his support, encouragement and patients in the course of this study. In addition, I would like to thanks my family in my home country for their moral support and encouragement. Finally, to my smile in this life, my daughter Sema, I dedicate this work, God bless you.

Zainab Al-Touama

Abstract

Paratuberculosis is a chronic enteric disease that affects ruminants. It is caused by *Mycobacterium avium subsp. paratuberculosis* (MAP) an obligated intracellular acid-fast bacilli that usually infect macrophages in the intestine and lymph nodes and is able to survive and replicate inside phagosomes by inhibition of the phago-lysosome maturation. Paratuberculosis is characterized clinically by progressive emaciation and chronic diarrhea and causes major economic losses in many countries by reduced milk production, weight loss and death.

The protective immunity against paratuberculosis is incompletely defined. However, it is well known that Th1 CD4⁺ T cells have a central role by activating the infected macrophages by producing IFN- γ during the antigen recognition process through the MHC class II- TCR interaction. On the other hand, there is a very little knowledge about other subgroups of CD4⁺ T cells like Th2, Treg and Th17, which produce cytokine like IL-4, IL-10 and IL-17A, respectively and their role in the protective immunity against paratuberculosis.

The main aim of this thesis was to study the characteristics of reactive CD4⁺ T cells under MAP infection by optimising a method to isolate T cell lines and T cell clones from naturally infected goats. Different methods were tried to obtain a maximum growth of CD4⁺ T cells. Positive selection of CD4⁺ T cells isolated by MACS or Dynal magnetic beads increased the yield of cultivated CD4⁺ T cells and minimized the overgrowth of CD8⁺ and $\gamma\delta$ T cells in the cultures. Media supplemented with 10% goat serum (GS) was better for the growth of T cells than the same concentration of foetal calf serum (FCS) and was further used for the culture of goat T cells. PHA at 1 μ g/ml was used as a mitogen, and the CellTiter-Glo assay was used instead of ³H-thymidin incorporation assay as a read out for cell proliferation in T cell recall response tests (T cell proliferation assay).

Cytokine production was tested by different methods. A routine plasma ELISA method was used to measure IFN- γ production by PBMC after PPD-J stimulation of whole blood for 20 hours and confirmed the presence of MAP responsive T cells in the blood. Intracellular staining by flow cytometry demonstrated that both CD4⁺ and CD8⁺ T cells could secrete IFN- γ , and that the highest production after PPD-J stimulation was in CD4⁺ T cells. RT-PCR for mRNA encoding IL-10, IFN- γ and IL-17 was established and an increase of IFN- γ and IL-17 mRNA expression after PPD-J stimulation was noted in one T -cell line. No increase of IL-10 mRNA

was detected after PPD-J stimulation of T cell lines by either Q-RT-PCR or intracellular cytokine staining.

The ability to culture antigen specific T cells was demonstrated after immunization with MAP specific peptides. Five CD4⁺ T cell lines had responses to three peptide pools that contained 20 peptides in each. Furthermore, responses to 11 individual peptides were demonstrated.

In conclusion, we have established a method for cultivation of CD4⁺ T cells in vitro. This method can be used for detailed characterisation of both specificity and phenotype of this T cell in MAP infection.

Abbreviations

| | |
|------------------------|---|
| $2^{-\Delta\Delta Ct}$ | delta-delta Ct |
| APC | Antigen Presenting Cell |
| ATP | Adenosine Tri Phosphate |
| CD | Cluster of Differentiation |
| CD | Crohn's disease |
| cDNA | complementary DNA |
| CMI | Cell Mediated Immunity |
| ConA | Concanavalin A |
| CR | Complement Receptor |
| Ct value | Cycle threshold |
| DC | Dendritic Cell |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleotide Triphosphates |
| DTH | Delayed-type hypersensitivity |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FCS | Foetal Calf Serum |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| GS | Goat Serum |
| HrIL-15 | human recombinant IL-15 |
| IFN- γ | Interferon gamma |
| IL- | Interleukin |
| IMS-PCR | Immunomagnetic separation-PCR |
| IS900 | Insertion Sequence 900 |
| MAP | <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> |
| MHC | Major Histocompatibility Complex |
| Min | minute |
| moAbs | monoclonal antibodies |
| mRNA | messenger RNA |
| ND | Not determined, not done |

| | |
|-----------------------|--|
| NVI | Norwegian Veterinary Institute |
| OD | Optical Density |
| P | Peptide |
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phosphate Buffer Saline |
| PHA | Phytohaemagglutinin |
| PP | Peptide Pools |
| PPD-J | Purified Protein Derivative (Johnin) |
| Q-RT-PCR | Quantitative Real Time-Polymerase Chain Reaction |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| RT-PCR | Real Time-Polymerase Chain Reaction |
| TCR | T cell receptor |
| TGF- β | Transforming Growth Factor beta |
| Th cell | Helper T cell |
| TLR | Toll-like Receptor |
| TNF- α | Tumor Necrosis Factor alpha |
| Treg | Regulatory T cell |
| ZN | Ziehl-Neelsen |
| $\alpha\beta$ T cell | Cell has alpha-beta T cell receptor |
| $\gamma\delta$ T cell | Cell has gamma-delta T cell receptor |

1 Introduction

1.1 Taxonomy and characteristic of MAP

There are more than 130 species of mycobacteria. They belong to the order: *Actinomycetales*, family: *Mycobacteriaceae* and genus: *Mycobacterium*. The obligate pathogenic mycobacteria include the *Mycobacterium tuberculosis complex* and *M. leprae*. The other species of mycobacteria range from obligate or facultative pathogens to harmless environmental organisms collectively referred to as Mycobacteria Other Than Tuberculosis (MOTTs), nontuberculous mycobacteria (NTM) or atypical mycobacteria. One important species of the atypical mycobacteria are the *M. avium* complex. All mycobacteria are acid fast, which means that they are resistant to decolourization by acids during staining procedures. The most common technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain. The mycobacteria are nonmotile, aerobic or microaerophilic rods, they contain mycolic acid in their cell wall and their genomes have a high GC content of 59-66% GC⁽¹⁻³⁾. The cell wall is made up of a waxy mixture of lipids and polysaccharides^(4,5). The thick waxy cell wall does not only give the bacteria its properties of acid-fastness, but it also creates hydrophobicity and an increased resistance to low pH, high temperature, and different types of chemicals, and increase their lifespan and disease-causing potential in harsh and diverse environments^(6,7).

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an obligate pathogenic bacterium in the *M. avium* complex.^(8,9) Genetically MAP is 99 % related to the other *M. avium* subspecies, but has different phenotypic characteristics such as slower growth. MAP also requires the addition of an iron transport molecule called mycobactin when grown in vitro and it forms a rough colony when grown on a solid agar medium^(2,10). MAP causes a distinct disease called paratuberculosis mainly in ruminants. Paratuberculosis is a chronic inflammation of the intestinal tract. Other important subspecies of *M. avium* are *M. avium* subsp *avium* and *M. avium* subsp. *hominissuis*. *Mycobacterium avium* subsp *avium* causes tuberculosis in birds while *M. avium* subsp *hominissuis* causes granulomatous lesions in lymph nodes of pigs and humans. Disseminated disease and lung infection can be seen in immunocompromised individuals.

1.2 Routes of infection

MAP has ability to survive outside the host organism for a considerable period of time: in the river water for up to 270 days, in faeces and black soil for 11 months and in manure-water at 5°C for up to 252 days (¹⁰). Survival of MAP in the farm environment can be aided by its ability to be established and persist in biofilms on wet surfaces until intake by a suitable host (⁷). Also, the extremely hydrophobic cell wall structure of these organisms increases their ability to adhere to surfaces (¹¹)

Commonly animals are infected through the faecal–oral route by ingestion of contaminated milk or food products or by accidental ingestion of the organism from contaminated surfaces (¹²). MAP can be excreted in colostrum and milk (¹³), and MAP has been detected by PCR in raw goat milk (¹⁴). *In utero* infections occur in cattle (¹⁵) and have been reported in goats as well (¹⁶). Spreading of MAP between herds is often a result of trading animals with unknown infection status, but spread due to contaminated faeces on pastures also occurs (¹⁷). Considering the fact that paratuberculosis has been found in several wild animals including badger, fox, primates, rabbits, swine, and weasels (^{6,18}), it has been proposed that wild animals can be another way of transfer for the MAP bacteria between farms and herds.

1.3 Development of clinical disease

The incubation period is usually months or years; periods ranging from 4 months to 15 years have been reported (¹⁹). It is believed that young animals less than six months of age are more susceptible to infection (²⁰). Animals are usually infected shortly after birth, but rarely show clinical signs before they are two years old (¹⁹). Clinical signs include a decrease in milk production, diarrhea, rough hair or alopecia, sub-mandibular oedema (bottle jaw), wasting and weakness, followed by death after a course of several weeks to months. However, the annual mortality rate is usually low and may be less than 1% despite the fact that up to 50% of the animals in the herd may be infected. Furthermore, only approximately 10-15% of the infected animals develop clinical disease. In some animals, mastitis and infertility may occur as secondary complications (²¹⁻²³).

Infected animals can be classified into four groups according to clinical symptoms, faecal shedding of bacteria and immunological responses: (i) silent infection; (ii) subclinical disease; (iii) clinical disease; and (iv) advanced clinical disease (²³).

When ingested, MAP established itself in the lymphoid tissue of the small intestine (²⁴). In the stage of silent infection, there are no clinical signs. There is usually no detectable bacterial shedding or circulating antibodies, but evidence of a cellular immune responses may be seen (^{25,26}).

During the phase of subclinical disease, there are still no clinical signs of paratuberculosis.

However, the animals can shed low numbers of bacteria in stool and there may be signs of both cellular and humoral immune responses. Goats may start faecal shedding of MAP one year after infection and can be persistent faecal shedders for a long period without showing any clinical signs of paratuberculosis (²⁶⁻²⁸).

Throughout this long sub-clinical phase (estimated at 2-10 years) when the ruminant is apparently healthy, it is able to transfer the infection through the shedding of MAP intermittently in milk and faeces (²⁹).

During the third stage of infection, referred to as clinical disease, the only consistent symptom is the loss of weight despite apparently normal appetite (¹⁶). Cattle, may develop diarrhea while this is rarely seen in goats (³⁰). At this stage, bacteria are usually found in the stool, and animals usually have antibodies against MAP. Most animals, if not killed, enter stage four.

In advanced clinical disease, animals develop a scaly skin and bad hair coat, and finally progressive emaciation, dehydration, anemia with submandibular oedema and depression are seen. At this stage of the infection, diarrhea, or more generally a clumping of stool, can be seen (¹⁶). The inflammatory cells that respond to such infection cause thickness of the intestinal wall until it no longer functions, thus leading to malabsorption and protein-losing enteropathy. When the animals had reached an advanced clinical stage, they usually die within a few weeks (⁶).

Macroscopical lesions are mainly seen in the intestine and draining mesenteric lymph nodes. Intestinal lesions can be segmented or diffuse, and is found most often in the terminal ileum, but may occur throughout the length of the intestinal tract. Thickening and corrugation

of the intestinal mucosa with transverse folds, and dilated and thickened serosal and mesenteric lymphatic vessels (lymphadenopathy) are common (^{25,31}). Intestinal mucosa is often redness, show crevices, and has a granular appearance. The mesenteric lymph nodes are pale, swollen and oedematous. In goats, nodular foci of caseous necrosis and calcification may be present both in the mucosa and in the lymphnodes (^{32,33}). Morin, M. (1982), noted caseous necrosis of Peyer's patches with ulceration of the overlying mucosa in the ileum of two from eight goats on his study (³²).

The histopathological lesions caused by MAP in goats were characterized by accumulations of macrophages in the intestinal mucosa. These macrophages had a prominent, foamy or vacuolated cytoplasm and many of them were packed with acid-fast bacilli. According to the clinical signs, the histopathological lesion can be described as:

Mild lesions were characterized by focal collections of macrophages and lymphoid cells in the upper parts of the lamina propria of the intestinal villi, with few or no acid-fast bacilli. This form is associated with strong cell-mediated immune responses.

In the more severe lesions, macrophages were infiltrated in the deeper zones of the lamina propria and finally, the whole lamina propria was invaded by massive numbers of macrophages which compressed the crypts of Lieberkuhn; in these areas, the villi were severely atrophic and blunted. The macrophages containing large numbers of acid-fast bacilli. This form is associated with strong humoral immune responses (^{25,32}).

In sheep carcasses; emaciation, oedema, ascites and hydropericardium, in addition to atrophy and necrosis of fat tissue, were also noted (³¹).

1.4 Possible role in Crohn's disease

Crohn's disease (CD), also known as regional enteritis is a kind of inflammatory bowel disease which may affect any part of the digestive tract from the mouth to the anus, but significantly affect the lower part of the small intestine, called the ileum. It causes a granulomatous inflammation of the intestine which anatomically resembles granuloma that occurs in intestinal tuberculosis. The aetiology of CD is unknown, but it is thought to be a dysregulated immune response to intestinal bacteria. However, there are contradictory data about the involvement of different organisms in CD. Several infectious agents, including MAP, adherent invasive *E. coli*, *Yersinia*, and *Pseudomonas* have been suggested as triggering

agents of CD (³⁴⁻³⁷). Because of the pathological similarities between paratuberculosis and CD, MAP has been the most durable infectious candidate to be proposed as a causative agent of CD (^{38,39}). In 1998 the British Medical Journal published a paper describing the case of a little boy who developed *M. paratuberculosis* infection in the lymph nodes of his neck, and after an incubation period for five years, developed disease in the bowel that was indistinguishable from CD (⁴⁰).

Humans can be exposed to MAP through many sources. Animals infected with MAP can excrete the live bacteria in both faeces and milk. Recent studies have shown that MAP present in milk can survive pasteurization, which has raised concerns about human health due to the nature of the spread of MAP in modern dairy herds. It is resistant to heat and is able to isolate itself inside white blood cells, which may contribute to its persistence in milk. It has also been reported to survive chlorination in municipal water supplies (^{29,41}). Infected animals can contaminate their surrounding environment, increasing the risk of spread of paratuberculosis at the farm level and potentially polluting of waterways used for the extraction of drinking water.

If MAP is involved in CD development, shedding of MAP by infected animals has implications for food and water safety, as illustrated in figure 1-1(⁴²). Consideration should thus be given to the animals with paratuberculosis and the risk of contamination of food and water sources until the situation is clarified (⁴³).

MAP infections, like most of the mycobacteria, are difficult to treat. It is resistant to antituberculosis drugs (which use generally to treat infection with *Mycobacterium tuberculosis*), but can only be treated with a combination of antibiotic such as rifabutin and a macrolide such as clarithromycin. Treatment regimens can last years (^{29,44})

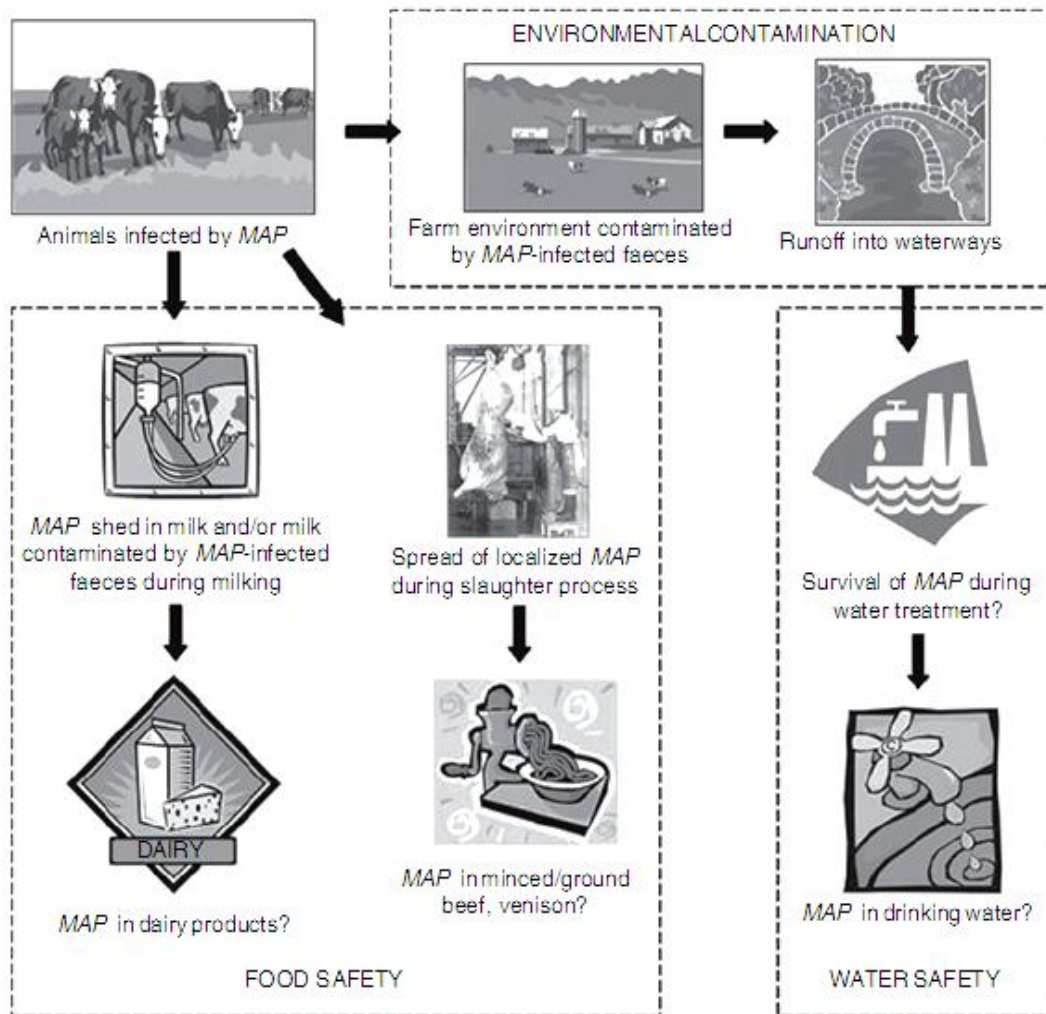


Figure 1.1: Spread of MAP shed by infected animals in faeces and milk, and potential routes of human exposure to MAP via animal-derived foods and water (use with permission from the publisher (CAB International, Wallingford, UK).

1.5 Diagnostic assays

The diagnosis of MAP infection is difficult, especially in the early stages of disease. This is due to the long incubation period, the variable lag phase associated with bacterial proliferation, and the slow progression of the lesions (²¹). In the early stages of the disease, it is thus impossible to identify all infected individuals. Therefore testing usually is performed on a herd level. In a herd, there will be animals at different stages of the disease and the likelihood to identify correctly an infected herd is quite high. The optimal testing strategy will include a combination of diagnostic tests (^{45,46}). Unfortunately, all forms of testing requires a lot of time and costs (⁴⁷) and often an optimal testing strategy is not feasible.

Diagnostic tests can be divided into two categories; test for the organism or tests measuring the animal's response to the infection.

1.5.1 Organism-based tests

There are two types of these assays; either culture of the living organism from manure, tissue or environmental samples, or PCR that amplifies the MAP genetic material from live or dead MAP.

MAP cultivation is a highly specific method, but it is expensive and requires approximately 8-16 weeks of incubation to produce visible colonies on solid media⁽²⁵⁾. If the sample is heavily contaminated with MAP a positive result can sometimes be seen within a few weeks, however a negative results requires at least 4 months. Some strains are also extremely difficult or impossible to culture in the laboratory.

Different methods have been used to detect MAP by PCR based on IS900 or other MAP specific genes from different samples of infected animals. The PCR can be performed directly on the samples. The sensitivity of this direct PCR is sometimes low due to low number of bacteria or the presence of PCR inhibitors in faeces. Different methods have thus been established to increase the sensitivity. One such method is immunomagnetic separation-PCR (IMS-PCR)⁽⁴⁸⁾. In this method, magnetic beads are coated with specific antibodies and incubated with samples to allow extraction of the bacteria from the samples. PCR is much quicker than cultivation and most of the laboratories provide a result in less than a week⁽⁴⁹⁾.

PCR is also performed on paraffin blocks when the tissue is collected at autopsy. The pathologist is looking for MAP specific lesions and for acid fast bacteria⁽⁵⁰⁾. PCR on this section can then be used to identify the bacterial species. Finally, most laboratories also use a PCR to confirm that the organism isolated in culture is in fact MAP.

1.5.2 Immunological methods

A paradigm has been that the infected animal elicit a strong cell-mediated immune response in the early stages of the infection and a strong humoral immune response in the later stages^(31,51). This paradigm has turned out to be too simplistic. It is clear, however, that different immunological assays have their advantage at different stages of the infection.

The detection of a systemic cell-mediated immune response generally comes before the detection of antibody production. Animals that have a silent infection are often not able to respond on serological tests but can react positively to tests that measure cell mediated immunity (⁵²). The Cell Mediated Immunity (CMI)-based methods like cutaneous testing for delayed-type hypersensitivity (DTH with johnin PPD), gamma-interferon assay and lymphocyte stimulation test thus have the highest sensitivity in animals with silent or sub-clinical infection (^{27,53,54}). However, the specificity of these tests is variable. They are also expensive, and the in vitro-tests require live cells, and can thus be a challenge when testing animals from remote areas.

Antibody based tests look for antibody produced by an infected animal. There are three common types of blood test: include complement-fixation test, the agar gel immunodiffusion (AGID) test and enzyme-linked immunosorbent assay (ELISA). The latter is sensitive in clinical infection but performs poorly in subclinical infection (⁵⁵⁻⁵⁷). A number of ELISA kits have been approved for use in milk from individual cows (not bulk tank) as well as blood samples.

Due to the biology of MAP infection, it is usually only possible to identify adult animals infected with MAP. This means that even though calves, kids, lambs etc. are infected while very young, they do not shed the organism with any frequency (so the organism detection assays will be negative) and they do not produce antibody (so the blood/milk tests will be negative). This is why it is recommended that diagnostic tests are used only for the animals of at least 18 months of age (^{58,59}).

1.6 Control

Paratuberculosis is endemic among the goats in 6 of 19 counties in Norway. Infection in sheep and cattle can be attributed either to import from countries with endemic disease or close contact with infected goats. Importation of live cattle in Norway has been very limited from around 1996 and has largely been replaced with imported semen and embryos. However the presence of infected flocks of goats constitute a risk for spreading infection to other ruminants (⁶⁰). Infection has been found to readily spread between and across species with no restrictions, making the disease hard to control. Movement of animals between herds through trading is probably the most common way that infection spread from farm to farm. However, wild animals are also proposed to be able to spread the disease between farms or herds (⁶).

The economic losses due to paratuberculosis are considerable, due to death of clinical cases and subclinical effects like reduced weight gain, milk yield and fertility. Therefore, control programs have been established in several countries. The programs vary from country to country, and their designs are based heavily on the species of animals, prevalence of the disease and the aim of the program (eradication or control).

Control programs are invariably expensive and in many countries vaccination, may be the only affordable alternative at this time. Different types of vaccines have been tried and used against paratuberculosis. For example; live (non-attenuated and attenuated) and killed whole cell (⁶¹), subunit vaccines consisting of sonicated bacteria, bacterial cell fractions or recombinant MAP antigens (^{62,63}), and recently, DNA vaccines, consisting of the inoculation of mammalian expression vectors containing MAP genes (^{64,65}).

Many studies have discussed the advantages and disadvantages of paratuberculosis vaccines. The advantages were: The number of animals with clinical symptoms was decreased in cattle, sheep and goats; faecal shedding was reduced, as well as the number and severity of bacteriological isolations and histological lesions in the intestines. On the other hand, there are also disadvantages of vaccination. The existing vaccines can delay the onset of clinical symptoms but do not protect against infection. This makes it difficult to identify infected animals. The vaccinated animals develop antibodies that interfere with existing serodiagnostic tests for paratuberculosis, and they become reactive in the tuberculin skin test, used for the control of bovine tuberculosis (⁶⁶). Kalis, C.H. *et al* (2001), found that the killed vaccine in cattle does not reduce faecal shedding or MAP transmission and the improvement of management procedures is more effective (⁶⁷). Other studies have shown that changing in the management and hygiene practices and good sanitation are important (⁶⁸).

In summary, the perfect vaccine has to have the following qualities: it causes minimal tissue reaction; there is no interference with diagnostics test for tuberculosis and paratuberculosis; it can discriminate between infected and vaccinated animals; it eliminates faecal shedding of bacteria; and prevents the occurrence of clinical disease (⁶⁹).

1.7 Pathogenesis

Ingested MAP is taken up through the wall of the intestinal tract through “M” cells in the Peyer’s patches and establish residence within the local macrophages in that region (^{21,70}). By complex and not completely understood mechanisms, MAP affects cellular signalling and other bacteria-fighting mechanisms of the macrophages and creates a hospitable environment for themselves within these cells. They replicate slowly and may stimulate inflammatory and immunological responses (^{29,33,71}). These microscopic infection of macrophages in the small intestine can continue for years without provoking any detectable reaction from the immune system of the animal i.e., Infected animals are not sick and do not react to the infection in any measurable way (²⁹).

While some exposed individuals may develop resistance to chronic infection, many infected goats subsequently carry the infection in a dormant state in the Peyer’s patches of the intestine and the mesenteric lymph nodes for a variable period into adulthood. At some point, triggered by stress or other ill-defined factors, some infected animals begin to shed the organism in the faeces (⁷²). Clinical symptoms of paratuberculosis usually begin to appear at this point. More macrophages are recruited to the site of infection and an ill-defined type of granuloma is formed constituting an aggregate of living, dying and dead MAP and macrophages. The lesions progress and regress, but remains a localized battle in the gastrointestinal tract and draining lymph nodes. As more MAP enter and replicate in the macrophages however, and as more cells are recruited to fight them, the lesion expands. This granulomatous inflammation spreads, and the infected macrophage may then depart the gastrointestinal tract for the neighbouring lymph nodes, and be spread through the blood to other organ systems (²⁹).

1.8 Immune response

The study of host immune responses to MAP is complicated by several factors, including long-term nature of the disease, the insidious nature of the organism and the size of the infective dose and the immunity of the host.

The major route of infection in ruminates are via ingestion of contaminated food or water with MAP organism. MAP uptake by M cells may be mediated by number of microbial pattern recognition receptors like B1 integrin (⁷³), Toll-like receptor-4 (TLR-4) and platelet

activating factor receptor (⁷⁴). The local intestinal macrophages are the target cells for MAP infection, which phagocyte the organism and have several processes to degrade phagocytosed material. The activation of macrophages upon phagocytosis of MAP is dependent upon the interaction of MAP with surface receptors, including TLRs, which are important for initiation the adaptive immune response (⁷⁵), regulation of bactericidal agents like reactive nitrogen and oxygen species and lysosomal peptides (⁷⁶).

Taylor, D.L. *et al* (2008), concluded that six TLRs were expressed in ileal and jejunal tissues and their associated lymph nodes from naturally infected sheep (TLR1, TLR2, TLR3, TLR4, TLR5, TLR8) (⁷⁷). Ferweda, G. *et al* (2007), suggested that TLR2 has the greatest impact on cellular activation and subsequent induction of cytokines (⁷⁸). Other receptors on macrophages that have potential modes of entry of MAP into macrophages are; complement receptors (CR), CR1, CR3 and CR4; mannose receptors, Fc receptors and CD14 (⁷⁹⁻⁸¹).

However, as an intracellular organism, MAP has ability to survive in the macrophage. Mycobacterial survival is enhanced by the bacteria's ability to prevent acidification of phagosomes and inhibiting the maturation of the phagosome (⁴³). The lipid rich layer of MAP may play an important role in resistant to intracellular killing mechanism (⁸²). Once the infected macrophage activated by MAP, they secreted numbers of cytokines such as tumor necrosis factor- α (TNF- α), IL-1, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (⁸³), which have an autocrine bactericidal effect or may be stimulated the production of other pro-inflammatory cytokines like IL-6, IL-8 and IL-10, that usually generated due to response to mycobacterial lipoarabinomannan (LAM), peptidoglycans or heat shock proteins (⁸⁴).

The innate immune system can sometimes control the infection; however, usually an induction of the adaptive immune response occurs. The induction of the adaptive immune response happens when macrophage and dendritic cells (DC) take up the bacteria and present antigens to the naïve T cells. There are two major groups of T cells carrying the alpha/beta T cell receptor on the surface. The CD8+ T cells recognize antigen presented on MHC class I, while the CD4+ T cells recognize antigen presented on MHC class II. The CD4+ T cells are of major importance for protection against mycobacterial infections including paratuberculosis. The CD4+ T cells, also called T helper (Th) cells, can develop into different subtypes dependent on the cytokine environment.

Studies over several years have shown that the effective resistance to progressive infection and disease due to MAP is associated with a cell-mediated rather than humoral immunity, and local rather than systemic responses are believed to be most important in the early stage of infection (³¹). In the early, subclinical infection stage, activated CD4+ T helper cell biased towards a Th1 response is dominating. This response is characterized by production of cytokines like gamma interferon (IFN- γ), IL-2 and tumor necrosis factor alpha (TNF- α). These cytokines are assumed to orchestrate the cell-mediated immune functions, which are crucial for containing such intracellular infections. Especially IFN- γ plays an important role in mycobacterial infections in the activation of T lymphocytes and macrophages, dendritic cell (DC) maturation, upregulation of MHC class I and II molecules and production of reactive oxygen and nitrogen species by macrophages (^{85,86}).

IFN- γ also induces the secretion of IL-12 by antigen presenting cells (APCs), which results in Th1 induction through a paracrine pathway, and it also works to directly complement the Th1 polarization through an autocrine mechanism that does not involve IL-12 (⁸⁷). It appears that CD4+ T cell (Th1) is the primary source of IFN- γ in the early stages of paratuberculosis infection, however CD8+ T cell and $\gamma\delta$ T cell can also produce IFN- γ (⁸⁸). The amounts of IFN- γ secreted from $\gamma\delta$ T cell in response to MAP Ag is lower than the amounts that is secreted from CD4+T cells and CD8+T cells (^{89,90}).

Another subgroup of CD4+ T cells are the Th17 cells. These cells produce IL-17 in response to IL-23 secreted from activated macrophages. IL-17 enhances inflammation by stimulating production of other proinflammatory cytokines, like IL-1, IL-6 and TNF- α . They also produce chemokines that mediate recruitment of macrophages and neutrophils to the site of infection, and these cells are involved in forming organized granulomatous lesions (^{31,91}). Although IL-23 and IL-17 seem to play roles throughout mycobacterial infection, the most crucial period, seems to be in the control of inflammation in the more advanced stage of tuberculosis (⁹²). The role of Th17 cells in MAP infection is not known, but higher gene expression of IL-17 have been seen in animals with severe disease compared to individuals with minimal or moderate disease (^{93,94}).

When the animal begin to exhibit the non-specific, clinical signs of paratuberculosis, such as weight loss, and in some species diarrhea, a switch from Th1 to Th2 immune responses is seen in many, but not all animals (^{91,95}). This stage is characterized by secretion of Th2 cytokines like IL-4 and IL-5 that promote B cell differentiation and antibody secretion.

The balance between Th1-Th2 is influenced by IL-4 and IL-10 tending to suppress Th1 responses, and by IFN- γ that act against Th2 responses (⁹⁶). Karcher, E.L. *etal* (2008), reported that IL-4 secretion by PBMC that were stimulated with whole cell sonicate of MAP, was higher in clinically infected cows compared to subclinically infected cows, which is fitting the paradigm of a shift to Th2 mediated immunity in clinical disease (⁹⁷).

As well as a Th1-Th2 shift during the late stage of MAP infection, another subpopulation of T cell known as T regulatory (Treg) cells further modulates host immune response to mycobacterial antigens. These Treg cells are responsible for controlling immune responses during infection via secretion of the immune suppressive cytokines, IL-10 and transforming growth factor- β (TGF- β) (⁹¹). An upregulation of IL-10 has been observed in ileum, mesenteric lymph nodes and cultured PBMC, from naturally infected cattle and sheep with paratuberculosis (^{98,99}). Another study showed that addition of exogenous IL-10 to bovine cells cultures before infection with live MAP reduced the IFN- γ secretion (¹⁰⁰). TGF- β also plays a regulatory role in host immunity through inhibition of T cell activation and proliferation which is associated with decreased IFN- γ production (¹⁰¹). However, the role of Treg cells in paratuberculosis remains unclear.

Animals that fail to control the disease will often develop a humoral immune response concurrent with increased shedding of bacteria in the stool, possibly followed by the onset of clinical symptoms (³³). Humoral responses in paratuberculosis seem to indicate an inverse correlation with cell-mediated immunity (CMI) in cattle and sheep. The role of B cells in mycobacterial infection is not well understood. However Begara-McGorum, I. *etal* (1998), found that the numbers of B cells in the mesenteric lymph nodes of lambs experimentally infected with MAP were significantly reduced (¹⁰²).

In contrast, the proportion of B cells in PBMC fraction isolated from cattle naturally infected with MAP was significantly higher for animals with clinical signs of disease comparing to control cows or subclinical infected cows (¹⁰³). As well as to develop into antibody secreting plasma cells, B cells act as APC and may play a role in the activation of Th2 cells (⁹¹). A recent report also documented a regulatory role for B cells in chronic inflammatory pathogenesis, which is mediated through IL-10 secretion (¹⁰⁴).

Often both humoral and cell mediated immune responses can be detected at the same time in peripheral blood of an infected animals reflecting that local immune responses in the

various foci may differ. It has previously been reported that infected animals are able to completely recover from paratuberculosis infection, but experimental infections in goats indicate that reactivation of bacteria in small foci is possible (²⁷). What drives this reactivation or what makes an infection developing to clinical disease in a proportion of animals, is still unclear. In some aspects, MAP can be regarded as an opportunistic pathogen since the majority of animals are indeed able to control the infection. It thus seems obvious that the variations in the immune response of the host are more important than the infectivity of the MAP bacilli.

It is thus of major importance to gain more knowledge about the immune response in paratuberculosis.

Aim of the study

To isolate and characterize *Mycobacterium avium* subspecies *paratuberculosis* reactive T cells.

The sub goals are

- 1) To optimization a method for T-cell cultivation in vitro
- 2) To characterise the phenotype of MAP reactive T cells
- 3) To perform initial screening for antigen specificity of the T cell lines
- 4) To isolate CD4+ T cell clones from PPD-J reactive T cell lines

The project is linked to an EU funded project: Aiming to develop a new vaccine against MAP infection.

2 Materials and Methods

2.1 Animals and standard diagnostic testing

2.1.1 Animals

The present study included four female goats, 4 years of age from Norwegian dairy goat herd in Etne i Hordaland that was naturally infected with MAP and tested positive in the Bovine IFN- γ EASIA kit (see below) test before arrival and housing at the Norwegian Veterinary Institute (NVI) in February 2011. One of the goats was pregnant and delivered twins three months after arrival at the institute.

After 6 months at the NVI the goats were immunized intramuscularly twice (4 weeks apart) with 118 MAP specific peptides in the CAF04 adjuvant (Statens Serum Institute, Copenhagen, Denmark). The peptides were designed as part of the ParaTBvaccine project and the sequences and design is not a part of this thesis. There were two aims of the vaccination. The first was to identify immunogenic peptides, while the second was to see if we could cultivate peptide specific T cells in vitro. The vaccination was approved by the Norwegian Animal Research Authority.

2.1.2 Detection of MAP

Five samples for bacteriological culture were obtained from the stool of these 4 goats to control shedding of MAP bacteria (20 samples in total). The samples were analysed by real time PCR (RT-PCR) and culture for MAP. Culturing was done according to standard procedures at the section for bacteriology at NVI, following the internal protocol (ME 0039 ‘‘*Mycobacterium avium* subsp. *paratuberculosis* påvisning’’’) (Tone Johansen, personal communication, 2012). MAP was cultured on selective and non-selective Dubos medium (¹⁰⁵). Briefly, approximately one gram of stool from each goat were homogenized, decontaminated by 4% sodium hydroxide and 5 % oxalic acid with 0.1% malachite green, centrifuged and redissolved in saline water. The samples were inoculated on the different Dubos media with mycobactin (2 $\mu\text{g/ml}$) (Allied Monitor, MO, USA) and pyruvate (4 mg/ml) and incubated at 37°C for approximately 16 weeks.

Bacterial colonies with typical morphology were confirmed as MAP by positive result on Ziehl-Neelsen staining, testing for mycobactin dependence and by detection of the MAP specific IS element IS900 by RT-PCR. (¹⁰⁶).

Real time PCR was performed on faecal samples using Adiavet[®]Paratb real time kit (Adiagène, Saint-Brieuc, France), detecting IS900. The company's new protocol for DNA isolation, where 3-10 g of stool can be analysed was used, increasing the sensitivity of the method.

Briefly, $3 \pm 0,2$ g of stool was suspended in 20 ml sterile distilled water using a stomacher and left overnight for rehydration as recommended by Adiagène. The supernatant was filtrated using chemfilter (Adiafilter, Adiagène) for removing of PCR inhibiting substances, the pellet redissolved and the bacterial cells mechanically disrupted by bead-beating. DNA was extracted with the QIAamp[®] DNA Mini Kit (Qiagen, Hilen, Germany) as recommended by the manufacturer. RT-PCR was performed using Adiavet[®]Paratb real time kit (Adiagène) as recommended with the EPC-Extraction as an internal control for each sample. The PARA positive control that was included with the kit was run for quality control of each assay, and Milli-Q water was run as negative control. RT-PCR was performed using Stratagene Mx3005P (Stratagene, La Jolla, CA, USA) (¹⁰⁷).

2.1.3 IFN- γ assay

One whole-blood sample was collected from vena jugular by the evacuated blood collection tubes (5 ml) with lithium heparin as anticoagulant (Terumo Europe N.V., Leuven, Belgium) from each animal and tested for the presence of IFN- γ production in response to MAP proteins by using the Bovine IFN- γ EASIA kit, manufactured by the (BioSource Europe S.A., Nivelles, Belgium), which is based on Competitive ELISA technique and used to semi-quantify protein levels of IFN- γ in plasma and supernatant.

The reference materials were represented by positive and negative control sera supplied by the manufacturer. Briefly, 1 ml whole-blood was incubated per well in 24-well plate (Corning, NY, USA) with 10 μ g/ml Purified Protein Derivative (Johnin) PPD-J (Norwegian Veterinary Institute (NVI), Oslo, Norway) for 24 hour at 37°C in humidified air with 5% CO₂. In addition, wells with no antigen added were set up for each animal as control wells. The next day plasma was collected and stored at -70°C. Testing was performed by

adding 100µl in duplicate to 96-well ELISA plate precoated with an anti-bovine IFN-γ monoclonal antibody (moAb) in 50 µl of incubation buffer. After 1 hour incubation at room temperature on a horizontal shaker, plate was washed three times and incubated with horseradish peroxidase anti-bovine IFN-γ monoclonal antibody conjugate (HRP) solution; the plate was incubated with shaking for another hour. Colour development was achieved by adding Chromogen tetramethylbenzidine (TMB) solution and was stopped after 15 min of incubation. Plate was read at 450 nm. The results are given as OD in the PPD-J stimulated well – OD in the non-stimulated well. A difference above 0.3 is considered positive.

2.2 Cultivation of T cells

2.2.1 Isolation of peripheral blood mononuclear cells (PBMC)

Blood was collected from the jugular vein into evacuated blood collection tubes (9ml)/EDTA as anticoagulant (Terumo), and PBMCs were isolated with Lymphoprep (Axis-Shield PoC, Oslo, Norway) by gradient centrifugation as previously described (¹⁰⁸). Equal volumes of blood were mixed with phosphate-buffered saline (PBS) with 2mM EDTA. 10 ml lymphoprep was pipetted underneath 35 ml blood-PBS and centrifuged at 1800xg for 30 min at room temperature, no brake. PBMCs were collected from the interface and washed twice. The PBMC pellet was resuspended in RPMI 1640 glutamax (GIBCO, Invitrogen, NY, USA) supplemented with gentamycin 50µg/ml (GIBCO), funigzone 1µg/ml as final concentration and 10% fetal calve serum (FCS) (PAA-gold, Austria) or 10 % goat serum (GS) (NVI) preheated to 37°C. The cells numbers were determined by the use of hemocytometer chamber (Bürker, Germany).

2.2.2 T cell cultivation

2.2.2.1 Antigens

PPD-J was made from MAP strain 2E at NVI and had a stock concentration of 1mg/ml. Synthetic peptides were kindly provided from Statens Serum Institute, Copenhagen, Denmark.

2.2.2.2 Cultivation of goat T cell lines:

The protocol for cultivating T cell lines from goat is a modified version of an established protocol for cultivation of human T cell lines from human blood or small intestine biopsies from patients with Crohn's disease (¹⁰⁹). Briefly, blood collection, PBMC isolation and cell suspension was done as described in 2.2.1. The PBMC cell suspension was adjusted to $1.6-2 \times 10^6$ cells/ml. PPD-J 5-10 μ g/ml was added to the cell suspension to stimulate PPD-J recognizing T cells and to increase the yield of MAP reactive T cells. Ovine IL-2 (1:200) (Norwegian School of Veterinary Science (NVH), Oslo, Norway) and Human recombinant (Hr) IL-15 1 ng/ml (eBioscience) were added to cell suspension, to stimulate T cell growth. MEM non-essential amino acid 100x (Gibco, Invitrogen, UK) and 2-Mercaptoethanol (Gibco) at 50 μ M/ml were added to T cell culture media when we started to use dynabeads, see below.

One hundred twenty five of the cell suspension was added into 8 wells on a 96 well U-bottom plate. The plate was incubated at 37°C/ 5% CO₂ for 4 days and checked for cell growth under microscope. At day, four or five the cells were fed with RPMI 1640 media supplemented with 10% FCS or 10 % GS and cytokines ovine IL-2 and Hr IL-15. If good growth in the wells, the cells were suspended and split 1:2.

2.2.2.3. Restimulation of Expanding T cell lines

After one week, the cultivated T cell lines were restimulated to increase the number of reactive cells for later testing. Briefly, PBMCs from another individual was used as feeder cells to stimulate T cell growth.

Isolated PBMC feeder cells were treated with Mitomycin C (10 μ g/ml) (Sigma-Aldich,) for 1.5-2 hours to inhibit further proliferation. The treated cells were washed 3 times with PBS, centrifuged at 500 x g for 5min at 20°C and further resuspended in RPMI 1640 media supplemented with 50 μ g/ml gentamycin and 10% FCS or GS preheated to 37°C. The cells were adjusted to (1.0-1.3 x 10⁶ cell/ml). Phytohaemagglutinin 1 μ g/ml (PHA16, Remel, Lenexa, KS, USA), 1 ng/ml HrIL-15 and 1:200 ovine IL-2 were added to the feeder mix.

Five hundred microliters of feeder mix was added into 4 wells of 48 well plate and approximately two wells of the 96 well U- bottom plate were added into 1 well on a 48 well plate. The plates were incubated at 37°C/ 5% CO₂ for 4 days and checked for cell growth under the microscope. At day, four or five the cells were fed with RPMI 1640 media

supplemented with gentamycine 50µg/ml, 10% FCS or 10 % GS and cytokines ovine IL-2 and Hr IL-15. Usually 16 wells on the 96 wells plate were transferred to 8 wells on the 48 wells plate. These eight wells were from now on treated and labelled as four separate T cell lines. If good growth in the wells, the cells were suspended and split 1:2 or 3 into a neighbouring empty wells. After 2 weeks of expansion the T cells lines were collected and prepared for freezing down. The harvested cell lines were tested by T cell proliferation assay after antigen stimulation and flow cytometry.

2.2.3 Negative and positive selection of CD4+ T cells

2.2.3.1. Negative selection of CD4+ T cell (Depleting $\gamma\delta$ T cell)

PBMC was prepared as described earlier (2.2.1). $\gamma\delta$ T cells were depleted from PBMC, by using MACS anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA USA). Briefly, 10×10^6 cells suspension was incubated with GB21A (mouse anti-bovine $\gamma\delta$ TCR mAb, VMRD, Pullman, WA, USA) ($1 \mu\text{g Abs}/ 10^6$ cell suspension) in PBS w/ 2 mM EDTA for 30 min on ice. After washing with PBS w/ 2 mM EDTA and 0.5% BSA, the cell pellet was resuspended to 1×10^6 cells and 20 µl of IgG-microbeads (MACS anti-mouse IgG microbeads, Miltenyi Biotec) were added and incubated for 10-15 min at 4°C with gentle shaking. The cells were thoroughly washed and loaded on the MACS separation columns (LD-columns for depleting, Miltenyi Biotec). Labelled cells were retained on the column within a magnetic field. Unattached cells were washed through, collected and resuspended in RPMI 1640 media supplemented with gentamycin 50 µg/ml and 10 % FCS into 1.2×10^6 cell/ml. The T cell cultures were stimulated, and incubated as described earlier in 2.2.2.2.

2.2.3.2 Positive selection of CD4+ T cell

Two methods were tested for positive selection of CD4+ T cells

2.2.3.2.1 Miltenyi MACS beads positive selection of CD4+ T cells

The first method used the Miltenyi MACS bead cell selection kit. Briefly, 10×10^6 cell/ml were incubated with GC1A (mouse anti-caprine CD4+ mAb, VMRD) ($1 \mu\text{g Abs}/ 10^6$ cell suspension), after incubation for 30 min on ice, the cells were washed and resuspended to 1×10^6 cells and 20 µl of IgG-microbeads (MACS) were added and incubated for 10 min at 4°C with gentle shaking. The cells were thoroughly washed and loaded on the MACS separation

LS-columns for positive selection (Miltenyi Biotec). Labelled cells were retained on the column in the magnetic field. The column was removed from the magnetic field, and the positively selected cells were eluted by passing PBS w/ 2 mM EDTA and 0.5% BSA thorough the column. The selected cells were washed and resuspended to 1.2×10^6 cell / ml and mixed with PBMC feeders as described earlier (2.2.2.3), antigens (PPD-J and/or MAP peptides) were added to the feeder mix. T cells were seeded out in 96 well U bottom plates as described earlier (2.2.2.2)

2.2.3.2.2 Dynal Dynabeads positive selection of CD4+ T cells

The second method tested for CD4+ T cell positive selection was using Dynabeads conjugated with Pan Mouse Ig (Dynal, Oslo, Norway). Briefly, PBMC were isolated (2.2.1), the cells were washed twice and incubated with GC1A (VMRD) ($1 \mu\text{g Abs/ } 10^6\text{-cell suspension}$) for 30 min on ice. After incubation, the cells were washed and 4×10^6 Dynabeads (Dynal) were added pr 1 ml of cell suspension and incubated for 30 min at 4°C with gentle shacking. Isolated CD4+ T cells (cells attached to the beads) with a magnetic particle concentrator (Dynal) for 15 ml tubes. The selected cells were washed and resuspended as described in the previous method.

2.2.4 T cell proliferation assay

T-cell activation assay measures the ability of T lymphocytes to proliferate in vitro as a recall response to earlier encountered antigen.

The protocol previously described in ⁽¹⁰⁹⁾ with some modifications. Autologus PBMC from infected goats were prepared and resuspended to 1.0×10^6 cell/ml and seeded into 96 wells flat plate and incubated for 1.5-2 hours for adherent cells to stick to the plastic.

The plate was washed 2-3 times with RPMI 1640 media supplemented with 2% FCS to wash away non adherent APCs. The antigens or mitogens were diluted by RMPI 1640 media supplemented with 10% FCS and added to the adherent APCs in triplicates and incubated overnight at $37^\circ\text{C/ } 5\% \text{ CO}_2$.

Next day, 1.0×10^6 cells/ml T cells (Approx 70000 cells/well) were added to the stimulated APCs and incubated for 72 hours before addition of 50 μl CellTiter-Glo® Reagent (Promega, Madison, USA) that use adenosine triphosphate (ATP) in a luminescence reaction

producing a stable luminescent signal via luciferase catalyzed luciferin+ATP reaction. The light signal is measured (i.e. luminescent signal) by a Wallac 1420 /Victor 2 multiwell scanning luminometer (Perkin Elmer, Wallac oy, Turku, Finland). Amount of ATP is linear correlated to the number of cells, thus it is possible to give an estimate of the cell proliferation. The data were expressed as percentage proliferative change of stimulated cells versus the control that consisted of unstimulated cells.

Alternatively the plate is incubated for 48 hours and 20 µl of ³H-thymidine (0.5µCi/well) (Hartman analytic, Germany) was added and incubated another 24 hours for incorporation of ³H-thymidine into the cell DNA. Thus, the proliferation and cell activation can be measured as the amount of radioactive labelled DNA by using a scintillation beta-counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter and Luminometer, Perkin Elmer). The results were expressed as a stimulation index (SI = mean optical values of cell stimulated cultures/ mean optical values of unstimulated cell culture (control)).

2.2.5 Freezing and thawing of cells

2.2.5.1. Thawing of T-cells

The cryovial containing cells was thawed in water bath at 37°C. Just before the last of the ice had melted, the content of the vial was transferred to the 50 ml tube. Eight ml RMPI 1640 media supplemented with 20% FCS preheated at 37°C was added drop wise, mixed carefully and spun down at 500xg for 7min at room temperature. The supernatant was poured off and the cell pellet was resuspended in preheated RMPI 1640 media supplemented with 10% FCS for further use.

2.2.5.2. Freezing T cell

Prior to freezing, 1.8-ml cryovials (Nunc, Denmark) were labelled and keep at 4°C in a freezing rack (Stratagene). The cell suspension was spun down and resuspended with half volume of total freezing volume with pre-cooled 50% FCS in RMPI 1640 media on ice. Then the last half of total freeze volume was added by dropwise adding RPMI 1640 media (60%) supplemented with 20% FCS and 20% dimethylsulfoxide (DMSO) (Sigma, MO, USA). In total 1 ml of cell suspension was added pr vial (5×10^6 cell/1ml in each vial). The cell

suspension was added immediately to the prechilled cryovials and stored in a -80°C freezer. Long term storage of cell vials was provided in a liquid N2 tank.

2.3 Flow cytometry

The monoclonal antibodies (moAbs) used in the present study are shown in table 2-1.

Table 2-1: Monoclonal antibodies used in single and triple colour flow cytometry

| moAbs | Final concentration | Specificity | Source | ****Ig isotype/fluorochrome | Source | Final concentration | Method used |
|-----------|---------------------|-------------------------|-------------------------|-----------------------------|--|---------------------|-------------|
| GC1A* | 5 µg/ml | CD4 | VMRD, Pullman, WA, USA | IgG2a / PE | Southern Biotechnology associates, Birmingham, AL, USA | 2µg/ml | Single |
| CACT80C** | 1µg/ml | CD8 | | IgG1/PE | | 2µg/ml | |
| GB21A** | 5µg/ml | γδ T δ chain specific | | IgG2b/PE | | 2µg/ml | |
| GC1A* | 7µg/ml | CD4 | VMRD | IgG2a / Alexia633 | Southern Biotechnology | 2,5µg/ml | Triple |
| 38.65*** | 2µg/ml | CD8 | AbD Serotec, Oxford, UK | IgG2a /Alexia633 | Invetrogen, Oregon, USA | 1,4µg/ml | |
| GB21A** | 4µg/ml | γδ TCR δ chain specific | VMRD | IgG2b/FITC | Southern Biotechnology | 11,9µg/ml | |
| CC302▣ | 7µg/ml | IFN-γ | AbD Serotec | IgG1/PE | AbD Serotec | 14µg/ml | |
| CC320▣▣ | 4µg/ml | IL-10 | Serotec | IgG1/PE | | 14µg/ml | |

* mouse anti-caprine / ** cross-reacting mouse anti-bovine / *** cross-reacting mouse anti-ovine / **** goat anti-mouse conjugated fluorescent antibody

▣ cross-reacting mouse anti-bovine IFN-γ / ▣▣ cross-reacting mouse anti-bovine IL-10

Single-colour flow cytometric analysis for surface markers was performed with unconjugated primary monoclonal antibodies (Table 1). In brief, 2×10^5 - 5×10^5 cells were added to 96 well round bottom plates and washed twice with PBS containing 1% BSA, and 10mM NaN_3 by centrifugation at 400xg for 3min at 4°C (washing buffer). Thereafter, cells were incubated with primary antibodies for 30 min at 4°C. After incubation the cells were washed twice and subsequently incubated with isotype specific goat anti-mouse secondary antibodies conjugated with phycoerythrin (PE) (Table 1) for 30 min at 4°C. Following incubation, cells were washed twice and fixed in 150 µl FACS Lysing Solution (Becton Dickinson, San Jose, CA, USA). Cells incubated with secondary antibodies only were used as negative controls.

Intracellular cytokine production by T cell lines was assessed by three-colour flow cytometry. The combination of moAbs used in triple colour flow cytometry are shown in table 2-2.

Table 2-2: Combination of moAbs used in triple- colour flow cytometry

| Unstimulated T cells | | | Stimulated T cell line (PPD-J 5µg/ml) | | | Positive control (SED) |
|------------------------|---------------------------|------------------------|---------------------------------------|---------------------------|------------------------|------------------------|
| CD4GC1/IgG2a-Alexia633 | γδGB21A/IgG2b-FITC | CD4GC1/IgG2a-Alexia633 | CD4GC1/IgG2a-Alexia633 | γδGB21A/IgG2b-FITC | CD4GC1/IgG2a-Alexia633 | CD4GC1/IgG2a-Alexia633 |
| IFNγ/IgG1-PE | CD8-38.65/IgG2a-Alexia633 | IL-10/IgG1-PE | IFNγ/IgG1-PE | CD8-38.65/IgG2a-Alexia633 | IL-10/IgG1-PE | IFNγ/IgG1-PE |
| | IFNγ/IgG1-PE | | | IFNγ/IgG1-PE | | |

Briefly, approximately between $1.8 - 4 \times 10^6$ cells/ well of T cells were incubated in 24 well plates containing adherent autologous cells with or without PPD-J at a final concentration of 5 µg/ml. Staphylococcus aureus enterotoxin D (SED) (Toxin Technology, Sarasota, Florida, USA) was used as a positive control at a final concentration of 0.1µg/ml. The plates were incubated for 8 hours before Brefeldin A (10 µg/ml) (Sigma) was added to the wells to block cytokine secretion. After a total incubation of 18 hour, the cells were transferred to 96 well staining trays and washed twice. After the washing step, cells were incubated with unconjugated primary monoclonal antibodies against surface markers for 30 min. on ice, (Table 1). After incubation, the cells were washed twice and incubated for 30 min. on ice with isotype specific goat anti-mouse conjugated secondary antibodies (Table 1). Thereafter, cells were washed twice before fixation and permeabilization by incubation for 20 min. on ice in Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA, USA). Cells were washed twice with PermWash solution (BD Biosciences).

Staining for intracellular cytokine was performed in PermWash with unconjugated monoclonal primary antibodies for 30 min. on ice (Table 1), followed by washing 3 times in PermWash. The cells were finally incubated 30 min. on ice with isotype specific goat anti-mouse secondary antibodies conjugated with PE against IFN-γ, or IL-10 (AbD Serotec, Oxford, UK) (Table 1). Following incubation, cells were washed 3 times and stored in Perm/Wash solution. Cells incubated with secondary antibodies only were used as negative controls.

The cell samples were run on a FACSCalibur flow cytometer. The lymphocytes were identified according to their specific forward- and side-scatter. Data from ten thousand cells were collected per run when performing for surface staining alone and a total of 100,000

gated cells were analysed for intracellular staining. Data analysis was performed using the CellQuest software version 6.0. Positive fluorescence gates were set with reference to the negative controls.

2.4 Quantitative real-time polymerase chain reaction (Q-RT-PCR)

2.4.1 Stimulation of T cell lines

T cell lines that had a high response to PPD-J were used in this experiment. Briefly, frozen autologous PBMCs were thawed as previously described in 2.2.5.1. 1×10^6 cell/ml were seeded in 96 wells plate for generation of adherent APC as described in 2.2.4.1 and incubated for 1.5 hours. After incubation, $10 \mu\text{g/ml}$ of each PPD-J and Concanavalin A (ConA) (1mg/ml) (Sigma-Aldrich, Norway) or medium only (as negative control) were added to the adherent APCs and incubated overnight at $37^\circ\text{C}/5\% \text{CO}_2$.

Next day, frozen expanded CD4^+ T cell lines were thawed and resuspended into approximately 2.0×10^6 cells/ml T cells, $50 \mu\text{l}$ of cell suspension (approximately 10×10^4 cells/well) were added to stimulated APCs including control wells and incubated for 20 hours.

After 20 hours, the stimulated cells were harvested and pooled, spun down at $3000 \times g$ for 5 min at room temperature, and resuspended in $350 \mu\text{l}$ lysis buffer (RLT buffer from the Qiagen RNeasy Mini Kit (Qiagen) with $1\% \beta$ -mercaptoethanol). Buffer RLT contains highly denaturing guanidine-thiocyanate which inactivates RNases and ensures purification of intact RNA. The chaotropic salts in the buffer RLT is critical for lysis, but also for the later binding of RNA to the silica membrane of the column (¹¹⁰).

Lysed cells were homogenized by 5 times passage through a 21-gauge needle fitted to a syringe to reduce the viscosity of the lysate sample. RNA was isolated directly after the lysis step or the lysate was stored at -70°C until use.

2.4.2 Isolation of RNA

Total RNA was extracted from stimulated and unstimulated T cell lines using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The RNeasy Mini spin column kit is based on selective binding properties of RNA to a silica membrane combined with speed microspin technology.

The frozen lysate was thawed at 37°C in a water bath and homogenized for a second time before RNA isolation.

One volume of 70% ethanol was mixed with the RNA sample. Ethanol is added to the lysate to enhance selective binding of RNA to the column. After centrifugation, the spin column was washed several times by adding washing buffer RW1 and washing buffer RPE, respectively. These two steps of washing were done to remove genomic DNA, proteins and other contaminants which may be retained on the column and cause reduction of RNA purity and yield.

The purified RNA was released from the membrane by adding RNase-free water directly to the spin column membrane, and RNA was eluted into a new tube by centrifugation. Elution of RNA using RNase-free water is important to reduce the risk of RNA being degraded by RNases.

The RNA concentration was measured by spectrophotometry using a nanodrop instrument (Thermo Fischer Scientific, Waltham, MA, USA). The 260/280 ratio and the 260/230 ratio were used as indicators of RNA purity. The purified RNA samples were frozen down at 70°C.

2.4.3 Complementary DNA (cDNA) synthesis

Reverse transcription of RNA into single stranded cDNA combined with genomic DNA (gDNA) elimination was performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

The first step is gDNA elimination to remove contaminating gDNA from the RNA and thereby minimize the detection of gDNA in the later qRT-PCR. Briefly, RNA template was mixed with the gDNA wipeout buffer from the kit and incubated for 2 min at 42°C. After gDNA elimination, the RNA template was mixed with a reverse transcription master mix that

includes Quantiscript reverse transcriptase, Quantiscript RT buffer and RT primer mix. The reaction mix for cDNA synthesis was incubated for 15 min at 42°C followed by 3 min. at 95°C to inactivate the reverse transcriptase.

Quantiscript reverse transcriptase is enzymatic mixture, which contains two types of enzyme that in combination are used to synthesis single-strand cDNA. The first enzyme is a RNA-dependent DNA polymerase that has the ability to synthesis DNA from a RNA template; the second type is RNase H activity that is specific to degrade RNA in RNA: DNA hybrids. RT primer mix is blend of two types of primers (oligo-dt and random primer). The oligo-dT which is a short sequence of deoxy-thymine nucleotides have advantage to bind to the poly-A tail providing a free 3'-OH end of the mRNA that can be extended by reverse transcriptase to create cDNA strand and produce full length transcript (¹¹¹). The second one; random primers; are oligonucleotides usually contain 6 nucleotides (hexamer) that consist of every possible combination of bases which can prime all along the RNA with or without poly-A tail (¹¹²).

The RT primer mix is use as a starting point to ensure cDNA synthesis from all regions of RNA transcripts, even from 5' regions. Quantiscript RT buffer includes Mg²⁺ and dNTPs, and in combination with Quantiscript reverse transcriptase and the RT primer mix, it has the ability to synthesis high cDNA yields. The cDNA was stored at -20°C.

2.4.4 Primer and probe design:

The Primer3Plus free software (<http://www.pubmed.de/cgi-bin/primer3/primer3plus.cgi>) was used to design oligonucleotides (primers and probes). Spidey tool (<http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/spideyweb.cgi>) was used to align genomic DNA of *Bos taurus* sequences with mRNA *capra hircus* to identify areas suitable for the design specific PCR primers and probes. The BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to confirm that none of the selected oligonucleotides recognized any registered DNA sequence other than the target. Sequences used for primers and probes design were obtained from public databases (GenBank, National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/nucleotide?term=>). Primers and probes were purchased from DNA Technology A/S (Denmark). Table 2-3.

Table 2-3: primers and probes sequences used for qRT-PCR conformation

| Gene | Primer | Sequences 5`—3` | Accession no. | Primer location | Product size (bp)** |
|----------------|---------|--|---------------|-----------------|---------------------|
| <i>IL-17A</i> | Forward | CTC CAC CGC AAT GAG GAC | GU269912.1 | Exon 3 | 138 |
| | Reverse | GAC CAG GAT CTC TTG CTG GA | | | |
| | Probe* | 6CT GGG AGG CCA AGT GCA GCC X | | | |
| <i>IFN-γ</i> | Forward | CAG GAG CTA CCG ATT TCA GC | U34232.1 | Exon 1 | 120 |
| | Reverse | CCT GGC CAT AAG AAC CAG AA | | | |
| | Probe* | 6CC GGC CTA ACT CTC TCC TAA ACG ATG AX | | | |
| <i>IL-10</i> | Forward | GCG CTG TCA TCG TTT TCT G | DQ837159.1 | Exon 4,5 | 117 |
| | Reverse | ATG TCA AAC TCA CTC ATG GCT TT | | | |
| | Probe* | 6TC TTC AAT ATG CTC CAA GAG AGG GGT GTC TX | | | |
| <i>bActine</i> | Forward | CGT GAG AAG ATG ACC CAG AT | AF481159.1 | Exon 1,2 | 122 |
| | Reverse | CCA GAG TCC ATG ACA ATG C | | | |
| | Probe* | 6CA CTC CTG CCA TGT ATG TGG CCX | | | |

* 6: 5` labelled with 6-FAM. X: 3` labelled with TAMRA

** bp: base pair

2.4.4.1 Test of primers

To test the designed primers and probes, two step real time PCR was performed using Maxima SYBP Green /ROX qPCR master mix (2x) (Fermentas, Maxima™, Fermentas GmbH, Germany). Maxima master mix is a ready-to-use solution that includes Maxima® Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer. It contains SYBR Green dye that allows DNA detection and analysis without using sequence-specific probes and supplemented with ROX as passive reference dye. dUTP is included in the mix for optional carryover contamination control using uracil DNA glycosylase (UDG). Only cDNA template of ≤500 ng and primers (Forward and Reverses) 10μM need to be added to the master mix. For negative controls, RNase free water was added.

All reactions were performed in duplicate. Reactions were run on Stratagene Mx3005P (Agilent Technologies, USA). For SYBR Green/ROX qPCR master mix, cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles with denaturation for 10 min at 95°C and annealing/elongation for 45 min at 60–72°C. Melt curves were run from 95°C to 72°C. All reactions were performed in duplicate.

2.4.5 Running of samples Q-RT-PCR

To quantify the expression of the selected gene, two steps Q-RT-PCR was performed using TaqMan® gene expression master mix (AB Applied Biosystems, Foster city, USA).

TaqMan® Gene Expression Master Mix is an appropriate mixture of components (except primers, probes, template, and water) which is necessary to perform qRT-PCR. It's contains UP (Ultra Pure) AmpliTaq Gold® DNA Polymerase, which is type of hot stable enzyme that activated only at temperatures where the DNA is fully denatured , Uracil-DNA Glycosylase (UDG) it's type of enzyme that use to prevent any generation of carryover-PCR product by eliminating any uracil integrated into single or double stranded amplicons, deoxyribonucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP) and ROX™ Passive Reference which is used for normalization in data analysis.

All reactions were performed in duplicate. The reaction mixture for each well of PCR plate was contain: 2x TaqMan Gene Expression Master mix, each primer (Forward and Reverse) (50µM), Probe (5µM), RNase free water, and ≤500 ng of template cDNA. In the negative controls, RNase free water was added.

Reactions were run on Stratagene Mx3005P (Agilent Technologies). For TaqMan® gene expression master mix, cycling conditions were as follows: 10 min at 95°C, followed by 45 cycles with denaturation for 15 min at 95°C and annealing/elongation for one hour at 59°C. Melt curves were run from 95°C to 59°C.

Q-RT-PCR assays were analyzed by using the 2-(ΔΔCt) method with Office Excel 2007 (Microsoft) as described previously (¹¹³). To assess the effect of stimulation (PPD-J or ConA) versus un-stimulated cell on cytokines expression in PBMCs within a goat, the bActin gene was used as housekeeping gene. Q-RT-PCR assays with CT values above 40 were considered negative (the lowest CT value; the highest ΔRn value).

2.5 T cell cloning

The protocol previously described in (^{109,114}) with few modifications. T cell clones were generated from PPD-J reactive T cell lines (CD4⁺ sorted T cell lines) with three days stimulation with PPD-J before seeding out potentially activated T cells in Terasaki plate (Nunc, Denmark). PBMCs were prepared as earlier described (2.2.1) and resuspended into 1×10^6 cells/ml, seeded in the 96 flat wells plate, and incubated at 37°C/ 5%CO₂ for 1,5-2 hours for adherent APCs. PPD-J antigen was diluted to 10µg/ml in RPMI 1640 media supplemented with gentamycin 50µg/ml, fungizone 1µg/ml and 10% GS added to the adherent APCs and incubated overnight. CD4⁺ T cells were added to APC fed with PPD-J and incubated for three days at 37°C/5%CO₂.

After three days, the restimulated T cells were collected and resuspended with autologous PBMCs feeder mix prepared from two or three donors as previously described (2.2.2.3). The T cells were cloned by limiting dilution method at a concentration of 1, 3 and 10 cell/well in Terasaki plate (Nunc) and incubated for 11 days at 37°C/ 5%CO₂. If the cells remain viable (feeder cell layers and optimal medium are usually required because of apparent low cell density / well) and proliferates, then an isolated clone of cells will have been established in the well.

After 11-14 days, plates were screened by microscopy for growing T cell clones. Wells with homogenous layer of “cobblestone like” cells were picked and transferred to 24 or 48 well plates and re-stimulated with feeder mix as for restimulation of T cells (2.2.2.3).

Growing T cell clones were tested for antigen-specific proliferation 10 days after restimulation as described earlier (2.2.4).

3 Results

3.1 MAP culture

MAP culture and RT-PCR for quantification was performed at the section for bacteriology at NVI to confirm the infection and shedding status of the animals. A total of 20 samples from the four naturally infected goats were tested. RT-PCR was able to detect MAP in all 20 samples, while 15 of 20 samples were positive by culture (Table 3-1). The lowest Ct value was observed in goat no. 7037, which also had five out of five samples positive on culture, indicating that this goat was heavily infected with MAP. The goat was an advanced case with clinical symptoms. The cut-off Ct value was set to 40.

Table 3-1: Culture and real-time PCR results of each goat that naturally infected with MAP.

| Goat no. | Day | Culture | Ct value (real time PCR) |
|----------|-----|---------|--------------------------|
| 7029 | 1 | + | 32,60 |
| | 2 | - | 34,88 |
| | 3 | + | 32,27 |
| | 4 | - | 34,33 |
| | 5 | + | 35,03 |
| 7037 | 1 | + | 17,94 |
| | 2 | + | 16,44 |
| | 3 | + | 17,15 |
| | 4 | + | 18,49 |
| | 5 | + | 18,60 |
| 7041 | 1 | + | 31,66 |
| | 2 | + | 34,86 |
| | 3 | - | 29,80 |
| | 4 | + | 33,96 |
| | 5 | + | 35,13 |
| 7257 | 1 | + | 38,10 |
| | 2 | - | 34,90 |
| | 3 | - | 38,55 |
| | 4 | + | 33,33 |
| | 5 | + | 34,76 |

3.2 IFN- γ assay

This experiment was performed as a routine diagnosis combined with bacteriological culture to confirm the diagnosis the paratuberculosis in naturally infected goats and to confirm the presence of PPD-J reactive T cells in the blood. As seen in figure 3-1, all animals had an IFN- γ response in June 2010, while the response declined below cut off value in one goat after arriving at the institute in February 2011.

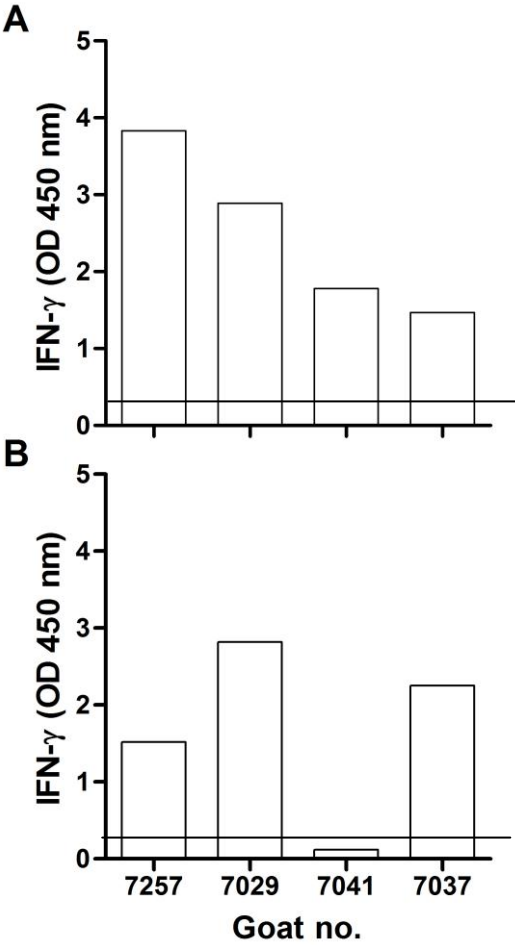


Figure 3-1: IFN- γ production presented as optical density OD responses following specific stimulation with PPD-J Ag. The cut-off point is represented as a horizontal line (OD 0.3). (A) June 2010 and (B) February 2011

3.3 T cell cultivation

3.3.1 CellTiter-Glo assay as read-out

For practical reasons we decided to use the CellTiter-Glo assay as our standard read-out for proliferative cell response. The CellTiter-Glo assay was compared to the standard assay for T-cell proliferation; incorporation of ^3H -thymidine (Figure 3-2). The two methods gave a similar profile in a dose response test using PBMCs stimulated with PHA that serial diluted 1:4. A parallel test of a PPD-J responsive T cell line was also performed at a later stage to ensure that similar results were obtained when assessing antigen specific responses. Figure 3-3

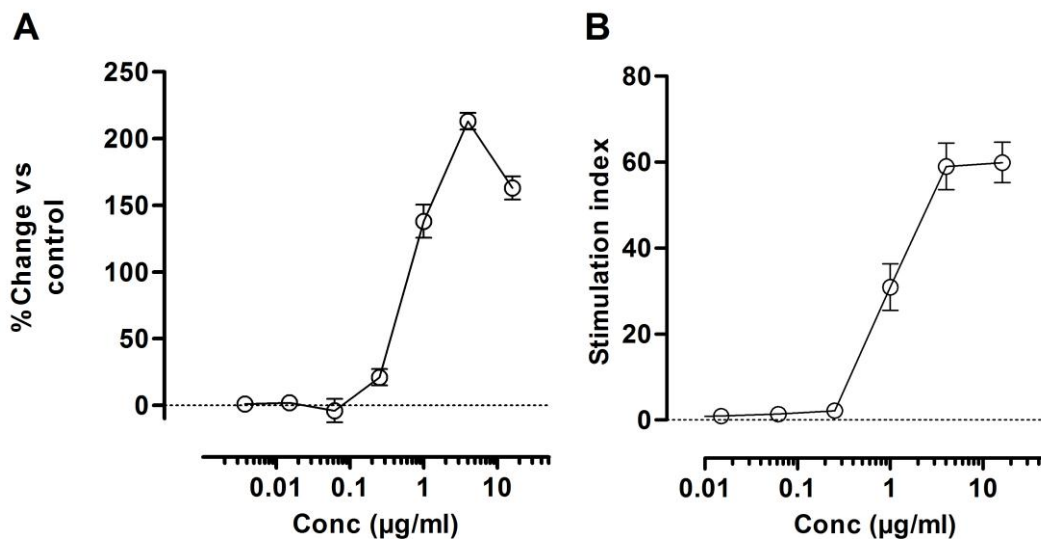


Figure 3-2: Comparison between CellTiter-Glo assay (A) and ^3H -thymidine incorporation assay (B) by using serial dilution of PHA mitogen (1:4) on PBMC. Data are expressed as SI (mean optical values of cell stimulated cultures/ mean optical values of unstimulated cell culture (control) for ^3H -thymidine incorporation assay and change % of stimulated cells vs unstimulated cells for CellTiter-Glo assay

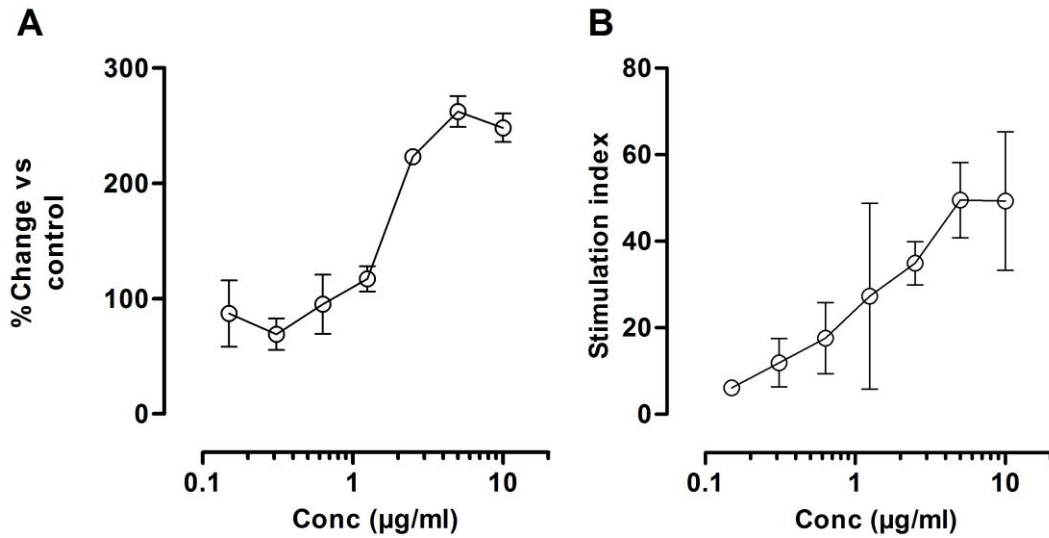


Figure 3-3: Comparison between CellTiter-Glow assay (A) and ³H-thymidine incorporation assay (B) by using serial dilution of PPD-J (1:2) on T cell lines. Data are expressed as SI (mean optical values of cell stimulated cultures/ mean optical values of unstimulated cell culture (control) for ³H-thymidine incorporation assay and change % of stimulated cells vs unstimulated cells for CellTiter-Glo assay.

3.3.2 Test of mitogen

To expand human T cells *in vitro*, PHA is used to agglutinate and trigger the cells. A titration of PHA and ConA was performed on PBMC to determine the optimal concentration of the mitogen, and to compare that PHA and ConA as mitogen for cultivation of caprine T cells. The proliferative mitogenic response to PHA for the PBMC gave both a higher response than with Con A and a response at lower concentration. PHA optimal concentration was approximately between 0.1µg/ml and 1µg/ml (Figure 3-4). According to these results PHA was chosen for restimulation of T cells and it was used at a concentration of 1µg/ml.

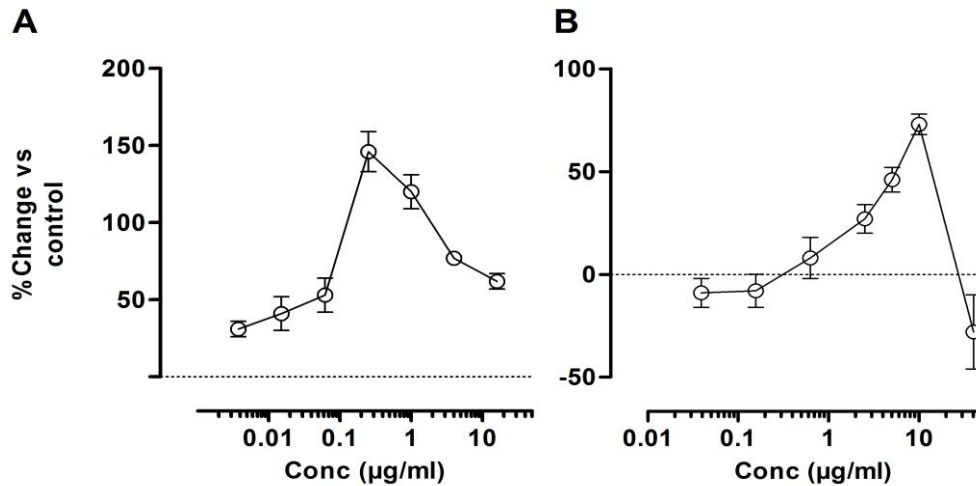


Figure 3-4: PBMC responses to serial diluted (1:4) mitogens PHA (A) and ConA (B). The results are expressed as change % of stimulated cells vs unstimulated cells by using CellTiter-Glo assay

3.3.3 Growth of T cells

The growth of T cell was subjectively assessed based colour of the medium, number of times they needed splitting and on how the cells looked in the microscope. A summary of all the results are given in Table 3-2. A less than expected growth of T cells was observed when using FCS (heat inactivated 56°C for 30 min), and we thus decided to see if pooled GS (heat inactivated 56°C for 30 min) would improve the growth of the cells. For the non-sorted T cell lines six out of nine lines grew relatively ok, while all the eight lines expanded with GS grew well.

Table 3-2: T cell growing score in media supplemented with 10% FCS or 10% GS

| Serum | FCS | | | | GS | | | |
|------------|-------|----------------|-----------|----------------------|-------|---------|-----------|---------------------|
| | Times | Growing | Lines no. | PPD-J resp. | Times | Growing | Lines no. | PPD-J resp. |
| Non-sorted | 9 | 6/9 | 35 | 22/35 G. 12/35 L. | 8 | 8/8 | 15 | 7/15 G. 4/15 M. |
| -gd T cell | 4 | Slow* (PHA) | 19 | 13/19 G. 2/19 M. | ND | ND | ND | ND |
| MACS CD4+ | 2 | 1/2 | 4 | 2/4 L. | 2 | 2/2 | 4 | 2/4 L. |
| Dynal CD4+ | ND | ND | ND | ND | 13 | 13/13 | 64 | 61/64 G. 3/64 M. |

-gd T cell: depleting $\gamma\delta$ T cell

*slow: added diluted PHA

ND: not done

G: good response (with cut-off $\geq 90\%$ change vs control%)

M: medium response (with cut-off 10 - 90 %change vs control%)

L: low response (with cut-off $\leq 10\%$ change vs control%)

3.3.4 T cell responses and phenotype of T cell lines

The T cell lines were tested in the CellTiter-Glo assay for responses to PPD-J and a variable response was observed (Table 3-2, and Figure 3-5). To try to find some explanations for this variation, we performed flow-cytometry on the expanded T cell lines to see the percentages of CD4+ T cells in the different lines. A huge variation was observed (Figure 3-6) with the percentage of CD4+ T cell ranging from only 0.06% - 29%.

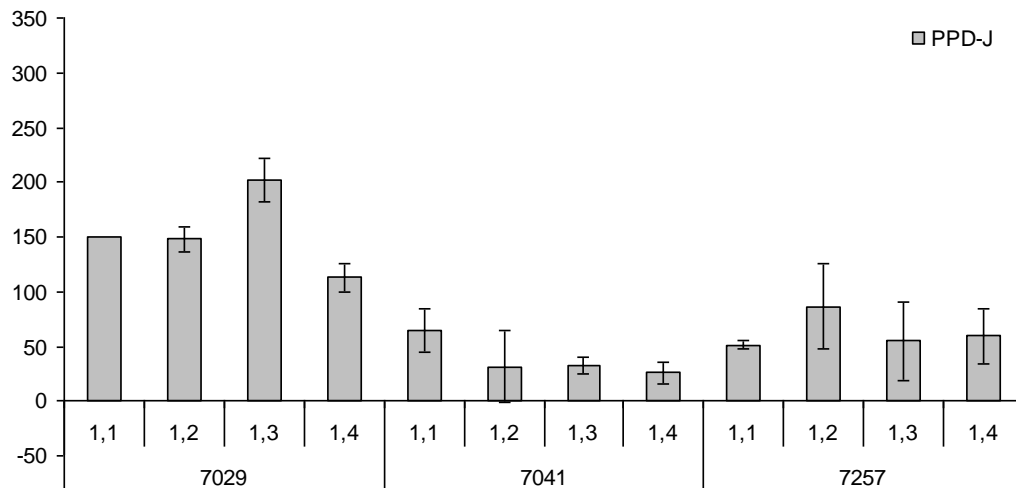


Figure 3-5: Proliferation of non-sorted T cell lines in response to PPD-J stimulation. Autologous PBMCs were used as APC and were incubated with antigens overnight. T cell lines were added followed by three days incubation. CellTiter-Glo reagent was added. Each T cell line was tested in triplicates. The results are expressed as change % of stimulated cells vs unstimulated cells by using CellTiter-Glo assay

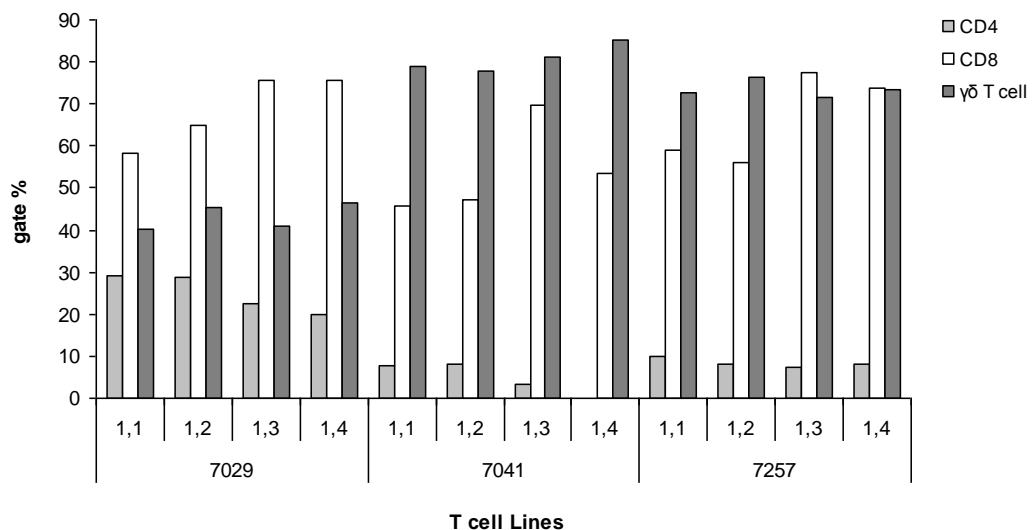


Figure 3-6: Non-sorted T cell lines after one week restimulation. Most T cell lines have high gate % of γδ T cell lines. The results are expressed as a gate % by using single colour flow cytometry.

Because of the overgrowth of $\gamma\delta$ T cells, we decided to deplete these cells by using MACS beads before the expansion of the T cells. The yield of CD4+ T cell was increased to 35% (range 3-59%) while the percentage of $\gamma\delta$ T cell lines was decreased to 0.4% (0.08% - 0.97%). However, we still noted a high percentage of CD8+ T cells in the culture, 59% (34 - 81%), Figure 3-7

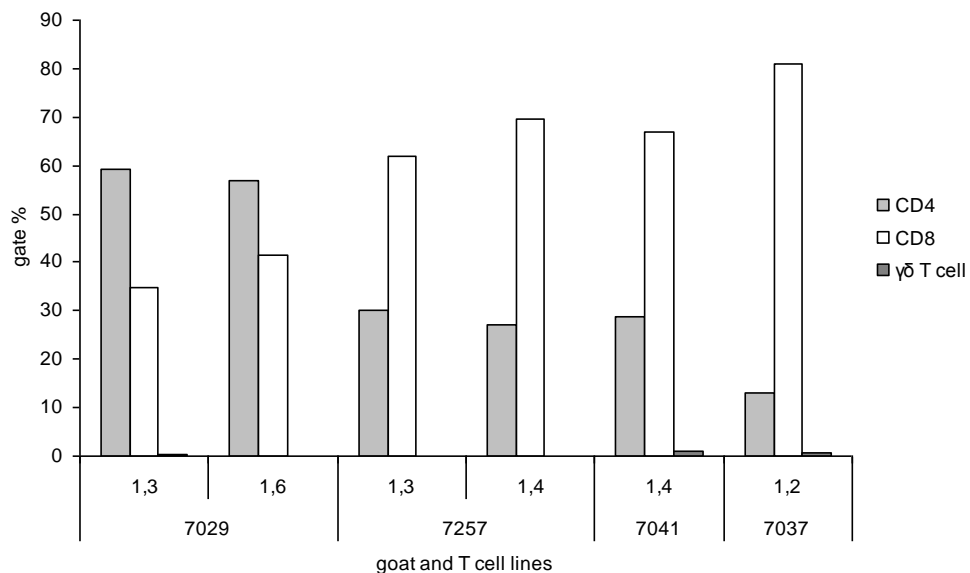


Figure 3-7: Gate percentage of T- cell subsets after depletion of $\gamma\delta$ T cells using MACS beads (negative selection). The results are obtained by using single-colour flow cytometry.

To increase the yield of CD4+ T cell and to avoid overgrowing of CD8+ and $\gamma\delta$ T cells, we choose to cultivate CD4+ T cell after positive selection by either MACS beads or dynabeads. Figure 3-8 illustrate that the yield of CD4+ T cells in lines expanded after positive selection using dynabeads were higher than in the non-sorted T cell lines, 79 % (78-81%) and 35% (20 -56%), respectively. Cultivation of CD4+T cells isolated by MACS beads gave approximately the same results (data not show).

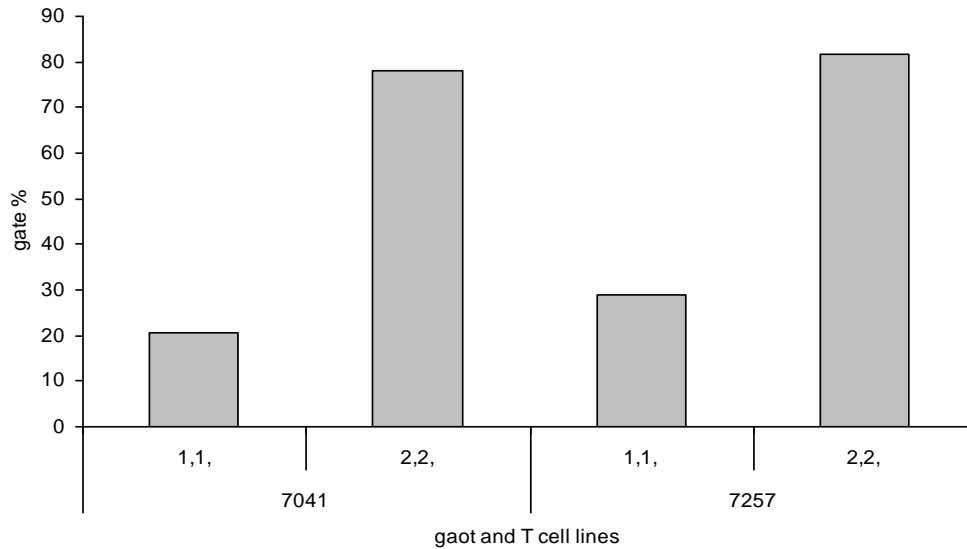


Figure 3-8: Percentage of CD4+ T cell in lines in non-sorted T cell lines and T cell lines expanded after positive selection using dynabeads. Non-sorted T cell lines are labelled 1.1 and sorted T cell lines are labelled 2.2. The data are expressed as gate % of CD4+ T cells tested by single-colour flow cytometry

By increasing the number of CD4+ T cell, PPD-J response increased in some lines only. In goat no. 7257 there is an increase in the PPD-J response in sorted T cells 2.1 (127% \pm 7%) and 2.2 (285% \pm 4%) compared with the non-sorted lines 1.1 (93% \pm 3%) and 1.2(112% \pm 5%) from the same animal, (change % response of T cell lines group \pm SD%). Figure 3-9. The increased response could be explained by an enrichment of antigen specific T cells.

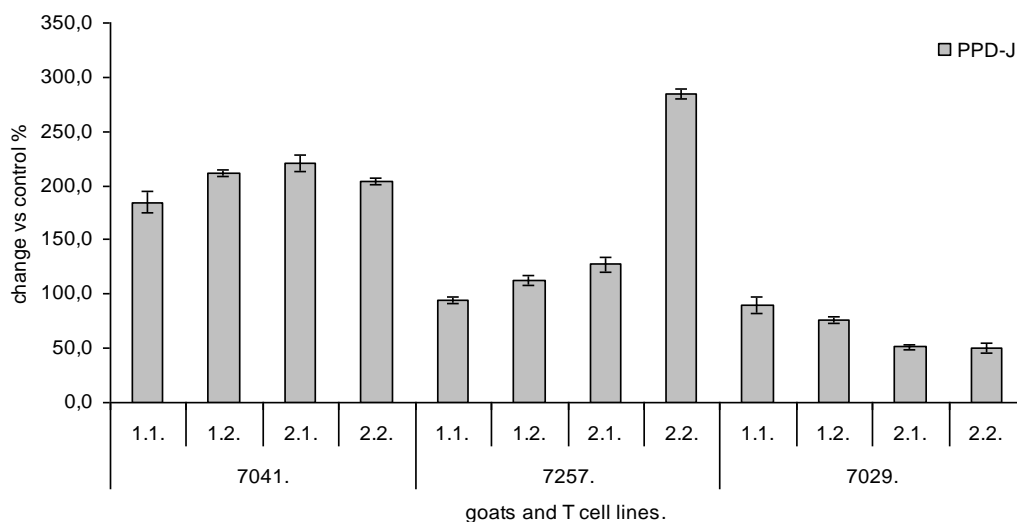


Figure 3-9: Proliferation of T cell lines in response to PPD-J stimulation. Autologous PBMCs were used as APC and were incubated with antigens overnight. T cell lines were added followed by three days incubation. CellTiter-Glo reagent was added. Each T cell lines were tested in triplicates. The results are expressed as change % of stimulated cells vs unstimulated cells by using CellTiter-Glo assay. Non-sorted T cell lines (1.1, 1.2) and T cell lines after positive selection using dynabeads are shown (2.1, 2.2)

Some lines were tested for the presence of the various T cell subsets after a second expansion of the lines. The percentages of CD8⁺ and $\gamma\delta$ T cells increased while the yield of CD4⁺ T cells decreased to 71% (69-72%) in sorted T cell lines and to 50% (40-56%) in non-sorted T cell lines. There were also a high percentage of double positive T cells expressing both CD8⁺ and $\gamma\delta$ T cell receptor in some lines 15% (3-31%). Figure 3-10.

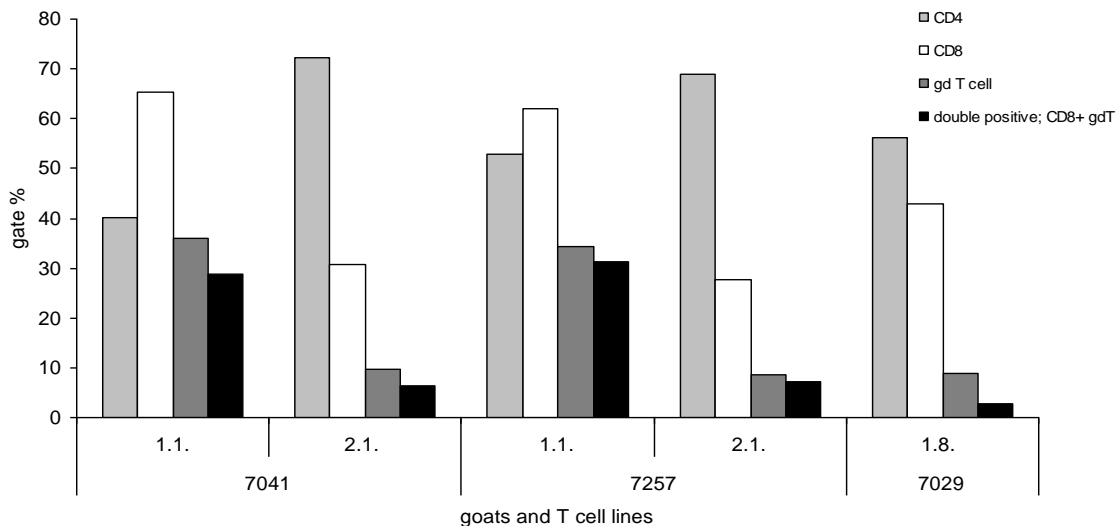


Figure 3-10: Overgrowing of CD8⁺ T cells and $\gamma\delta$ T cells after second expanding of sorted (2.1) and non-sorted T cell lines (1.1 and 1.8). T cell lines were stimulated with PPD-J at 5 μ g/ml and incubated with autologous PBMCs as APC in triplicates well for each stimulate and unstimulated T cell lines. Data expressed as gate % by using triple-colour flow cytometry

3.3.5 Peptide response

The goats were tested for IFN- γ production against MAP specific peptide pools before and after vaccination. No responses to any peptides were detected before immunization and minimal responses were seen after immunisation (results not shown). To see if we could cultivate peptide responsive T cells in vitro, T cell lines were made in the presence of pools of peptides. Five T cell lines from goat 7041 (3.1, 5.1) and 7257 (3.1, 4.1, 5.1) had response to three pools of peptides as well as a PPD-J response. T cell lines 3.1 from both goat 7257 and 7041 had significant response to PP 3, T cell lines 4.1 of goat 7257 had response to PP 5, while T cell lines 5.1 from both goat 7257 and 7041 had mediocre response to PP 8 (data not shown)

These T cell lines were expanded further and tested against the individual peptides in each of these three pools. The stimulated T cell lines had high response to numbers of individual peptide, Figure 3-11. The highest responsiveness was to peptides No.: p 27, 28, 29, 48, 50, 63, 69, 70, 74, 78, and 79.

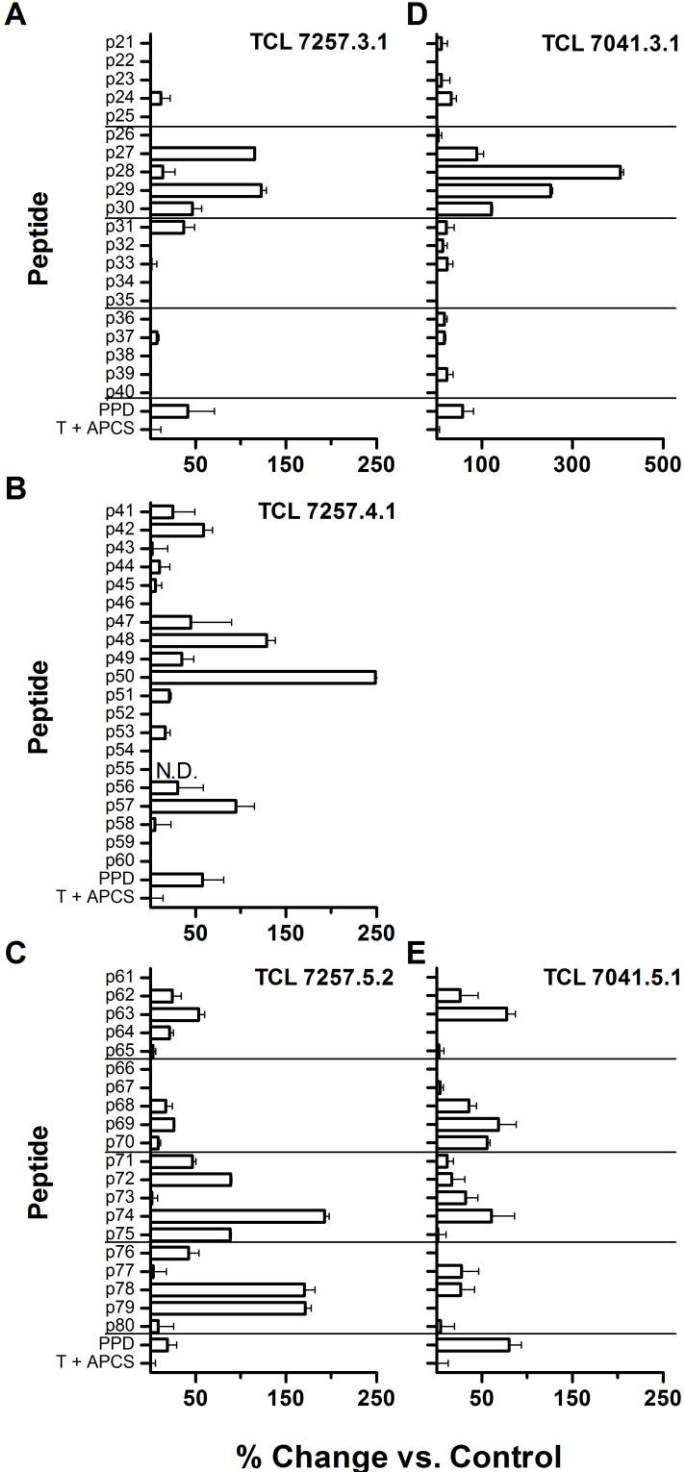


Figure 3-11: Responses of expanded T cell lines after stimulation with PPD-J at concentration 10µg/ml as well as individual peptides. Autologous PBMCs were used as APC and were incubated with antigens overnight. T cell lines were added followed by three days incubation. CellTiter-Glo reagent was added. Each T cell line was tested in triplicates. A and D T cell lines response to PP3, B PP5, C and E PP8. The highest responsiveness was to individual peptides No.: p 27, 28, 29, 48, 50, 63, 69, 70, 74, 78, and 79. Results are expressed by change vs control % by using CellTiter-Glo assay. ND; not determined.

3.4 Cytokine expression by Q-RT-PCR and flow cytometry

Relative expression of the genes encoding IFN- γ , IL-17A and IL-10 were measured in three T cell lines. The lines were stimulated with PPD-J, ConA or left unstimulated. A high relative expression of IFN- γ after stimulation with ConA was seen in all lines, while an increase in IL-17 production was detected only in goat 7029. A high expression of mRNA encoding IFN- γ and IL-17 was detected in the T cell line from goat 7029 after stimulation with PPD-J. The relative expression ratios of mRNA encoding IFN- γ and IL-17A were 85 (range 71-103) and 37 (range 31-44), respectively. No increase in IL-10 production was detected after stimulation in any of the non-sorted T cell lines. Figure 3-12.

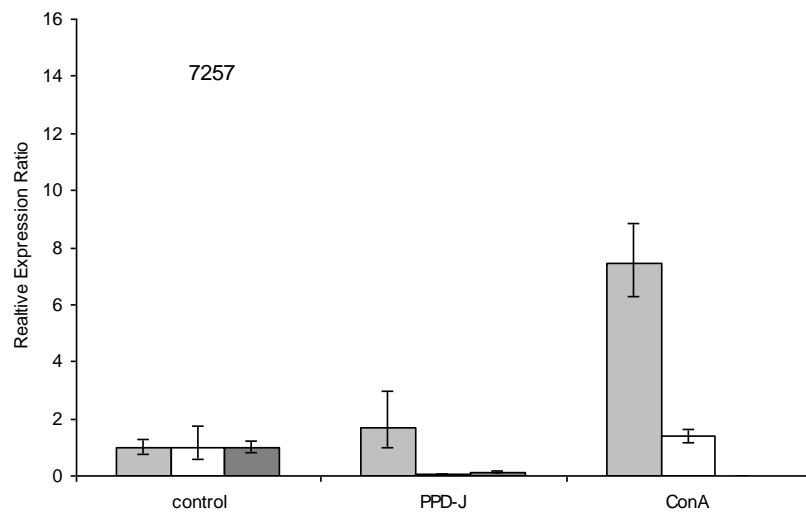
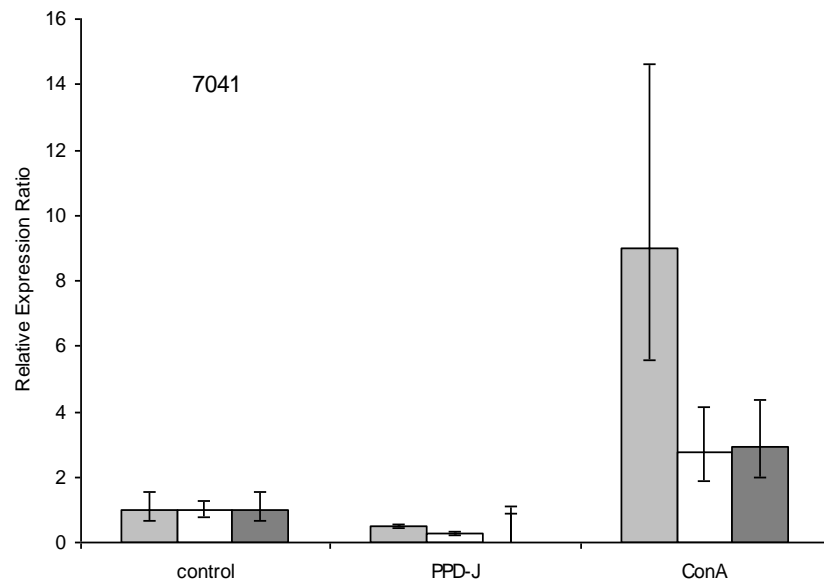
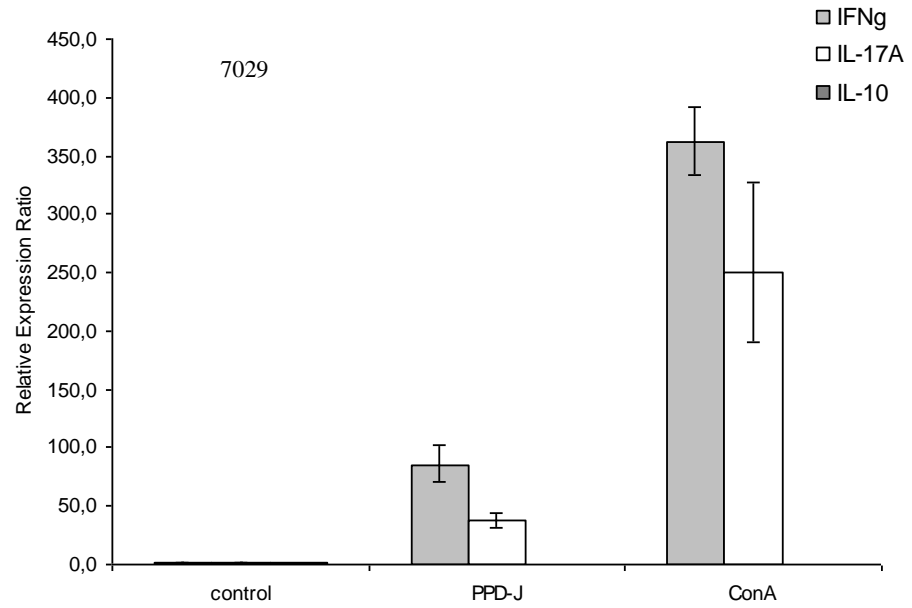


Figure 3-12: Cytokine mRNA expression for IFN- γ , IL-17A and IL-10 in non-sorted T cell lines were stimulated with PPD-J and ConA for 20 hours. The levels of mRNA were normalized to the housekeeping gene bActin. Non-stimulated T cells were used as calibrators by using $\Delta\Delta C_t$ method. The results were expressed as mean relative expression and rang (lower, higher)

To obtain additional data on cytokine expression intracellular cytokine staining for IFN- γ and IL-10 was performed on sorted and non-sorted T cell lines after stimulation with PPD-J. An increase in IFN- γ production was detected in both CD4+ and CD8+ T cells after the PPD-J stimulation. However, the increase was most pronounced for the CD4+ T cells. Table 3-3 and Figure 3-13. None of T cell subsets produced IL-10 (Data not show).

Table 3-3: Percentage of T cell subsets producing IFN- γ .

| Goat | T cell lines | CD4+ T cell | | CD8+ T cell | | $\gamma\delta$ T cell | |
|------|--------------|--------------|-------|--------------|-------|-----------------------|-------|
| | | unstimulated | PPD-J | unstimulated | PPD-J | unstimulated | PPD-J |
| 7029 | 1.8 (NS) | 0.05 | 0.61 | 0.27 | 0.57 | 0.04 | 0.15 |
| 7041 | 1.1 (NS) | 0.15 | 0.36 | 0.50 | 0.56 | 0.29 | 0.29 |
| | 2.1 (CD4) | 0.42 | 1.62 | 0.20 | 0.38 | 0.11 | 0.10 |
| 7257 | 1.1 (NS) | 0.06 | 1.11 | 0.30 | 0.61 | 0.13 | 0.17 |
| | 2.1 (CD4) | 0.09 | 0.64 | 0.23 | 0.45 | 0.07 | 0.11 |

NS: non-sorted T cell lines
 CD4: sorted T cell lines

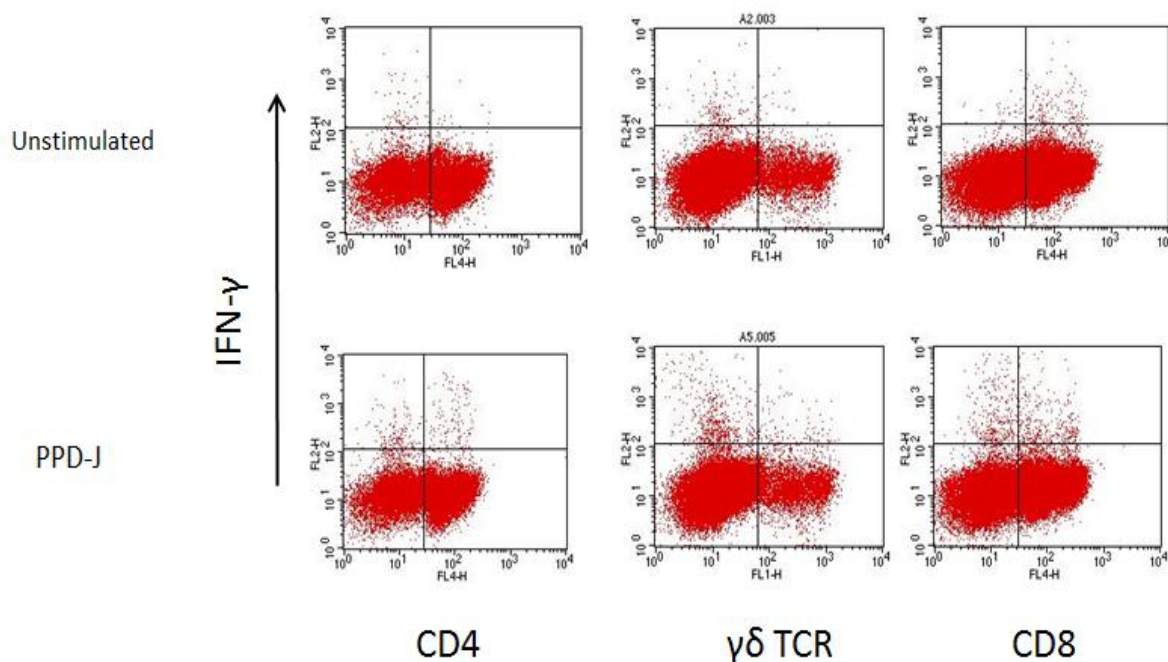


Figure 3-13: Flow cytometric dot plots (triple colour staining) showing the phenotypes and IFN- γ expression of unstimulated T cell lines (upper panels) and PPD-J-stimulated T cell lines (lower panels) from one representative infected goat 7029 (1.8).

3.5 T cell cloning

Eight attempts to generate T cell clones were by limiting dilution from CD4+ T cell lines that have strong PPD-J response was performed. Only a few clones grew sufficiently well to study the T cell clones further and attempt another round of expansion and determine their epitope specificity. Only three expanded T cell clones gave good response to PPD-J at 10 µg/ml, two clones from 1 cell/well and one from 3 cell/well. Figure 3-14. Other T cell clones lost their reactivity after expansion.

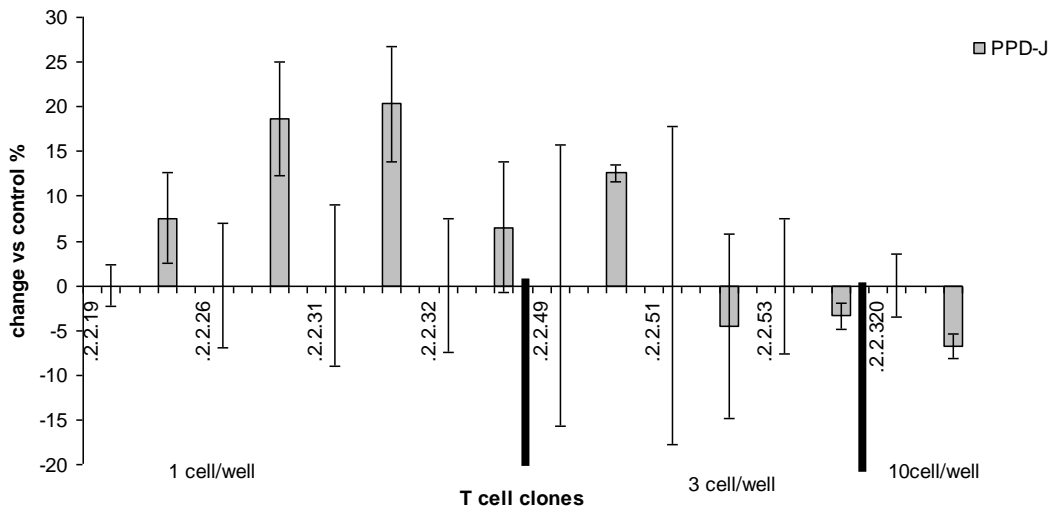


Figure 3-14: Responses of expanded T cell clones after stimulation with PPD-J at concentration 10µg/ml. autologous PBMCs were used as APC and were incubated with PPD-J overnight. T cell clones were added followed by three days incubation. CellTiter-Glo reagent was added. Each T cell clone was tested in triplicates. Three expanded T cell clones gave good response to PPD-J at 10 µg/ml, two clones from 1 cell/well and one from 3 cell/well. Data expressed as change vs control % by using CellTiter-Glo assay

4. Discussion

The success of cell culture depends upon the cell type and the culture condition. Here we systematically studied various common culture conditions including sera, mitogens and described a reliable culture method of caprine T cells.

Serum is routinely added to the cell culture as a source of nutrition that stimulate the cell proliferation (¹¹⁵). It also contains several essential factors and attachment factors that enhance the proliferation of cells (¹¹⁶). Among several sera types that are available; FCS is most frequently used in media for cell culture. However, in the present study we noted that the T cells grew less than expected in 10% FCS and did not seem to thrive on visual inspection. We thus decided to try pooled GS instead, since in our hands human pooled serum is superior to FCS when cultivating human T cells. We observed that the T cells cultured in media supplemented with 10% GS grew better and looked healthier on visual examination. It is not clear if this is due to the fact that we used serum from the same species or because the serum are from adult goats instead of foetuses. Serum from adult animals is likely to contain other growth factors, cytokines etc that may be beneficial for growth of T cells. The serum did not, however, affect the response to PPD-J in the T cell lines or the percentages of CD4+ T cells in the lines. In conclusion we decided to use 10% GS in our standard protocol for T cell expansion, while FCS was used for freezing and thawing of the cell and also for the short term tests like T cell proliferation assays. Several studies were in agreement with our result to replace FCS with GS as a source of nutrition in cell culture (¹¹⁷⁻¹¹⁹).

It is presumed in paratuberculosis infection that CD4+ T cells play a central role in the controlling of the infection. Mogue, T. *etal* (2001), investigated that mice lacking MHC class II or depleted of CD4+ T cells by monoclonal antibodies succumb faster to infect with *Mycobacterium tuberculosis* than wild-type mice, and *M. tuberculosis*-infected IFN- γ knockout mice have significantly less survival times than their infected wild-type counterparts (¹²⁰). The aim of this study was therefore to establish a method for cultivation of CD4+ T cells from goats. In our experiments, we noted that the CD8+ and $\gamma\delta$ T cells generally grew much better than the CD4+ T cells, and these cells frequently overgrew the cultures. Smyth, A.J. *et al* (2001), studied the kinetics of mycobacterial antigen responsiveness in *M. bovis* infected cattle, and showed that $\gamma\delta$ T cells remained significantly activated for at least 7 days

in culture, while activation of $\alpha\beta$ T cells (CD4⁺ and CD8⁺ T cells) declined during that period. This can explain the predominance of $\gamma\delta$ T in non-sorted T cell lines (¹²¹).

Three isolation methods were performed to enrich for CD4⁺ T cells. In the present study, the lowest yield of CD4⁺ T cells were generally in T cell lines that were non-sorted or depleted for $\gamma\delta$ T cells, while positive selection using MACS or dynabeads gave higher percentages of CD4⁺ T cells. The yield of CD4⁺ T cells by the latter two methods was similar. However, dynabeads were more practical and faster and appeared to be gentler on the cells. The cells looked more viable and the dynabead method was therefore chosen as the standard protocol. Despite the fact that the purity was >98% after positive selection, the percentages of CD4⁺ T cells in the lines progressively declined after expansion. This demonstrated the superior growth of both CD8⁺ T cells and $\gamma\delta$ T cells when using our protocol. The PPD-J response varied between the lines and was not consistently higher in positively selected CD4⁺ T cell lines, but some lines did have a significantly higher response than non-sorted lines. We did not identify which subsets of cells that proliferated after PPD-J stimulation and cannot exclude that the CD8⁺ T cells or $\gamma\delta$ T cells also responded to PPD-J in some lines. Other studies have, however, demonstrated that CD4⁺ T cells were the predominant reactive T cells with high PPD-J response (^{88,122-124}). A higher number of CD8⁺ T cells or $\gamma\delta$ T cells will lead to less CD4⁺ T cells in the line, and the chance of having a CD4⁺ T cell responsive to specific antigen (PPD-J) in the cell suspension decreases.

The simplest explanation for a lower response in non-sorted T cells are the lower number of CD4⁺ T cells in these lines, and that CD8⁺ and $\gamma\delta$ T cell completely over grow the responsive CD4⁺ T cells. An alternative explanation is that the CD8⁺ and $\gamma\delta$ T cells can have a suppressive effect on CD4⁺ T cells. Chiodini, R.H. and Davis, W.C. (1992) concluded that the presence of $\gamma\delta$ T cell *in vitro* suppresses the response of CD4⁺ T cell to PPD-J, and may have an immunoregulatory effect on CD4⁺ T cell (¹²⁵). Navarro, J.A. *et al* (1998), indicated that the progression of paratuberculous lesions may be due to an ineffective host immune response attributable to CD8⁺ T lymphocyte subset that "downregulate" the activities of the CD4⁺ T lymphocytes (¹²⁶). This was not in agreement with Chiodini, R.H. and Davis W.C. (1993), who demonstrated that CD8⁺ T cells might have a contrasuppression role to the CD4⁺ T cells by suppressing the $\gamma\delta$ T cells (¹²⁷). One interesting observation with the work of T cells isolated from goats compared with T cells from human, is that the amount of willingly growing CD8⁺ and $\gamma\delta$ T cells is much greater in the goats. Potentially is this also reflecting

the important role of these cells in ruminants, and thus even more focus should be put on these subsets to understand their role in the immune response during MAP infection.

In the present study ³H-thymidine incorporation assay was replaced with CellTiter-Glo assay. The present read out method (Celltiter-Glo assay) was easy to use (Add-mix-measure), safe and non-radioactive. Our results also showed that the titration curves for both mitogen and antigen were similar and eliciting the same sensitivity. This result was in agreement with many researches that replaced ³H-thymidine incorporation assay with CellTiter-Glo assay or other kind of non-radioactive assay (¹²⁸⁻¹³¹).

A definitive diagnosis of paratuberculosis currently requires demonstration of MAP by routinely mycobactin dependent bacteriological culture, acid fast stain by ZN's method and detection of IS900 by PCR amplification. To confirm the presence of MAP reactive T cells in peripheral blood a whole blood IFN- γ assay was performed. All five goats had high level of IFN- γ and were shedding bacteria in three or more of five faecal samples. These results were in agreement with other studies that indicated faecal shedding and high level of IFN- γ in the same animals (^{132,133}).

Many studies using vaccines that consist of either live attenuated or inactivated whole-cell, have demonstrated that the incidence of clinical symptoms and tissue colonization can be reduced, but infection is not prevented (^{134,135}). These whole-cell vaccines also interfere with diagnosis of bovine tuberculosis and paratuberculosis (^{136,137}). This has led to development of new paratuberculosis vaccines candidates consisting of specific MAP antigens, potentiated with adjuvants to stimulate the adaptive immunity against paratuberculosis (^{90,138-140}). The present study is related to an EU funded project that aims to develop a new vaccine against MAP infection, which does not interfere with diagnostic tests for bovine tuberculosis and paratuberculosis. The EU project has thus identified potentially immunogenic MAP specific peptides. These pools of peptides were injected intramuscularly to the infected goats to boost any immune response present in these animals. The specific aim of the present work was mainly to see if we were able to cultivate peptide specific CD4+ T cells, but we also wanted to obtain preliminary data on the immunogenicity of these peptides. Sorted CD4+ T cell were expanded in the presence of peptide pools and subsequently tested for responses against the peptides. Some of the CD4+ T cell lines had a response to PP 3,5 and 8 and we identified the response to individual peptides in these T cell lines. These preliminary findings confirmed that we are indeed able to cultivate specific CD4+ T cells in

vitro. We have also confirmed that some of the selected peptides induced a CD4+ T cell response, and these peptides are potentially candidates to be included in future vaccines.

IFN- γ is Th1 cell type cytokine that important in the control of paratuberculosis infection by activation of macrophages and clearance of mycobacterial infections (¹⁴¹). Some studies demonstrated that mice and humans with a deficiency in the ability to produce or respond to IFN- γ are very susceptible to mycobacterial infections such as tuberculosis (^{142,143}). On the other hand IL-17A is critical for enhancement of memory responses to mycobacterial infections, and after challenge with *M. tuberculosis*, IL-17-inducible chemokine expression mediates the recruitment of Th1 lymphocytes to lung tissue (¹⁴⁴). Many studies have recorded higher expression of IFN- γ in PBMCs of cattle infected with paratuberculosis (^{98,100,145}), especially in subclinical infected cattle (^{94,146,147}). Overnight stimulation with PPD-J is standard in IFN- γ testing and results in significant production of this cytokine (¹⁴⁸). While IL-10 is an anti-inflammatory cytokine that downregulates Th1 type immune response. IL-10 has been demonstrated to be higher in the late or clinical stage of paratuberculosis infection to those of subclinically affected animals (⁹⁹). IL-10 has an inhibitory effect on the killing of mycobacteria and suppresses T cell functions (^{97,149}). Because of the relevance of IFN- γ , IL-17A and IL-10 in the immune response to paratuberculosis, we decided to look at the expression of these cytokines in some of the established T cell lines.

To characterise the cytokine expression of the MAP reactive T cell lines, two methods were used; Q-RT-PCR and intracellular staining. The Q-RT-PCR was established for IFN- γ , IL-17A and IL-10 and the response in T cell lines to stimulation with PPD-J and ConA were compared to unstimulated cells. A PPD-J specific response with expression of IFN- γ and IL-17A was seen in one of the tested T cell lines, while IL-10 was not detected. These results were in agreement with other studies that demonstrated no or a low amount of expression of IL-10 as a response to *in vitro* mitogen stimulation (^{97,150,151}).

Intracellular IFN- γ and IL-10 levels were also measures and co-staining for CD4+, CD8+, and $\gamma\delta$ T cell subpopulations were performed to identify which cells produced the cytokines. The CD4+ T cell subset appeared to be the primary cellular source of IFN- γ during infection with MAP, with little to no IFN- γ produced by the $\gamma\delta$ T cell subset. Some IFN- γ production was also seen in the CD8+ T cells. Furthermore, IFN- γ production was also seen in the unstimulated cells. This might explain why we could not detect any difference in the RT-PCR where the PPD-J response is compared to the response in the unstimulated T-cells.

These results were in agreement with other studies that demonstrated that CD4+ cells are the predominant T cell that secreted IFN- γ during MAP infection, and that no significant expression level IL-10 was detected^(62,88).

5. Conclusions

1. In this study, we have made several different attempts and tried to optimize the cultivation of MAP reactive CD4⁺ T cells isolated from blood of MAP infected goats.
2. We were able to isolate MAP reactive T cells that responded to PPD-J after three weeks of cultivation.
3. To maintain reactivity we found that it was important to sort out the CD4⁺ T cells, to reduce the chance of overgrowth from CD8⁺ and $\gamma\delta$ T cells.
4. The isolated T cells had the hallmarks of Th1 reactive T cells by expressing IFN- γ after PPD-J stimulation.

6. Future perspectives

The long-term aim of this research project is to develop a vaccine that are safe and protective and give minimal side effects for the animals and thus can be accepted by the livestock industry. In addition, this vaccine should not compromise the diagnostic tests so it is possible with simple biological test to separate infected from vaccinated animals. To reach these goals we need to understand the development of protective immunity in the animals and will focus on the key players providing this protection. This means that the work will focus on understanding the role of the different subsets of T cells taking part in the immune response, CD4+, CD8+ and $\gamma\delta$ -T cells. By developing a panel of MAP reactive T cell lines and clones from several animals, we would also be able to determine a number of essential epitopes that should be included in either a vaccine or a diagnostic test. By studying the immunology of paratuberculosis we also hope to be able to give clues about the treatment of the human equivalent Crohn's disease.

7. References

- 1 Cosma, C. L., Sherman, D. R. & Ramakrishnan, L. The secret lives of the pathogenic Mycobacterium. *Annu. Rev. Microbiol.* **57**, 641-676 (2003).
- 2 Madigan, M. T., Martinko, J. M., Dunlap, P. V. & Clark, D. P. *Brock Biology of Microorganisms*. 12th edn, (Pearson Benjamin Cummings, 2009).
- 3 Olsen, I., Barletta, R. G. & Thoen, C. O. in *Pathogenesis of bacterial infection in animals* (eds C.L. Gyles, J. F. Prescott, J. G. Songer, & C. O. Thoen) Ch. 7, 113-132 (Wiley- blackwell, 2010).
- 4 Collins, M. Update on paratuberculosis: 1. Epidemiology of Johne's disease and the biology of Mycobacterium paratuberculosis. *Irish Veterinary Journal* **56**, 565-574 (2003).
- 5 NCBI. *Mycobacterium avium subsp. paratuberculosis K-10 k10 project at Univ. Minnesota*, <http://www.ncbi.nlm.nih.gov/sites/entrez?db=bioproject&Cmd=Retrieve&list_uids=91> (2004).
- 6 Elizabeth, J. B. & Manning, M. P. H. Mycobacterium avium subspecies paratuberculosis: a review of current knowledge. *Journal of Zoo and Wildlife Medicine* **32**, 293-304 (2001).
- 7 Rowe, M. T. & Grant, I. R. Mycobacterium avium ssp. paratuberculosis and its potential survival tactics. *Letters in Applied Microbiology* **42**, 305-311 (2006).
- 8 Greenwood, M., Seymour, R. & Meehan, J. *Textbook of human disease in Dentistry* 1st edn, (Wiley-Blackwell, 2009).
- 9 Ryan, K., Ray, C. & Sherris, J. *Sherris Medical Microbiology* 5th edn, (McGraw Hill., 2010).
- 10 Chiodini, R., Van Kruiningen, H. & RS, M. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. *Cornell Vet* **74**, 218-262 (1984).
- 11 Steed, K. A. & Falkinham, J. O., 3rd. Effect of growth in biofilms on chlorine susceptibility of Mycobacterium avium and Mycobacterium intracellulare. *Appl Environ Microbiol* **72**, 4007-4011, doi:10.1128/aem.02573-05 (2006).
- 12 Harris, N. B. & Barletta, R. G. Mycobacterium avium subsp. paratuberculosis in veterinary medicine. *Clinical Microbiology Reviews* **14**, 489-512 (2001).
- 13 Streeter, R. N., Hoffsis, G. F., Bech-Nielsen, S., Shulaw, W. P. & Rings, M. Isolation of Mycobacterium paratuberculosis from colostrum and milk of subclinically infected cows. *American Journal of Veterinary Research* **56**, 1322-1324 (1995).
- 14 Djønné, B., Jensen, M. R., Grant, I. R. & Holstad, G. Detection by immunomagnetic PCR of Mycobacterium avium subsp. paratuberculosis in milk from dairy goats in Norway. *Veterinary Microbiology* **95**, 135-143 (2003).
- 15 Whittington, R. J. & Windsor, P. A. In utero infection of cattle with Mycobacterium avium subsp. paratuberculosis: a critical review and meta-analysis. *The Veterinary Journal* **179**, 60-69 (2009).
- 16 Stehman, S. M. Paratuberculosis in small ruminants, deer, and South American camelids. *Vet Clin North Am Food Anim Pract* **12**, 441-455 (1996).
- 17 Radunz, B. & Fitzpatrick, S. *Johne's Disease*, <http://www.nt.gov.au/d/Content/File/p/Anim_Dis/630.pdf> (2009).
- 18 Beard, P. M. *et al.* Paratuberculosis infection of nonruminant wildlife in Scotland. *Journal of clinical microbiology* **39**, 1517-1521, doi:10.1128/jcm.39.4.1517-1521.2001 (2001).
- 19 Paratuberculosis, Johne's Disease. 1-6 (Iowa State University, 2007).

- 20 Shulaw, W. P. Johne's Disease in Sheep and Goats. <<http://ohioline.osu.edu/vme-fact/0003.html>>.
- 21 Sigurdardóttir, Ó. G., Press, C. M., Saxegaard, F. & Evensen, Ø. Bacterial Isolation, Immunological Response, and Histopathological Lesions during the Early Subclinical Phase of Experimental Infection of Goat Kids with Mycobacterium avium subsp. paratuberculosis. *Vet Pathol* **36**, 542–550 (1999).
- 22 Carrigan, M. J. & Seaman, J. T. The pathology of Johne's disease in sheep. *Aust Vet J* **67**, 47-50. (1990).
- 23 Whitlock, R. H. & Buergelt, C. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet Clin North Am Food Anim Pract* **12**, 345-356 (1996).
- 24 Chacon, O., Bermudez, L. E. & Barletta, R. G. Johne's disease, inflammatory bowel disease, and Mycobacterium paratuberculosis. *Annu Rev Microbiol* **58**, 329-363, doi:10.1146/annurev.micro.58.030603.123726 [doi] (2004).
- 25 Djønne, B. in *Paratuberculosis: Organism, Disease, Control* (eds M.A. Behr & D. M. Collins) 169-178 (CAB International USA,UK, 2010).
- 26 Tiwari, A., VanLeeuwen, J. A., McKenna, S. L., Keefe, G. P. & Barkema, H. W. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *The Canadian veterinary journal. La revue veterinaire canadienne* **47**, 874-882 (2006).
- 27 Storset, A. K. *et al.* Subclinical paratuberculosis in goats following experimental infection. An immunological and microbiological study. *Veterinary immunology and immunopathology* **80**, 271-287 (2001).
- 28 Stehman, S. Q & A: *Johne's Disease (Paratuberculosis)*, <<http://kinne.net/johnesqa.htm>> (1994).
- 29 Collins, M. & Manning, E. *General Pathology-JOHNE'S INFORMATION CENTER*, <<http://www.johnes.org/general/pathology.html>> (
- 30 Manning, E. J. B. & Collins, M. T. Mycobacterium avium subsp. paratuberculosis: pathogen, pathogenesis and diagnosis. *Rev. sci. tech. Off. int. Epiz.* **20** 133-150 (2001).
- 31 Clarke, C. J. The pathology and pathogenesis of paratuberculosis in ruminants and other species. *Journal of comparative pathology* **116**, 217-261 (1997).
- 32 Morin, M. Johne's Disease (Paratuberculosis) in Goats: A Report of Eight Cases in Quebec. *The Canadian veterinary journal. La revue veterinaire canadienne* **23**, 55-58 (1982).
- 33 Olsen, I., Sigurgardottir, G. & Djønne, B. Paratuberculosis with special reference to cattle. A review. *The Veterinary quarterly* **24**, 12-28 (2002).
- 34 Bull, T. J. *et al.* Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *Journal of clinical microbiology* **41**, 2915-2923 (2003).
- 35 Darfeuille-Michaud, A. *et al.* High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* **127**, 412-421 (2004).
- 36 Lamps, L. W. *et al.* Pathogenic Yersinia DNA is detected in bowel and mesenteric lymph nodes from patients with Crohn's disease. *The American journal of surgical pathology* **27**, 220-227 (2003).
- 37 Sutton, C. L. *et al.* Identification of a novel bacterial sequence associated with Crohn's disease. *Gastroenterology* **119**, 23-31 (2000).
- 38 Feller, M. *et al.* Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. *The Lancet infectious diseases* **7**, 607-613, doi:10.1016/s1473-3099(07)70211-6 (2007).

- 39 Sartor, R. B. Does *Mycobacterium avium* subspecies paratuberculosis cause Crohn's disease? *Gut* **54**, 896-898, doi:10.1136/gut.2004.055889 (2005).
- 40 Hermon-Taylor, J., Barnes, N., Clarke, C. & Finlayson, C. *Mycobacterium paratuberculosis* cervical lymphadenitis, followed five years later by terminal ileitis similar to Crohn's disease. *BMJ* **316**, 449-453 (1998).
- 41 Taylor, R. H., Falkinham, J. O., 3rd, Norton, C. D. & LeChevallier, M. W. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. *Appl Environ Microbiol* **66**, 1702-1705 (2000).
- 42 Grant, I. R. in *Paratuberculosis: Organism, Disease, Control* (ed M.A. Behr and D.M. Collins) Ch. 4, 30 (CAB International, www.cabi.org, 2010).
- 43 Kuehnel, M. P. *et al.* Characterization of the intracellular survival of *Mycobacterium avium* ssp. paratuberculosis: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cellular microbiology* **3**, 551-566 (2001).
- 44 Gui, G. P. *et al.* Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *J Antimicrob Chemother* **39**, 393-400 (1997).
- 45 Collins, M. T., Gardner, I. A., Garry, F. B., Roussel, A. J. & Wells, S. J. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. *Journal of the American Veterinary Medical Association* **229**, 1912-1919 (2006.).
- 46 Fernandez-Silva, J. A., Abdulmawjood, A. & Bulte, M. Diagnosis and Molecular Characterization of *Mycobacterium avium* subsp. paratuberculosis from Dairy Cows in Colombia. *Veterinary medicine international* **2011**, 352561, doi:10.4061/2011/352561 (2011).
- 47 Collins, M. T. Paratuberculosis: Review of present knowledge. *Acta veterinaria Scandinavica*. **44** 217-221 (2003).
- 48 Djonne, B., Jensen, M. R., Grant, I. R. & Holstad, G. Detection by immunomagnetic PCR of *Mycobacterium avium* subsp. paratuberculosis in milk from dairy goats in Norway. *Vet Microbiol* **92**, 135-143, doi:S0378113502003553 [pii] (2003).
- 49 Ikonomopoulos, J. *et al.* Comparative evaluation of positive tests to *Mycobacterium avium* subsp. paratuberculosis in clinically healthy sheep and goats in south-west Greece using molecular techniques, serology, and culture. *Veterinary journal (London, England : 1997)* **174**, 337-343, doi:10.1016/j.tvjl.2006.09.004 (2007).
- 50 Whittington, R. J., Reddacliff, L., Marsh, I. & Saunders, V. Detection of *Mycobacterium avium* subsp paratuberculosis in formalin-fixed paraffin-embedded intestinal tissue by IS900 polymerase chain reaction. *Aust Vet J* **77**, 392-397 (1999).
- 51 Perez, V., Tellechea, J., Corpa, J. M., Gutierrez, M. & Garcia Marin, J. F. Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis. *Am J Vet Res* **60**, 123-127 (1999).
- 52 PARATUBERCULOSIS (Johnes's disease), OIE Terrestrial Manual. 276-291 (World organisation for Animal Health Paris, France 2008).
- 53 Kalis, C. H., Collins, M. T., Hesselink, J. W. & Barkema, H. W. Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. *Vet Microbiol* **97**, 73-86 (2003).
- 54 Kurade, N. P., Tripathi, B. N., Rajukumar, K. & Parihar, N. S. Sequential development of histologic lesions and their relationship with bacterial isolation, fecal shedding, and immune responses during progressive stages of experimental infection of lambs with *Mycobacterium avium* subsp. paratuberculosis. *Vet Pathol* **41**, 378-387, doi:10.1354/vp.41-4-378 (2004).

- 55 Burnside, D. M. & Rowley, B. O. Evaluation of an enzyme-linked immunosorbent assay for diagnosis of paratuberculosis in goats. *Am J Vet Res* **55**, 465-466 (1994).
- 56 Gumber, S., Eamens, G. & Whittington, R. J. Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without Mycobacterium paratuberculosis infection. *Vet Microbiol* **115**, 91-101, doi:10.1016/j.vetmic.2006.01.003 (2006).
- 57 Whittington, R. J., Eamens, G. J. & Cousins, D. V. Specificity of absorbed ELISA and agar gel immuno-diffusion tests for paratuberculosis in goats with observations about use of these tests in infected goats. *Aust Vet J* **81**, 71-75 (2003).
- 58 Collins, M. & Manning, E. *Diagnosis-JOHNE'S INFORMATION CENTER*, <<http://www.johnes.org/general/diagnosis.html>> (
- 59 Gwozdz, J. M. Paratuberculosis (Johne's Disease). 1-38 (OIE and National Reference Laboratory for Johne's Disease, Department of Primary Industries, Australia, 2010).
- 60 Grøneng, G. M. & Djønné, B. The surveillance and control programme for paratuberculosis in Norway. (National Veterinary Institute, Norway, 2008).
- 61 Bastida, F. & Juste, R. A. Paratuberculosis control: a review with a focus on vaccination. *Journal of immune based therapies and vaccines* **9**, 8, doi:10.1186/1476-8518-9-8 (2011).
- 62 Kathaperumal, K. *et al.* Evaluation of immune responses and protective efficacy in a goat model following immunization with a cocktail of recombinant antigens and a polyprotein of Mycobacterium avium subsp. paratuberculosis. *Vaccine* **27**, 123-135, doi:10.1016/j.vaccine.2008.10.019 (2009).
- 63 Koets, A. *et al.* Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. *Vaccine* **24**, 2550-2559, doi:10.1016/j.vaccine.2005.12.019 (2006).
- 64 Huntley, J. F., Stabel, J. R., Paustian, M. L., Reinhardt, T. A. & Bannantine, J. P. Expression library immunization confers protection against Mycobacterium avium subsp. paratuberculosis infection. *Infection and immunity* **73**, 6877-6884, doi:10.1128/iai.73.10.6877-6884.2005 (2005).
- 65 Velaz-Faircloth, M., Cobb, A. J., Horstman, A. L., Henry, S. C. & Frothingham, R. Protection against Mycobacterium avium by DNA vaccines expressing mycobacterial antigens as fusion proteins with green fluorescent protein. *Infection and immunity* **67**, 4243-4250 (1999).
- 66 Rosseels, V. & Huygen, K. Vaccination against paratuberculosis. *Expert review of vaccines* **7**, 817-832, doi:10.1586/14760584.7.6.817 (2008).
- 67 Kalis, C. H., Hesselink, J. W., Barkema, H. W. & Collins, M. T. Use of long-term vaccination with a killed vaccine to prevent fecal shedding of Mycobacterium avium subsp paratuberculosis in dairy herds. *Am J Vet Res* **62**, 270-274 (2001).
- 68 Benedictus, G. & Kalis, C. J. Paratuberculosis: eradication, control and diagnostic methods. *Acta veterinaria Scandinavica* **44**, 231-241 (2003).
- 69 Benedictus, G. in *8ICP Workshop 4*.
- 70 Secott, T. E., Lin, T. L. & Wu, C. C. Mycobacterium avium subsp. paratuberculosis Fibronectin Attachment Protein Facilitates M-Cell Targeting and Invasion through a Fibronectin Bridge with Host Integrins. *Infection and immunity* **72**, 3724-3732 (2004).
- 71 Stevenson, K. Studying the pathogenesis of paratuberculosis: the enduring challenge. *Theme 2: Immunology, Pathology and Pathogenesis*, 88-97 (2005). <<http://pubs.paratuberculosis.org/index.php/paratb/article/viewFile/240/128>>.
- 72 Smith, M. C. & Sherman, D. M. in *Goat Medicine* 419-427 (Wiley Blackwell, 2009).

- 73 Sigurdardottir, O. G., Bakke-McKellep, A. M., Djonne, B. & Evensen, O. Mycobacterium avium subsp. paratuberculosis enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. *Comparative immunology, microbiology and infectious diseases* **28**, 223-230, doi:10.1016/j.cimid.2005.01.004 (2005).
- 74 Tyrer, P., Foxwell, A. R., Cripps, A. W., Apicella, M. A. & Kyd, J. M. Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium. *Infection and immunity* **74**, 625-631, doi:10.1128/iai.74.1.625-631.2006 (2006).
- 75 Krutzik, S. R. & Modlin, R. L. The role of Toll-like receptors in combating mycobacteria. *Seminars in immunology* **16**, 35-41 (2004).
- 76 Sieling, P. A. & Modlin, R. L. Activation of toll-like receptors by microbial lipoproteins. *Scandinavian journal of infectious diseases* **33**, 97-100 (2001).
- 77 Taylor, D. L., Zhong, L., Begg, D. J., de Silva, K. & Whittington, R. J. Toll-like receptor genes are differentially expressed at the sites of infection during the progression of Johne's disease in outbred sheep. *Veterinary immunology and immunopathology* **124**, 132-151, doi:10.1016/j.vetimm.2008.02.021 (2008).
- 78 Ferwerda, G. *et al.* Mycobacterium paratuberculosis is recognized by Toll-like receptors and NOD2. *Journal of leukocyte biology* **82**, 1011-1018, doi:10.1189/jlb.0307147 (2007).
- 79 Coussens, P. M. Mycobacterium paratuberculosis and the bovine immune system. *Animal health research reviews / Conference of Research Workers in Animal Diseases* **2**, 141-161 (2001).
- 80 Hostetter, J., Kagan, R. & Steadham, E. Opsonization effects on Mycobacterium avium subsp. paratuberculosis--macrophage interactions. *Clinical and diagnostic laboratory immunology* **12**, 793-796, doi:10.1128/cdli.12.6.793-796.2005 (2005).
- 81 Souza, C. D., Evanson, O. A., Sreevatsan, S. & Weiss, D. J. Cell membrane receptors on bovine mononuclear phagocytes involved in phagocytosis of Mycobacterium avium subsp paratuberculosis. *Am J Vet Res* **68**, 975-980, doi:10.2460/ajvr.68.9.975 (2007).
- 82 Tanaka, S., Sato, M., Taniguchi, T. & Yokomizo, Y. Relationship of acid phosphatase activity to ultrastructural features in mice inoculated with Mycobacterium paratuberculosis. *Journal of comparative pathology* **114**, 81-91 (1996).
- 83 Nathan, C. F. Secretory products of macrophages. *The Journal of clinical investigation* **79**, 319-326, doi:10.1172/jci112815 (1987).
- 84 Moreno, C. & Rees, A. J. Striking the right balance; the role of cytokines in mycobacterial disease. *Clinical and experimental immunology* **94**, 1-3 (1993).
- 85 Delvig, A. A., Lee, J. J., Chrzanowska-Lightowlers, Z. M. & Robinson, J. H. TGF-beta1 and IFN-gamma cross-regulate antigen presentation to CD4 T cells by macrophages. *Journal of leukocyte biology* **72**, 163-166 (2002).
- 86 Shankar, G., Pestano, L. A. & Bosch, M. L. Interferon-gamma Added During Bacillus Calmette-Guerin Induced Dendritic Cell Maturation Stimulates Potent Th1 Immune Responses. *Journal of translational medicine* **1**, 7, doi:10.1186/1479-5876-1-7 (2003).
- 87 Teixeira, L. K. *et al.* IFN-gamma production by CD8+ T cells depends on NFAT1 transcription factor and regulates Th differentiation. *Journal of immunology (Baltimore, Md. : 1950)* **175**, 5931-5939 (2005).
- 88 Bassey, E. O. & Collins, M. T. Study of T-lymphocyte subsets of healthy and Mycobacterium avium subsp. paratuberculosis-infected cattle. *Infection and immunity* **65**, 4869-4872 (1997).

- 89 Ferrick, D. A. *et al.* Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* **373**, 255-257, doi:10.1038/373255a0 (1995).
- 90 Shin, S. J. *et al.* In vitro cellular immune responses to recombinant antigens of *Mycobacterium avium* subsp. paratuberculosis. *Infection and immunity* **73**, 5074-5085, doi:10.1128/iai.73.8.5074-5085.2005 (2005).
- 91 Stabel, J. R. in *Paratuberculosis: Organism, Disease, Control* (eds M.A. Behr & D. M. Collins) 230-243 (CAB International USA, UK, 2010).
- 92 Khader, S. A. & Cooper, A. M. IL-23 and IL-17 in tuberculosis. *Cytokine* **41**, 79-83, doi:10.1016/j.cyto.2007.11.022 (2008).
- 93 Robinson, M. W., O'Brien, R., Mackintosh, C. G., Clark, R. G. & Griffin, J. F. Immunoregulatory cytokines are associated with protection from immunopathology following *Mycobacterium avium* subspecies paratuberculosis infection in red deer. *Infection and immunity* **79**, 2089-2097, doi:10.1128/iai.00779-10 (2011).
- 94 Shu, D. *et al.* Diverse cytokine profile from mesenteric lymph node cells of cull cows severely affected with Johne's disease. *Clinical and vaccine immunology : CVI* **18**, 1467-1476, doi:10.1128/cvi.05201-11 (2011).
- 95 Koets, A. P. *et al.* in *Sixth International Colloquium on Paratuberculosis* (eds E.J.B. Manning & M.T. Collins) 585-592. (Melbourne, Australia, 1999).
- 96 Mosmann, T. R. & Moore, K. W. The role of IL-10 in crossregulation of TH1 and TH2 responses. *Immunology today* **12**, A49-53 (1991).
- 97 Karcher, E. L., Beitz, D. C. & Stabel, J. R. Modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. paratuberculosis. *Veterinary immunology and immunopathology* **123**, 277-288, doi:10.1016/j.vetimm.2008.02.006 (2008).
- 98 Coussens, P. M., Verman, N., Coussens, M. A., Elftman, M. D. & McNulty, A. M. Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with *Mycobacterium avium* subsp. paratuberculosis: evidence for an inherent proinflammatory gene expression pattern. *Infection and immunity* **72**, 1409-1422 (2004).
- 99 Khalifeh, M. S. & Stabel, J. R. Upregulation of transforming growth factor-beta and interleukin-10 in cows with clinical Johne's disease. *Veterinary immunology and immunopathology* **99**, 39-46, doi:10.1016/j.vetimm.2004.01.009 (2004).
- 100 Khalifeh, M. S. & Stabel, J. R. Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of *Mycobacterium avium* subsp. paratuberculosis in monocyte-derived macrophages from naturally infected cattle. *Infection and immunity* **72**, 1974-1982 (2004).
- 101 Ludviksson, B. R., Seegers, D., Resnick, A. S. & Strober, W. The effect of TGF-beta1 on immune responses of naive versus memory CD4+ Th1/Th2 T cells. *European journal of immunology* **30**, 2101-2111 (2000).
- 102 Begara-McGorum, I. *et al.* Early immunopathological events in experimental ovine paratuberculosis. *Veterinary immunology and immunopathology* **63**, 265-287 (1998).
- 103 Waters, W. R. *et al.* Antigen-specific B-cell unresponsiveness induced by chronic *Mycobacterium avium* subsp. paratuberculosis infection of cattle. *Infection and immunity* **67**, 1593-1598 (1999).
- 104 Lampropoulou, V. *et al.* TLR-activated B cells suppress T cell-mediated autoimmunity. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 4763-4773 (2008).

- 105 Saxegaard, F. Isolation of Mycobacterium paratuberculosis from intestinal mucosa and mesenteric lymph nodes of goats by use of selective Dubos medium. *Journal of clinical microbiology* **22**, 312-313 (1985).
- 106 Green, E. P. *et al.* Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of Mycobacterium paratuberculosis. *Nucleic Acids Res* **17**, 9063-9073 (1989).
- 107 Johansen, T. B. *et al.* in *11th International Colloquium on Paratuberculosis*.
- 108 Masseyeff, R. F., Albert, W. H. & Staines, N. A. *Methods of Immunological Analysis*. Vol. 3 (Weinheim : VCH Publ., 1993).
- 109 Olsen, I. *et al.* Isolation of Mycobacterium avium subspecies paratuberculosis reactive CD4 T cells from intestinal biopsies of Crohn's disease patients. *PloS one* **4**, e5641, doi:10.1371/journal.pone.0005641 (2009).
- 110 Kennedy, S. *How Silica Spin Column DNA and RNA Preps Work*, <<http://bitesizebio.com/articles/how-silica-spin-column-dna-and-rna-preps-work/>> (2010).
- 111 Ghatak, K. L. 618-619 (Ghosh, A.K. , PHI learning private limited, New Delhi, 2011).
- 112 Gordon, P. M. & Sensen, C. W. Osprey: a comprehensive tool employing novel methods for the design of oligonucleotides for DNA sequencing and microarrays. *Nucleic Acids Res* **32**, e133, doi:10.1093/nar/gnh127 (2004).
- 113 *User Bulletin #2. ABI P RISM 7700 Sequence Detection System, AB Applied Biosystem*, <http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf> (1997(updated 10/2001)).
- 114 Abbott, R. J. *et al.* Structural and functional characterization of a novel T cell receptor co-regulatory protein complex, CD97-CD55. *The Journal of biological chemistry* **282**, 22023-22032, doi:10.1074/jbc.M702588200 (2007).
- 115 McKeehan, W. L. & Ham, R. G. Stimulation of clonal growth of normal fibroblasts with substrata coated with basic polymers. *J Cell Biol* **71**, 727-734 (1976).
- 116 Yamada, K. M. & Olden, K. Fibronectins--adhesive glycoproteins of cell surface and blood. *Nature* **275**, 179-184 (1978).
- 117 Nanda, P. *et al.* Goat serum as an alternative to establish cell culture from Indian major carp. 148-151, doi:10.1007/s11626-008-9160-7 (2009).
- 118 Paranjape, S. Goat serum: an alternative to fetal bovine serum in biomedical research. *Indian J Exp Biol* **42**, 26-35 (2004).
- 119 Sharma, G., Sharma, R. D. & Nichani, A. K. Successful long-term in vitro cultivation of Theileria annulata schizonts in media supplemented with homologous and heterologous sera. *Vet Parasitol* **79**, 135-141 (1998).
- 120 Mogues, T., Goodrich, M. E., Ryan, L., LaCourse, R. & North, R. J. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *The Journal of experimental medicine* **193**, 271-280 (2001).
- 121 Smyth, A. J., Welsh, M. D., Girvin, R. M. & Pollock, J. M. In vitro responsiveness of gammadelta T cells from Mycobacterium bovis-infected cattle to mycobacterial antigens: predominant involvement of WC1(+) cells. *Infection and immunity* **69**, 89-96, doi:10.1128/iai.69.1.89-96.2001 (2001).
- 122 Hasvold, H. J., Valheim, M., Berntsen, G. & Storset, A. K. In vitro responses to purified protein derivate of caprine T lymphocytes following vaccination with live strains of Mycobacterium avium subsp paratuberculosis. *Veterinary immunology and immunopathology* **90**, 79-89 (2002).

- 123 Koo, H. C. *et al.* Analysis of the immune response to *Mycobacterium avium* subsp. paratuberculosis in experimentally infected calves. *Infection and immunity* **72**, 6870-6883, doi:10.1128/iai.72.12.6870-6883.2004 (2004).
- 124 Waters, W. R. *et al.* Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. paratuberculosis infection of calves. *Infection and immunity* **71**, 5130-5138 (2003).
- 125 Chiodini, R. J. & Davis, W. C. The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent CD4+ activity. *Microb Pathog* **13**, 447-463 (1992).
- 126 Navarro, J. A., Ramis, G., Seva, J., Pallares, F. J. & Sanchez, J. Changes in lymphocyte subsets in the intestine and mesenteric lymph nodes in caprine paratuberculosis. *Journal of comparative pathology* **118**, 109-121 (1998).
- 127 Chiodini, R. J. & Davis, W. C. The cellular immunology of bovine paratuberculosis: immunity may be regulated by CD4+ helper and CD8+ immunoregulatory T lymphocytes which down-regulate gamma/delta+ T-cell cytotoxicity. *Microb Pathog* **14**, 355-367, doi:10.1006/mpat.1993.1035 (1993).
- 128 Promega. CellTiter-Glo® Luminescent Cell Viability Assay. *Madison, USA*, 1-13 (2011).
- 129 Hannah, R., Beck, M., Moravec, R. & Riss, T. Celltiter-Glo™ Luminescent Cell Viability Assay: A Sensitive And Rapid Method For Determining Cell Viability. *Cell Notes*, 11-13 (2001).
- 130 Zakowicz, H. Assessing Cell Viability In Primary Cultures Using A Luminescent, Atp-Based Assay: Applications For The Celltiter-Glo® Assay. *Cell Notes*, 14-15 (2007).
- 131 Ahmed, S. A., Gogal, R. M., Jr. & Walsh, J. E. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J Immunol Methods* **170**, 211-224 (1994).
- 132 Paolicchi, F. A. *et al.* Application of Different Methods for the Diagnosis of Paratuberculosis in a Dairy Cattle Herd in Argentina. *J. Vet. Med.* **B 50**, 20–26 (2003).
- 133 Stabel, J. R. & Whitlock, R. H. An evaluation of a modified interferon-gamma assay for the detection of paratuberculosis in dairy herds. *Veterinary immunology and immunopathology* **79**, 69-81 (2001).
- 134 Sweeney, R. W. *et al.* Effect of subcutaneous administration of a killed *Mycobacterium avium* subsp paratuberculosis vaccine on colonization of tissues following oral exposure to the organism in calves. *Am J Vet Res* **70**, 493-497, doi:10.2460/ajvr.70.4.493 (2009).
- 135 Uzonna, J. E. *et al.* Efficacy of commercial and field-strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* **21**, 3101-3109 (2003).
- 136 Kohler, H. *et al.* Immune reactions in cattle after immunization with a *Mycobacterium paratuberculosis* vaccine and implications for the diagnosis of *M. paratuberculosis* and *M. bovis* infections. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health* **48**, 185-195 (2001).
- 137 Muskens, J., van Zijderveld, F., Eger, A. & Bakker, D. Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. *Vet Microbiol* **86**, 269-278 (2002).
- 138 Koets, A. P. *et al.* Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis. *Veterinary immunology and immunopathology* **70**, 105-115 (1999).

- 139 Nagata, R., Muneta, Y., Yoshihara, K., Yokomizo, Y. & Mori, Y. Expression cloning of gamma interferon-inducing antigens of *Mycobacterium avium* subsp. paratuberculosis. *Infection and immunity* **73**, 3778-3782, doi:10.1128/iai.73.6.3778-3782.2005 (2005).
- 140 Rigden, R. C. *et al.* Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium* subspecies paratuberculosis. *Journal of medical microbiology* **55**, 1735-1740, doi:10.1099/jmm.0.46785-0 (2006).
- 141 Flesch, I. E. & Kaufmann, S. H. Attempts to characterize the mechanisms involved in mycobacterial growth inhibition by gamma-interferon-activated bone marrow macrophages. *Infection and immunity* **56**, 1464-1469 (1988).
- 142 Cooper, A. M. *et al.* Disseminated tuberculosis in interferon gamma gene-disrupted mice. *The Journal of experimental medicine* **178**, 2243-2247 (1993).
- 143 Jouanguy, E. *et al.* Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. *The Journal of clinical investigation* **100**, 2658-2664, doi:10.1172/jci119810 (1997).
- 144 Pappu, R., Ramirez-Carrozzi, V., Ota, N., Ouyang, W. & Hu, Y. The IL-17 family cytokines in immunity and disease. *Journal of clinical immunology* **30**, 185-195, doi:10.1007/s10875-010-9369-6 (2010).
- 145 Coussens, P. M., Colvin, C. J., Rosa, G. J., Perez Laspiur, J. & Elftman, M. D. Evidence for a novel gene expression program in peripheral blood mononuclear cells from *Mycobacterium avium* subsp. paratuberculosis-infected cattle. *Infection and immunity* **71**, 6487-6498 (2003).
- 146 Stabel, J. R. Production of gamma-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* **8**, 345-350 (1996).
- 147 Stabel, J. R. Transitions in immune responses to *Mycobacterium paratuberculosis*. *Vet Microbiol* **77**, 465-473 (2000).
- 148 Collins, M. T., Sockett, D. C., Ridge, S. & Cox, J. C. Evaluation of a commercial enzyme-linked immunosorbent assay for Johne's disease. *Journal of clinical microbiology* **29**, 272-276 (1991).
- 149 Weiss, D. J., Evanson, O. A. & Souza, C. D. Expression of interleukin-10 and suppressor of cytokine signaling-3 associated with susceptibility of cattle to infection with *Mycobacterium avium* subsp paratuberculosis. *Am J Vet Res* **66**, 1114-1120 (2005).
- 150 Karcher, E. L. *Evaluation of the Host Immune Response to Mycobacterium Avium Subsp. Paratuberculosis in Naturally Infected Periparturient Dairy Cows* Ph.D. thesis, Iowa State University., (2007).
- 151 Stabel, J. R., Robbe-Austerman, S. & Davis, W. C. in *9th International Colloquium on Paratuberculosis*.

